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Milk and serum proteomes in subclinical and clinical mastitis in Simmental cows

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A B S T R A C T

Bovine mastitis causes changes in the milk and serum proteomes. Here changes in both proteomes caused by naturally occurring subclinical and clinical mastitis have been characterised and quantified. Milk and serum samples from healthy dairy cows ($n = 10$) were compared to those of cows with subclinical ($n = 12$) and clinical mastitis (n = 10) using tandem mass tag (TMT) proteomics. Proteins that significantly increased or decreased in milk ($n = 237$) or serum ($n = 117$) were quantified and classified by the type of change in subclinical and clinical mastitis. A group of the proteins ($n = 38$) showed changes in both milk and serum a number of which decreased in the serum but increased in milk, suggesting a particular role in host defence for maintaining and restoring homeostasis during the disease. Proteins affected by bovine mastitis included proteins in host defence and coagulation pathways. Investigation of the modified proteomes in milk and serum was assessed by assays for haptoglobin, serum amyloid A and α_1 acid glycoprotein validating the results obtained by quantitative proteomics. Alteration of abundance patterns of milk and serum proteins, together with pathway analysis reveal multiple interactions related to proteins affected by mastitis. Data are available via ProteomeXchange with identifier PXD022595.

Significance: Mastitis is the most serious condition to affect dairy cows and leads to reduced animal welfare as well as having a negative economic effect for the dairy industry. Proteomics has previously identified changes in abundance of milk proteins during mastitis, but there have been few investigations addressing changes that may affect proteins in the blood during the infection. In this study, changes in the abundance of proteins of milk and serum, caused by naturally occurring mastitis have been characterised by proteomics using a quantitative approach and both subclinical and clinical cases of mastitis have been investigated. In both milk and serum, change in individual proteins was determined and classified into varying types of altering abundance, such as increasing in subclinical mastitis, but showing no further increase in clinical mastitis. Of special interest were the proteins that altered in abundance in both milk and serum which either showed similar trends - increasing or decreasing in both biological fluids or showed reciprocal change decreasing in serum but increasing in milk. As well as characterising proteins as potential markers of mastitis and the severity of the disease, these results provide insight into the pathophysiology of the host response to bovine mastitis.

1. Introduction

Bovine mastitis is still a major concern worldwide being a major cause of economic losses in the dairy industry due to reduced milk production, increased treatment costs, reduced fertility and increased culling of affected animals. A wide range of causative pathogens makes the treatment of mastitis demanding, with a high risk of the development of antimicrobial resistance. Moreover, mastitis poses a one-health concern due to a zoonotic potential of particular causative microorganisms, such as *E. coli, Salmonella* spp. or *Listeria* spp. and the potential for food-born infection with a high risk for human health. Apart from infectious microorganisms, the causes of mastitis could be, in some cases, a traumatic event or even some environmental factors (chemical or physical).

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Mastitis prevalence in dairy cows in Croatia has significantly dropped during the last two decades due to the adoption of European standards regulating somatic cell count (SCC) and total bacteria count in raw milk [1]. Market pressure through the milk conformity standards has caused either abandoning milk production by farmers or stronger orientation toward commercial production. In commercially oriented dairy production, more attention is paid to the management of mastitis. Hence the improvement in milk quality is visible through the lower prevalence of mastitis. While in some older reports regarding mastitis occurrence among dairy cows in Croatia the infection rate was above 50% of the total udder quarters examined, the most recent report [1] showed 13% of quarters were infected. The dominating pathogen was *Staphylococcus aureus* followed by members of genus *Streptococcus*, *Trueperella pyogenes*, *Corynebacterium* spp. and *Streptococcus uberis*, with other pathogens isolated only sporadically [1].

Diagnosis of clinical mastitis is relatively easy as a range of visible clinical signs of udder inflammation along with a disturbance in general health are present, but subclinical mastitis diagnosis is challenging due to the absence of any visible clinical sign. In daily practice, an increased number of SCC in the milk is the only evidence that helps in the diagnosis of subclinical mastitis and results in reduced payments to the dairy farmer because of reduced milk quality [2]. Somatic cells in milk mostly consist of macrophages, neutrophils, lymphocytes and in some degree to the presence of mammary epithelial cells. In healthy mammary gland secretions, macrophages are the major cell population, but during early inflammation neutrophils are the predominant cell type [3]. Upon the bacterial invasion of the mammary gland, macrophages respond to bacteria and their toxins by releasing cytokines, which recruit leukocytes at the site of infection and trigger the local and systemic acute phase response. Understanding the pathogenesis of mastitis, particularly the innate and specific immune responses at the systemic level and in the mammary gland, including the host-pathogen interaction, is crucial for development of reliable biomarkers that might contribute to the diagnosis of mastitis in its early stage.

Over the past decade it has become recognised that during mastitis there is a substantial change in the proteins in milk. Decreases are found in the more abundant proteins in healthy milk such as the caseins and β-lactoglobulin [4], while at the same time an increase occurs in proteins of the innate immune system such as haptoglobin (HP) and mammary associated serum amyloid A (MSAA3) which are known to be acute phase proteins (APP) [5–7]. There has been less focus on the effect of mastitis on serum proteins, but it is known that infection of the mammary gland does cause increases in serum APP with elevated HP detected in serum of dairy cows with naturally occurring mastitis [8] and with raised HP and MSAA3 in experimentally induced mastitis [6]. In our previous study, the serum proteome profile was investigated in cows with subclinical and clinical mastitis. Several APP, including HP, apolipoprotein A-I and inter-alpha-trypsin inhibitor heavy chain 4, were differentially expressed in subclinical and clinical mastitis compared to the control [9]. There have been further studies on the use of the APP as biomarkers of mastitis including studies where their concentrations were determined in both milk and serum [10–13]. Correlations between the same protein in serum and milk showed variable results with HP having a significant correlation but MSAA3 having no correlation between the levels in these fluids [14]. Inter-alpha-trypsin inhibitor heavy chain 4 had a moderate correlation between milk and serum concentrations which varied according to the severity and pathogen responsible for the mastitis [13].

As well as a growing interest in the acute phase proteins in milk, recent investigations have used the increasing power of proteomics to examine the change in milk during mastitis [15–22].

In contrast, there have been few studies in which proteomics has been used to examine both serum and milk from dairy cows with this condition. In one study, using two-dimensional electrophoresis (2-DE) coupled with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), the concurrent changes in the proteins in serum and milk whey in response to infection of the mammary gland were depicted [23]. Healthy and mastitis serum samples showed relatively similar protein composition with marked differences in expression levels, while healthy and mastitis milk whey showed a very different composition, likely due to extravasation of blood proteins to the mammary gland.

A study by Kim et al. [24] characterised immune protein responses to mastitis caused by different strains of *S. aureus* by monitoring serum and milk cytokine kinetics and the milk proteome profile. Results demonstrated differential expression of the cytokines predominately in serum, while milk proteomics showed 29 protein spots, which were differentially expressed during the acute or chronic phases of infection. Of these component-3 of the proteose peptone (CPP3) was identified as differentially expressed over time and also between the *S. aureus* challenge groups. In another study, a proteomic approach was used to characterise the changes in bovine plasma and milk during naturally occurring *E. coli* mastitis. The plasma protein content was not significantly different among cows with mild mastitis, severe mastitis, and healthy cows. Caseins levels were decreased, whereas the levels of anti-microbial and APP were increased in milk with cows suffering from severe mastitis demonstrating the greatest change compared with control cows [25].

From these investigations it is clear that using a proteomic approach to assess simultaneous change in serum and milk protein in response to mastitis might be advantageous. To overcome the shortcomings of the gel-based methods used in these previous studies and to increase the dynamic range and quantitative accuracy, gel-free and more sensitive high-throughput quantitative proteomics methods have been applied in this investigation. Thus, the objective of this study was to use a quantitative tandem mass tag (TMT) approach to determine the alteration of serum and milk proteomes that occur in natural cases of clinical and subclinical mastitis, while also comparing the proteomes to those of serum and milk from healthy animals. Acute phase protein concentrations were also determined in the serum and milk from the same groups to both validate the proteomic results and for comparative analysis of the innate immune response in both biofluids.

2. Material and methods

2.1. Samples: milk and serum and background on dairy cows

The study included milk and serum samples taken from thirty-two Simmental dairy cows kept on private farms located in the Zagreb County, Croatia. All cows were checked by physical examination. Somatic cell count and California mastitis test (CMT) were performed on the milk samples to differentiate samples from quarters with subclinical mastitis from healthy milk samples. A threshold of 400,000 cells/ml in the SCC was used to select samples for inclusion in the subclinical mastitis group. According to the results, cows were assigned into three groups: Group H (healthy control, $n = 10$) consisted of healthy cows with SCC below 400,000 cells/ml on the monthly check-up and a negative California mastitis test (CMT) on the day of sampling and without any clinical sign of mastitis. Group SC (subclinical mastitis, $n = 12$) comprised cows without clinical signs of mastitis but with SCC above 400,000 cells/ml on the basis of the monthly check and a positive CMT at the time of sampling. Group C (clinical mastitis, $n = 10$) consisted of cows with clinical signs of mastitis which include changes in milk appearance (flakes and clots in milk), different stages of udder inflammation (hyperaemia, oedema, pain, udder enlargement and elevated udder temperature) and disturbance of general health (depression, relaxed cold ears, dehydration, elevated body temperature, increased heart and respiratory rate, decreased ruminal contraction and decreased appetite).

Blood samples were taken from *v. coccygea* and centrifuged at 3000*g* for 15 min after clotting for two hours at room temperature. Serum samples were stored at −80 °C until analysis.

Milk samples were taken aseptically before the morning milking.

First few streams were discarded. Teat ends were disinfected with cotton swabs soaked with 70% ethanol. Samples were taken into sterile tubes and transported to laboratory on ice within a few hours. Milk samples were centrifuged at 13000 ×*g* for 30 min at 4 °C to remove cells and fat globules. From each sample, the middle clear portion was carefully transferred to a clean Eppendorf tube and used for subsequent proteomic analysis.

Bacteriological examination of milk was carried out according to the recommendations of the National mastitis council [26]. Aesculin agar with addition of 5% ovine blood was used for isolation of pathogens. Samples were inoculated on plates and incubated at 37 °C for 24 h. Grown colonies were identified taking into account morphological (size, colour) and physiological properties (Gramm staining, haemolysis, catalase and oxidase production). Depending on the initially observed properties, colonies were sub cultivated on Baird Parker agar, McConkey agar, Triple sugar iron agar (TSI) and Kanamycin aesculin azide agar. The ability of staphylococci to produce coagulase was checked using a tube coagulase test with rabbit serum. Streptococci were checked using a CAMP test. Finally, Gramm positive pathogens were submitted to a Micronaut–RPO system for identification based on biochemical reactions using 44 different substrates while Micronaut-GNE, based on 24 substrates was used to identify Gramm negative bacteria.

2.2. Proteomics: sample preparation of milk and serum; LC-MS/MS analysis

Relative quantification of proteins in milk and serum was performed using a shotgun Tandem Mass Tag (TMT)-based proteomic approach which has been described previously [27]. In brief, the total protein concentration of the samples was determined using the BCA assay (Thermo Scientific, Rockford, USA). Internal standards were prepared for both milk and serum samples by mixing equal amounts of all milk or all serum samples, respectively. An amount of 35 μg of total protein from individual samples and internal standards was diluted using 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA) and subsequently reduced with 20 mM dithiothreitol (DTT) for 60 min at 55 °C (Sigma Aldrich, St. Louis, MO, USA), alkylated with 30 mM iodoacetamide (IAA) for 30 min at room temperature in the dark (Sigma Aldrich, St. Louis, MO, USA) and acetone-precipitated (6 volumes, overnight, −20 °C). Protein digestion was performed using trypsin Gold (Promega, Madison, WI, USA) at 1:35 *w*/w, at 37 °C with an overnight incubation. Subsequently, peptides were labelled using freshly prepared TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA). For each sample type (milk and serum), differentially TMT-labelled samples were combined with the matching internal standard, aliquoted, dried and stored at −20 °C for further DDA LC-MS/MS analysis.

LC-MS/MS analysis was performed using the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) connected to a Q Exactive Plus mass spectrometer (MS) (Thermo Fisher Scientific, Bremen, Germany) as described elsewhere [30]. After loading onto the trap column, peptides were separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 μm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min at the flow rate of 300 nL/min. Mobile phase A was 0.1% formic acid in water. Ionization was achieved using a nanospray Flex ion source (Thermo Fisher Scientific). The MS was operated in positive ion mode using a DDA Top8 method with parameters set as follows: full scan MS spectra range from *m/z* 350.0 to *m/z* 1800.0, resolution of 70,000, injection time 120 ms, AGC target 1E6, isolation window±2.0 Da and with a dynamic exclusion of 30 s. For HCD fragmentation, resolution was set to 17,500 and AGC target to 2E5.

Raw data were analysed using Proteome Discoverer software (version 2.3., Thermo Fisher Scientific) with SEQUEST algorithm implemented. Database search against *Bos taurus* NCBInr FASTA files (downloaded 04/04/2019, 69,663 entries) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.05 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M) and TMT sixplex (K, peptide N-terminus) dynamic modifications. Proteins with at least two peptides (including 1 unique) and 5% FDR were selected as reliably identified. The internal standard was used to compare data between TMT experiments. The mass spectrometry proteomics data for the milk and serum investigations have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022595 [28].

2.3. Statistical and bioinformatic analysis

All statistics were performed using R v3.2.2 [29]. Sample outliers were detected and removed from further analysis per each group, for each of the protein using the Dixon's test from R package *outliers* v0.14 [30]. Normality was tested by Shapiro-Wilk test and as the majority of analysed proteins did not follow the normal distribution, the difference in protein abundance between groups was tested using Kruskal-Wallis test. For proteins shown to be significantly differentially abundant after *P*-value correction (false discovery rate, FDR < 0.05), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 [31]. Fold change between two groups was calculated as mean (Case) /mean (Control) and expressed on log2 scale. Principal component analysis (PCA) score plot, Volcano plots and validation plots were designed using R package *ggplot2* v3.1.1, the heatmaps were designed using R package *ggplot2* v3.1.1 and *pheatmap* 1.0.12 and Venn diagrams were designed using web tool InteractiVenn [32–34]. Proteins' GenInfo identifier (GI) accession numbers were converted into official gene symbol either by the DAVID conversion tool (https://david.ncifcrf.gov/conversion.jsp), UniProtKB ID mapping (https://www.uniprot.org/uploadlists/) or from the Mascot search engine implemented into Proteome Discoverer. Pathway enrichment analysis and networks of the relationship between desired KEGG pathway and proteins with significantly different abundances between groups were designed using Cytoscape v3.7.1 [35] plug-ins ClueGO v2.5.7 [36] and CluePedia v1.5.7. [37]. Gene ontology (GO) analysis was performed using Protein Analysis Through Evolutionary Relationship (PANTHER) classification tool (http://www.pantherdb.org/) which uses GO-Slim, a subset of terms from the full Gene Ontology database.

The results from proteomic analysis for HP, SAA1 and ORM1 were compared by immunoassay in order to validate obtained proteomic results. Difference between groups was determined using the Kruskal-Wallis test with Conover *post*-hoc test for pairwise comparisons with FDR *P*-value corrections, separately for each tested protein and validation method. To compare results of protein levels obtained with these validation methods, Spearman's rank correlation coefficient was calculated.

2.4. Validation of proteomic results

For validation of results from proteomics the concentrations of HP in milk along with SAA and ORM1 in both milk and serum were determined using Spatial Proximity Analyte Reagent Capture Luminescence (SPARCL™) immunoassays [38]. Bovine specific SPARCL assays were used for HP, SAA, and ORM1 (Life Diagnostics Inc., West Chester, USA) as previously validated [39]. The concentration of the HP in serum was determined by a haemoglobin-HP binding assay described by Eckersall et al. [40] as modified according to Brady et al. [41].

3. Results

3.1. Health status of cows with subclinical and clinical mastitis and healthy cows

The milk and serum were collected from groups of healthy cows (Group H, $n = 10$), cows with subclinical mastitis (Group SC, $n = 12$) and

clinical mastitis (Group C, n = 10). The health status of the cows including bacteriological findings in milk and CMT are presented in Table 1 along with their serum concentrations of HP, SAA and ORM1. The cows with clinical mastitis demonstrated visible signs of udder inflammation in a form of parenchymatous or catarrhal inflammation, with changes in milk appearance while cows with subclinical mastitis were without any visible sign of inflammation but had increased SCC on the monthly check-up and a positive CMT at the time of sampling. Causative agents of subclinical and clinical mastitis cases were *Streptococcus uberis, Klebsiella* spp., *Pasteurella* spp., *Staphylococcus* spp., and *E. coli.*

The serum concentrations of the APP are given in Table 1 demonstrate the range of concentration found in the different groups during mastitis. While there are significant differences between groups as shown in the validation section below (Section 3.5), there was a degree of overlap between groups, especially for the subclinical group in which the ranges from HP (7.4–501 μg/ml) compared to the healthy range (2.8–24 μg/ml) and for milk from clinical mastitis (8–842 μg/ml). There was a similar spread of results for SAA and ORM1 concentrations in serum.

3.2. Milk proteome in mastitis

In the milk samples there were a total of 237 unique proteins that showed significant differences in abundance between the healthy, subclinical and clinical mastitis groups. Within changes in protein abundance there were 6 main types of change observed, with majority of proteins (213 of the 237 unique proteins) showing one of these 6 main types of change in abundance. The types of change are summarized in Table 2. The largest number of milk proteins exhibiting a significant change ($n = 66$) were those that showed an increase in clinical mastitis compared to the healthy milk (Type 3) but did not show a significant increase compared to milk from cows with subclinical mastitis. This was followed by proteins ($n = 64$) that showed an increase in subclinical mastitis but no further increase in samples from cows with clinical mastitis (Type 1) while there were 23 proteins that increased in subclinical and then exhibited a further increase in clinical mastitis (Type 2). For proteins that decreased in abundance, the largest number ($n = 43$) were proteins that showed a decrease in clinical mastitis compared to the healthy milk but did not show a significant decrease in cows with subclinical mastitis compared to healthy (Type 6). This was followed by proteins ($n = 13$) that showed a decrease in subclinical mastitis and a further decrease in samples from cows with clinical mastitis (Type 5) whereas there were 4 proteins that decreased in subclinical and but exhibited no further decrease in clinical mastitis (Type 4). The 8 milk proteins showing the highest fold change in these types of change (Types 1–6) are listed in Table 3 while the full list of milk proteins in all these categories and in further minor types of change are given in Supplementary Table S1.

The results from proteomic analysis of milk samples between the

Table 1
Health status, reproductive status, California mastitis test (CMT) and bacteriology findings in milk and acute phase protein in serum of investigated dairy cows (Group: H – healthy control group; Group: SC – subclinical mastitis; Group: C – clinical mastitis). All milk samples in the healthy group had somatic cell count (SCC) of <400,000 cells/ml and all milk samples in the subclinical group had SCC of >400,000 cells/ml but no clinical signs of mastitis. All dairy cows in the clinical group showed clinical signs of mastitis (hyperaemia, oedema, pain, udder enlargement, elevated udder temperature and clots in the milk).

			Milk data		Reproduction data			Serum acute phase protein		
Group	Sample number	Udder inspection findings	CMT	Bacteriology test	Pregnant (YES/NO)	Duration of pregnancy (days)	Days of lactation	HP μg/ml	SAA μg/ml	AGP μg/ml
H:Healthy control	23		negative	negative	YES	60	136	5.9	1.7	815
	24		negative	negative	NO		35	3.8	5.7	271
	25		negative	negative	NO		40	8.6	1.0	352
	26		negative	negative	YES	102	105	18.3	0.1	274
	27		negative	negative	YES	60	172	13.8	0.2	208
	28		negative	negative	YES	50	106	<3	4.3	831
	29		negative	negative	YES	62	115	5.5	0.2	293
	30		negative	negative	NO		25	24.3	10	696
	31		negative	negative	YES	52	106	17.2	NA	288
	32		negative	negative	YES	101	152	24.2	NA	246
SC:Sub-clinical	2		+++	negative	NO		46	21.4	3.0	234
	3		++	negative	YES	32	96	17.8	3.2	274
	4		+	negative	YES	118	168	578	168	607
	5		+++	negative	NO		72	9.5	4.9	319
	10		+++	negative	NO		26	243	49	250
	11		+++	*Streptococcus uberis*	YES	62	117	7.4	4.3	360
	12		+++	*Streptococcus uberis*	YES	180	255	13	NA	NA
	16		+++	negative	YES	245	375	501	129	276
	17		+++	*Streptococcus* spp.	YES	20	62	10	20	828
	18		++	negative	NO		82	89	23	273
	19		+	negative	NO		75	7.4	0.3	138
	20		+++	negative	YES	210	282	19.1	0.4	177
C:Clinical	1	Parenchymatous mastitis	++	negative	NO		35	305	55	799
	6	Catarrhal mastitis	+	negative	YES	93	150	116	17	351
	7		+++	*Pasteurella* spp.	NO		72	17	42	381
	8	Catarrhal mastitis	+++	negative	NO		63	8	24	1111
	9	Catarrhal mastitis	+++	*Staphylococcus* spp.	YES	55	105	91	6	438
	13	Catarrhal mastitis	++	*Klebsiella* spp.	NO		150	159	29	444
	14	Catarrhal mastitis	++	negative	YES	155	224	143	41	326
	15	Parenchymatous mastitis	++	*E.coli*	NO		55	842	173	175

three groups of milk from healthy cows and from those with subclinical or clinical mastitis are additionally depicted using PCA score plots (Supplementary Fig. S1A) and Volcano plots (Supplementary Fig. S2A). The PCA demonstrated that for milk proteins, those that changed in subclinical mastitis were separate from the healthy milk proteins but still close while the milk proteins from clinical mastitis were substantially apart. The volcano plots for milk proteins show that a greater number of milk proteins were significantly increased than decreased in abundance especially in the clinical mastitis group when compared to the healthy group.

3.2.1. Differentially abundant proteins in milk during mastitis

Changes in abundance of individual milk proteins for each main type of change as given in Table 3 and shown as heat maps in Fig. 1 and Supplementary Fig. S3 show that of proteins that increased in abundance in milk during mastitis the greatest fold change (log_2) was in haptoglobin (HP) which had a FC of 2.128 in clinical mastitis compared to healthy milk, although this was not significantly raised above the level found in subclinical mastitis. This pattern of increase (Type 1) was shared with serum amyloid A (SAA1), β-defensin (DEFB1), and serpins B3 and B1 (SERPINB1, SERPINB3). Proteins showing a significant increase in subclinical mastitis compared to healthy and a further significant increase in clinical mastitis (Type 2) included α_2 macroglobulin (A2M), immunoglobulin heavy chain (IGHG), pregnancy zone protein (PXP) and cathelicidin 3 (CATH3). There were also proteins that did not increase in subclinical mastitis but were increased in clinical mastitis (Type 3) including a different variant (19) of A2M, calponin (CNN2) cathelicidin1 (CATHL1) and apolipoprotein A-1 and apolipoprotein A-2 (APOA1, APOA2).

For milk proteins that decreased in response to mastitis the largest group of proteins were those that showed no change in subclinical mastitis but demonstrated a significant decrease in abundance in clinical mastitis compared to milk from healthy cows (Type 6) with the greatest reduction being shown by thrombospondin (THBS1) with a FC of −1.522, along with α_{S2}-casein (CSN1S2), β-lactoglobulin (PAEP) and κ-casein (CSN3) among others. Proteins showing significant decreases in subclinical mastitis and further decreases in clinical mastitis (Type 5) included an ATP-binding transporter (ABCG2), mucin-1 (MUC1) and butyrophilin (BTN1A1). Only 4 proteins decreased in subclinical mastitis but did not show further change in clinical mastitis (Type 4).

Pathway analysis of the results for the milk proteome are shown in Supplementary Table S3 and Fig. 2 and indicated that complement and coagulation cascades, glucagon signalling pathway and glycine, serine and threonine metabolism were affected by mastitis. Gene ontology analysis (Supplementary Table S4) revealed that for differentially abundant milk proteins in molecular function category the largest percentage of genes had binding or catalytic activities, for biological process cellular, metabolic and regulation were the most common, while the highest percentage of genes in cellular component category were recognised as cellular anatomical entities. In addition, metabolite interconversion enzyme and cytoskeletal protein had the largest percent of genes present when looking at the protein class.

Table 2
The number of milk and serum proteins showing significant changes between Group H (healthy), Group SC (subclinical) and Group C (clinical) mastitis.

Change type	Change relative to healthy	Number of significant proteins	
		Milk	Serum
1	Increase in subclinical; no further increase in clinical	64	13
2	Increase in subclinical; additional increase in clinical	23	4
3	No change in subclinical; increase in clinical	66	5
4	Decrease in subclinical; no further decrease in clinical	4	48
5	Decrease in subclinical; additional decrease in clinical	13	24
6	No change in subclinical; decrease in clinical	43	6

3.3. Serum proteome in mastitis

In the serum samples there were a total of 117 unique proteins that showed significant differences between the healthy, subclinical and clinical mastitis groups. Within these proteins there were 6 main types of change observed, with majority of proteins (100 of the 117 unique proteins) showing one of these 6 main types of change in abundance. The types of change are shown in Table 2. The largest number of proteins increasing in serum ($n = 13$) were in proteins that showed an increase in subclinical mastitis compared to the healthy serum (Type 1) but did not show a further significant increase in serum from cows with clinical mastitis. There were 5 proteins that showed no increase in subclinical mastitis but did increase in samples from cows with clinical mastitis (Type 3) whereas 4 proteins that increased in subclinical did have a further increase in clinical mastitis (Type 2). For proteins that decreased in abundance, the largest number ($n = 48$) were proteins that showed a decrease in subclinical mastitis compared to the healthy serum but did not show a further significant decrease in cows with clinical mastitis compared to healthy (Type 4). This was followed by proteins ($n = 24$) that showed a decrease in subclinical mastitis and a further decrease in samples from cows with clinical mastitis (Type 5) whereas there were 6 proteins that decreased in subclinical and exhibited a further decrease in clinical mastitis (Type 6). The 8 proteins showing the highest fold change in these types of change are listed in Table 4 while the full list of serum proteins in all types of change are given in Supplementary Table S2.

The results from proteomic analysis of serum samples between the three groups of healthy cows and from those with subclinical or clinical mastitis are additionally depicted using PCA score plots (Supplementary Fig. S1B), and Volcano plots (Supplementary Fig. S2B). The PCA demonstrated that for serum proteins, those that changed in subclinical mastitis were separate from the healthy serum proteins but clustered closely with serum proteins from clinical mastitis (Supplementary Fig. S1B). The volcano plots for serum proteins show that a greater number of serum proteins were significantly decreased than increased in abundance when comparing the subclinical and clinical mastitis groups to the healthy group.

3.3.1. Differentially abundant proteins in serum during mastitis

Changes in abundance of individual serum proteins for each type of change are given in Table 4 and shown as heat maps in Fig. 3 and Supplementary Fig. S4. In contrast to differentially abundant protein in milk, more proteins reduced in abundance in serum ($n = 78$) than increased in abundance ($n = 22$). The majority of these were in the group of proteins that were reduced in subclinical mastitis but did not show a further fall in abundance (Type 4) in cows with clinical mastitis. Among these were immunoglobulin -l (VL1X) with the largest fall in abundance of a FC of −1.709, transthyretin (TTR), phospholipid transfer protein (PLTP) and β-casein (CSN2). Among proteins which were reduced in serum in subclinical mastitis but had a further significant fall in clinical mastitis were carboxypeptidase B2 (CPB2), inter-α-trypsin inhibitor heavy chain H1 (ITIH1), antithrombin-III (SERPINC1) and peptidoglycan recognition protein (PGYRP2). There were 6 proteins that were not reduced in abundance in subclinical mastitis but were significantly lower in serum from cows with clinical mastitis compared to healthy (Type 6) including immunoglobulin heavy chain-δ (IGHD), retinol binding protein (RBP4) and gelsolin (GSN). For serum proteins that increased in abundance in cows with mastitis, 13 proteins increased in subclinical mastitis with no further increase in serum from cows with clinical mastitis and included coagulation factor X (F10) and factor IX (F9), α1-acid glycoprotein (ORM1) and immunoglobulin heavy chains

Table 3
Changes in milk proteins in main categories of change listing up to the 8 proteins most increased or decreased in the category; groups abbreviation: H – control group; SC – subclinical mastitis; C – clinical mastitis. For proteins showed to be significantly differentially abundant after P-value correction (FDR < 0.05), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 [31]. Fold change between two groups was calculated as for example mean (C)/ mean(H) and expressed on log2 scale. NS = not significant

Accession Number	Gene	Description	FDR	log2 fold change		
				SC vs H	C vs SC	C vs H
Type 1 change: increase in SC and no further increase in C (n = 64)						
2144490	HP	Haptoglobin, bovine	0.009	1.484	NS	2.128
296471870	SAA1	Serum amyloid A protein	0.021	1.236	NS	2.069
298775	DEFB10	Beta-defensin (peptide BNBD-10)	0.010	1.138	NS	1.525
27819614	ACTA1	Actin, alpha skeletal muscle	0.013	1.045	NS	1.709
1387189648	SERPINB3	Serpin B3	0.021	0.941	NS	1.231
1387222928	ALDOA	Fructose-bisphosphate aldolase C	0.018	0.936	NS	1.596
1387238032	SERPINB1	Leukocyte elastase inhibitor	0.017	0.896	NS	0.989
296475914	CALM1	Calmodulin	0.013	0.882	NS	1.548
Type 2 change: increase in SC and further increase in C (n = 23)						
408689599	A2M	Alpha-2-macroglobulin variant 18	0.004	0.376	1.332	1.708
1150079692	IGHG	Immunoglobulin gamma heavy chain	0.005	0.371	1.145	1.516
1387270777	PZP	Pregnancy zone protein	0.005	0.181	0.960	1.141
296477366	ACTR2	Actin-related protein 2	0.016	0.645	0.913	1.559
1168625	CATHL3	Cathelicidin-3	0.009	0.984	0.900	1.884
296487402	LOC511240	Hypothetical protein LOC511240	0.005	0.348	0.809	1.157
73919851	TAGLN2	Transgelin-2	0.013	0.412	0.723	1.135
1708945	CATHL6	Cathelicidin-6	0.006	1.158	0.683	1.841
Type 3 change: no change in SC and increase in C (n = 66)						
408689601	A2M	Alpha-2-macroglobulin variant 19	0.004	NS	1.628	1.950
93204556	CNN2	Calponin-2	0.030	NS	1.116	1.618
296474766	CATHL1	Cathelicidin-1	0.009	NS	1.507	1.554
296480244	APOA1	Apolipoprotein A-I preproprotein	0.006	NS	1.376	1.545
114052298	APOA2	Apolipoprotein A-II	0.016	NS	1.123	1.471
1083056	HMG-2	Nonhistone chromosomal protein HMG-2, bovine	0.019	NS	0.796	1.371
296485395	PRTN3	Proteinase 3	0.007	NS	0.917	1.346
226373739	SERPINA3–6	Serpin A3–6	0.004	NS	1.254	1.306
Type 4 change: decrease in SC and no further decrease in C (*n* = 4)						
117306582	SCGB1D	Secretoglobin, family 1D, member 2	0.015	−0.233	NS	−0.544
50401067	MUC15	Mucin-15	0.012	−0.349	NS	−0.472
741933771	SIL1	Nucleotide exchange factor SIL1	0.007	−0.417	NS	−0.807
2323398	IGL	Immunoglobulin light chain variable region	0.005	−0.483	NS	−0.777
Type 5 change: decrease in SC and further decrease in C (n = 13)						
1765910	CD36	PAS-4	0.005	−0.430	−0.466	−0.896
49259423	PAEP	Beta-lactoglobulin	0.005	−0.447	−0.677	−1.124
2136760	N/A	Glycoprotein antigen MGP57/53, mammary gland, bovine	0.005	−0.509	−0.523	−1.033
157835743	MFGE8	Bovine lactadherin C2 domain	0.005	−0.529	−0.461	−0.990
547484	SLC34A2	Sodium-dependent phosphate transporter	0.004	−0.545	−0.646	−1.191
1387237809	BTN1A1	Butyrophilin subfamily 1 member A1	0.005	−0.589	−0.593	−1.182
528942106	MUC1	Mucin-1	0.004	−0.625	−0.334	−0.959
112496644	ABCG2	ATP-binding cassette transporter subfamily G member 2	0.010	−0.830	−0.323	−1.153
Type 6 change: no change in SC and decrease in C (n = 43)						
1391916210	CSN2	Beta-casein	0.005	NS	−0.942	−1.007
227993	FABP3	Fatty acid-binding protein	0.018	NS	−0.669	−1.007
163115	CLU	Glycoprotein III	0.005	NS	−1.096	−1.067
144953871	SELENOF	Selenoprotein F	0.015	NS	−0.896	−1.080
40311084	CSN3	Kappa-casein	0.005	NS	−1.020	−1.108
939231248	PAEP	Beta-lactoglobulin	0.009	NS	−1.484	−1.304
1486942190	CSN1S2	Alpha-S2-casein	0.005	NS	−1.179	−1.453
860883	THBS1	Thrombospondin	0.005	NS	−0.963	−1.522

(IGH, IGHE, IGG). There were 4 proteins which showed an increase in subclinical and a further increase in clinical mastitis, being serum amyloid A1 and A3 (SAA1, SAA3) and immunoglobulin heavy chains (IGHA, IGHD). There were 5 proteins that had no increase in subclinical but were increased in clinical mastitis among which were haptoglobin (HP) with the highest FC of 2.445, serum amyloid A (SAA3) and lipopolysaccharide binding protein (LBP). There were a number of proteins ($n = 9$) which showed an increase in serum from cows with subclinical mastitis, but which had a significantly lower abundance in serum from cases of clinical mastitis than those with subclinical mastitis. These included selenoprotein (SELENOP), CD59 molecule (CD59), complement C3 (C3) and complement C4 binding protein (C4BPA).

Pathway analysis of the results for the serum proteome are shown in Supplementary Table S3 and Fig. 4 and indicated that complement and coagulation cascades and cholesterol metabolism were among pathways affected by mastitis leading to change in serum proteins. Gene ontology analysis (Supplementary Table S4) revealed that grouping of genes identified for differentially abundant serum proteins were similar to milk proteins in the relevant categories. Thus, for molecular function the largest percentage of genes of serum proteins had binding or catalytic activities, cellular, metabolic and regulation were the most common biological process, while cellular anatomical entities were the most common in cellular component category. Regarding protein class, serum proteins were most abundant for protein-binding activity modulator class.

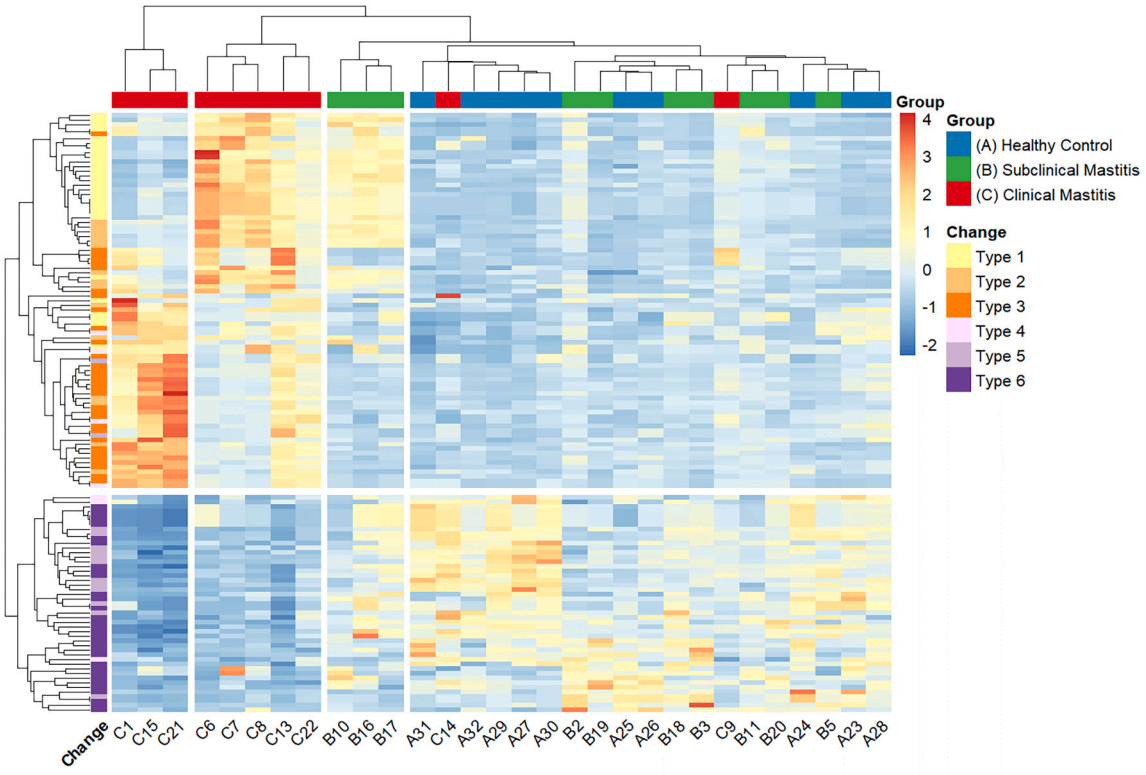

Fig. 1. Heatmap of all milk proteins (groups abbreviation: A – control group; B – subclinical mastitis; C – clinical mastitis). The heat map for milk proteins changing in each type of change listed in Table 2 (Type 1-Type 6) is shown in Supplementary Fig. S3. Dairy cow identification numbers are given at the base of the heat map and refer to the sample numbers given in Table 1.

3.4. Milk and serum proteomes compared

There were 38 proteins which were differentially abundant in both milk and serum (Table 5) and these proteins fell into four types of change, with 11 proteins that were increased in abundance in both milk and serum and 8 proteins that had lower abundance in both milk and serum from cows with mastitis than in healthy cows. A further 17 proteins had a higher abundance in milk but a lower abundance in serum from cows with mastitis while 2 proteins decreased in milk but increased in serum from affected cows.

The changes in abundance in milk and serum, when in the same direction were not always consistent in significant change with severity of the mastitis. Thus, HP had higher abundance in milk from both subclinical and clinical cases while in serum only in clinical cases of mastitis was an increase in abundance observed. In contrast, ORM1 was increased in serum from both subclinical and clinical mastitis cases but was only increased in abundance in milk from cows with clinical mastitis. Other proteins which were increased in both milk and serum were SAA1 and SAA3, serum albumin (ALB) and transferrin (TF). Proteins reduced in abundance in milk and serum included glycoprotein III (CLU), CSN2, CSN1S1 and α_2-glycoprotein1 (AZGP1). Among the proteins which were increased in abundance in milk but decreased in serum were α2-macroglobulin (A2M), α_1-microglobulin/bikunin precursor (AMBP), amine oxidase (AOC3), APOA2, ITIH2, complement factor B (CFB), PGLYRP and RBP.

Pathway analysis incorporating changes in both milk and serum identified only one pathway, the complement and coagulation cascades (Supplementary Table S3, Supplementary Fig. S5) being affected in relation to both biofluids. The interconnection between proteins increasing and decreasing in milk and serum is illustrated in the Venn diagrams (Supplementary Fig. S6) indicating the patterns of change in the milk and serum from the dairy cows in subclinical and clinical mastitis.

3.5. Validation

The relative abundance, shown by TMT proteomics of HP in milk from healthy cows and those with subclinical or clinical mastitis, are shown in Supplementary Table S5 and Supplementary Fig. S7 (a and b) along with the concentrations of HP determined by immunoassay for milk and by the haemoglobin binding assay for serum HP. For all methods of analysis there were significant increases in HP in subclinical compared to healthy with further reduced P value in comparison of clinical to healthy milk. However, while there was not a significant difference for abundance of HP between milk from subclinical compared to clinical mastitis, as determined by TMT proteomics, with biochemical or immuno-assays a significant increase was evident in the clinical samples compared to subclinical. Overall samples the HP abundance was significantly correlated to the concentration determined by assay in milk ($r = 0.897$, $P < 0.001$, $n = 30$) serum ($r = 0.553$, $P = 0.002$) as shown in Supplementary Fig. S8 (a and b).

For SAA in milk and serum the median and range of abundances for the healthy, subclinical and clinical are shown in Supplementary Table S5 and Supplementary Fig. S7 (c and d) with correlations of $r = 0.479$, $P = 0.038$ for milk and $r = 0.657$, $P < 0.001$ for serum between the methods used (Supplementary Fig. S8 c and d). For ORM1 (ORM1) in milk and serum the median and range abundances for healthy, subclinical and clinical cases are shown in Supplementary Table S5 and Supplementary Fig. S7 (e and f) with correlations of $r = 0.68$, $P < 0.001$ for milk and $r = 0.484$, $P = 0.007$ for serum between the methods used (Supplementary Fig. S7 e and f).

4. Discussion

4.1. Overview of findings

Microbiological findings in this study were in accordance with the

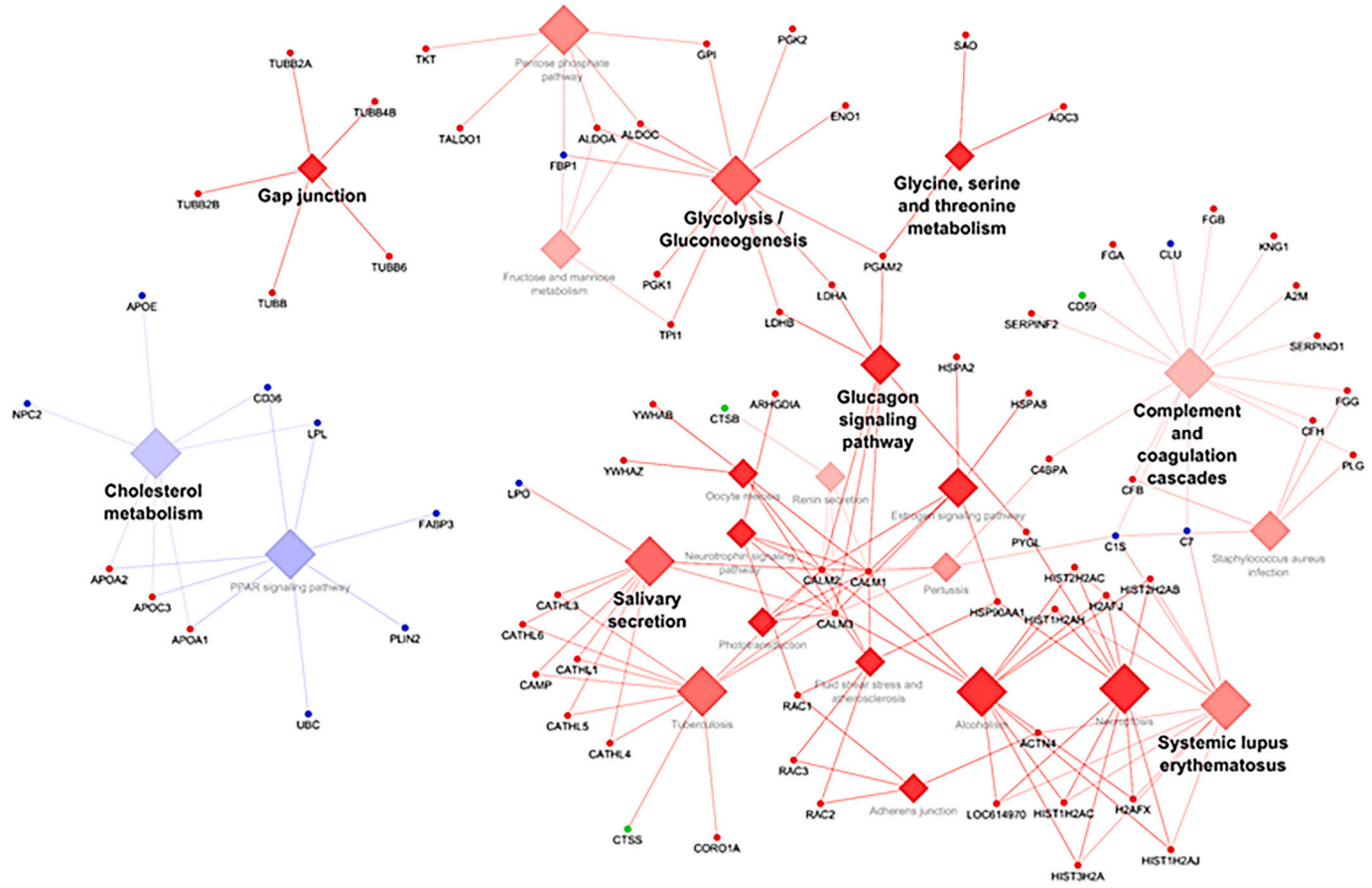

Fig. 2. Interactions of milk proteins with their enriched pathways.

observed clinical feature, that is the type of mastitis. Coliform bacteria such as *E. coli* and *Klebsiella* are environmental pathogens often associated with clinical mastitis. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is released from the bacteria following cell death and during multiplication. LPS is responsible for most pathophysiological reactions and clinical feature in *E. coli* mastitis. After elimination of the infection, signs of inflammation, such as increased milk SCC, can persist in the infected quarter for several weeks [42]. *Streptococcus uberis*, another environment-associated pathogen, was associated mainly with subclinical mastitis in our study. Cows acquire an infection with *S. uberis* mainly during the dry period and infections are manifested as acute mastitis, usually during the subsequent lactation [43]. However, in most cases of subclinical mastitis no pathogen was isolated, suggesting that changes in environmental conditions, poor management practices, or stressful conditions might have caused an increase of somatic cell count and further affected inflammation of the mammary gland [44].

Considering that the milk and serum samples were collected from natural cases of mastitis, cows may be represented at different stages of progression of subclinical and clinical mastitis. Furthermore, different pathogens (Table 1) were identified as causative agents and as the cows were from different farms with differences in environmental conditions these factors could be considered as a limitation of the study but only if the objective was to determine responses of the mammary gland to a particular pathogen. However, as an objective of the study was to identify biomarkers that could be used as diagnostic methods for detection and monitoring of mastitis on farms, it is important that changes in protein abundance should be applicable to a wider range of cases than if the study had been restricted to a single bacterial species being the causative pathogen. Nevertheless, it would in the future be valuable to assess change to the proteomes of milk and serum caused by different bacteria to determine if different patterns of biomarker change could be related to the pathogen causing the mastitis.

The proteomic analysis of milk from dairy cows with mastitis has been the subject of several reports, going back to the use of two dimensional gel electrophoresis through to the present day and the introduction of gel-free quantitative proteomics methods [20,21,45–49]. While there have been reports of alterations in serum proteins during mastitis, there have been few investigations that used the proteomic approach in both biofluids [24,25,50]. In this investigation, milk and serum samples from healthy dairy cows and those with either subclinical mastitis or clinical mastitis, have been subjected to quantitative proteomics. Changes in specific protein abundances in both biofluids have been compared based on the severity of the disease. This has led to fascinating insights into the protein changes in milk and serum, being stimulated during the differing severities of mastitis. For instance, some proteins such as HP and SAA1 were increased in abundance in milk in subclinical mastitis but did not show further elevations in milk from cows with clinical mastitis when measured by TMT quantitative proteomics. Other proteins such as CATHL3 were increased in subclinical and showed a further increase in clinical mastitis. Furthermore, HP increased in both biofluids, B2M decreased in both biofluids, A2M increased in milk and decreased in serum and CD59 decreased in milk but increased in serum.

Overall, of 237 proteins with significant differences in abundances between the healthy, subclinical and clinical mastitis groups in the milk proteome, the majority (153 proteins) showed some type of higher abundance in subclinical and clinical groups compared to healthy cows (change types 1–3). An opposite trend was found in the serum proteome, with most of the proteins (78 of 117) showing lower abundance in the subclinical and clinical groups compared to healthy group (change types 4–6). The increased abundance of proteins in milk could be explained by

Table 4
Changes in serum proteins in main categories of change listing up to the 8 proteins most increased or decreased in the category; groups abbreviation: H – control group; SC – subclinical mastitis; C – clinical mastitis. For proteins showed to be significantly differentially abundant after P-value correction (FDR < 0.05), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 [31]. Fold change between two groups was calculated as for example mean (SC)/ mean(H) and expressed on log2 scale. NS = not significant.

Accession Number	Gene	Description	FDR	log2 fold change		
				SC vs H	C vs SC	C vs H
Type 1 change: increase in SC and no further increase in C (n = 13)						
253723167	F10	Coagulation factor X	0.014	1.684	NS	1.142
229505	N/A	Factor X1 L	0.014	1.684	NS	1.142
163054	F9	Factor IX (Christmas factor)	0.015	0.886	NS	0.647
121957959	ORM1	Alpha-1-acid glycoprotein	0.014	0.724	NS	0.780
1000346	IGH	Immunoglobulin heavy chain variable region	0.022	0.621	NS	0.495
1059270845	N/A	Bovine Fab A01 heavy chain	0.027	0.616	NS	0.524
509264755	IGHE	Immunoglobulin epsilon heavy chain variable region	0.017	0.603	NS	0.898
89611	IGG	Ig gamma-2 chain C region, bovine	0.034	0.533	NS	0.620
Type 2 change: increase in SC and further increase in C (n = 4)						
620597439	SAA3	Serum amyloid A3	0.012	1.012	0.645	1.657
296471870	SAA1	Serum amyloid A protein	0.011	1.029	0.636	1.665
509264888	IGHA	Immunoglobulin alpha heavy chain variable region	0.014	0.667	0.195	0.861
509264513	IGHD	Immunoglobulin delta heavy chain variable region	0.014	0.667	0.195	0.861
Type 3 change: no change in SC and increase in C (*n* = 5)						
2144490	HP	Haptoglobin, bovine	0.029	NS	1.853	2.445
8574568	SAA3	Serum amyloid A protein	0.012	NS	1.280	1.553
14139702	N/A	Unnamed protein product	0.022	NS	0.787	1.534
108860784	LBP	Lipopolysaccharide-binding protein	0.025	NS	0.920	0.755
1059270816	N/A	Fab F08_B11 heavy chain	0.022	NS	0.785	0.749
Type 4 change: decrease in SC and no further decrease in C (n = 48)						
15866746	FN1	Fibronectin	0.008	−0.711	NS	−0.723
1387191381	IL1RAP	Interleukin-1 receptor accessory protein	0.025	−0.725	NS	−0.942
1387239638	C4A	Complement C4-A	0.024	−0.738	NS	−0.536
508834	IGHV	Anti-respiratory syncytial virus Ig heavy chain V region	0.025	−0.767	NS	−0.575
119388700	CSN2	Beta-casein	0.020	−0.846	NS	−0.426
1387203538	PLTP	Phospholipid transfer protein	0.021	−0.866	NS	−0.662
74267870	TTR	Transthyretin	0.011	−0.876	NS	−0.898
975864	VL1X	Immunoglobulin lambda light chain variable region	0.029	−1.709	NS	−1.542
Type 5 change: decrease in SC and further decrease in C (n = 24)						
1405680571	F13A1	Coagulation factor XIII A chain	0.017	−0.383	−0.232	−0.616
114050845	CPB2	Carboxypeptidase B2	0.006	−0.435	−0.437	−0.872
86438060	C1QB	Complement component 1, q subcomponent, B chain	0.019	−0.465	−0.336	−0.801
296486107	PGLYRP2	Peptidoglycan recognition protein L	0.006	−0.494	−0.150	−0.644
109940161	SERPINC1	Antithrombin-III	0.006	−0.521	−0.107	−0.628
296474790	ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	0.006	−0.523	−0.085	−0.608
1387235265	ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3	0.006	−0.560	−0.083	−0.642
1387201758	CPB2	Carboxypeptidase B2	0.029	−0.806	−0.417	−1.224
Type 6 change: no change in SC and decrease in C (n = 6)						
810213776	C3	Complement component 3	0.025	NS	−0.147	−0.198
296475776	C6	Complement component C6	0.037	NS	−0.158	−0.203
28189883	LDHB	Similar to lactate dehydrogenase B	0.037	NS	−0.143	−0.253
296484314	GSN	Gelsolin A	0.006	NS	−0.345	−0.359
157831280	RBP4	Retinol binding protein	0.019	NS	−0.279	−0.422
509264464	IGHD	Immunoglobulin delta heavy chain variable region	0.038	NS	−0.306	−0.456

a mix of local synthesis in the mammary gland and by extravasation of blood proteins to the mammary gland through blood-mammary gland barrier. The overall decrease in abundance of a number of serum proteins could be caused by consumption during systematic inflammatory response in a negative APP response and/or leakage and transfer of proteins to the mammary gland.

Gene ontology analysis gave similar patterns of changes in genes for the proteins of milk and serum for molecular function, biological process, and cellular component. This is likely to be due to the primary function of milk and serum in secretion of metabolites for growth and maintenance along with protection from environmental pathogens. Of relevance is that enzymic activity for metabolite interconversion, binding activities along with cytoskeletal proteins were among the functions of genes identified in both milk and serum, indicating the importance of metabolic change and transport functions in both, but also that cellular turnover in cytoskeleton is a feature of the response to mastitis.

4.2. Changes in the milk proteome during mastitis

The pathogenesis of mastitis involves a complex set of interactions between an invading pathogen and the immune systems of the host. Taking into account the complex nature and the high dynamic range of the milk proteome, a quantitative proteomic approach and the associated bioinformatics analysis, are considered as complementary tools for the comprehensive study of the dynamic interactions between the immune system and pathogens.

It has been shown by several groups that the milk proteome undergoes considerable modification during bovine mastitis. The findings here are in general agreement with previous studies with many of the protein responses to the infection of the mammary gland having been previously described [20,46]. For example HP, SAA1, DEFB10, SERPINB3 had been previously observed to increase in milk from cows with mastitis [51]. However it was found here that these proteins were increased in subclinical mastitis relative to healthy cow milk, but the

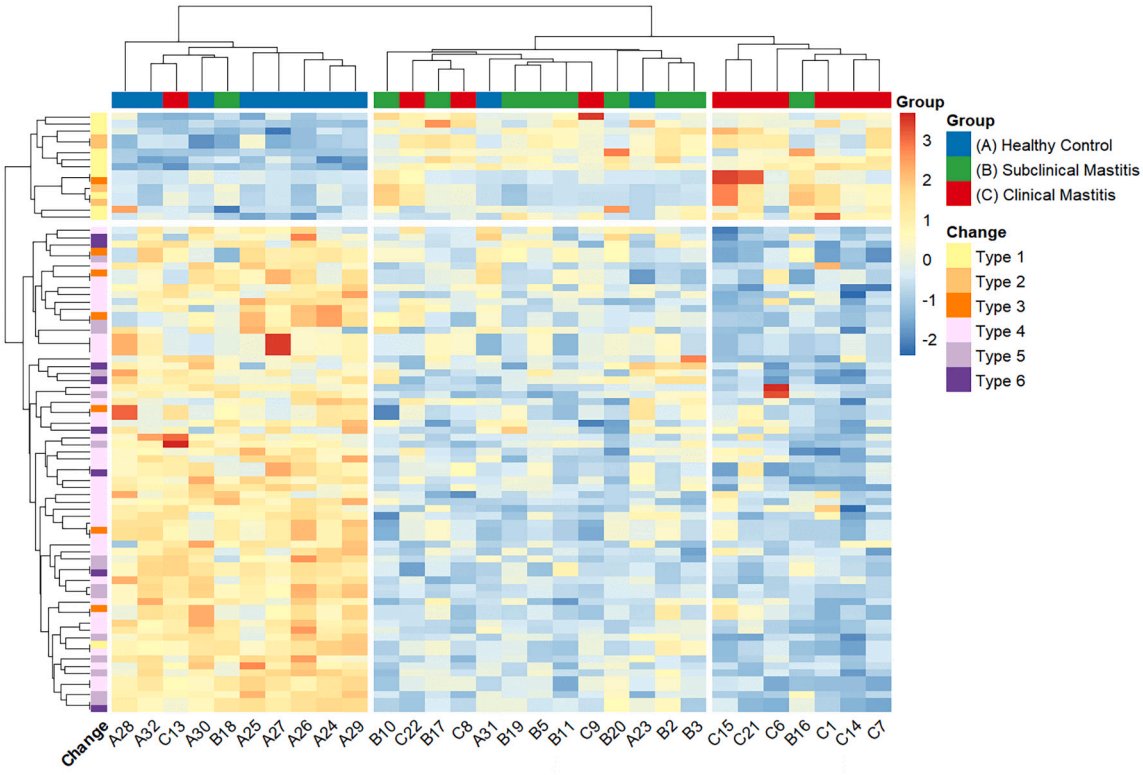

Fig. 3. Heatmap of all serum proteins (groups abbreviation: A – control group; B – subclinical mastitis; C – clinical mastitis). The heat map for serum proteins changing in each type of change listed in Table 2 (Type 1-Type 6) is shown in Supplementary Fig. S4. Dairy cow identification numbers are given at the base of the heat map and refer to the sample numbers given in Table 1.

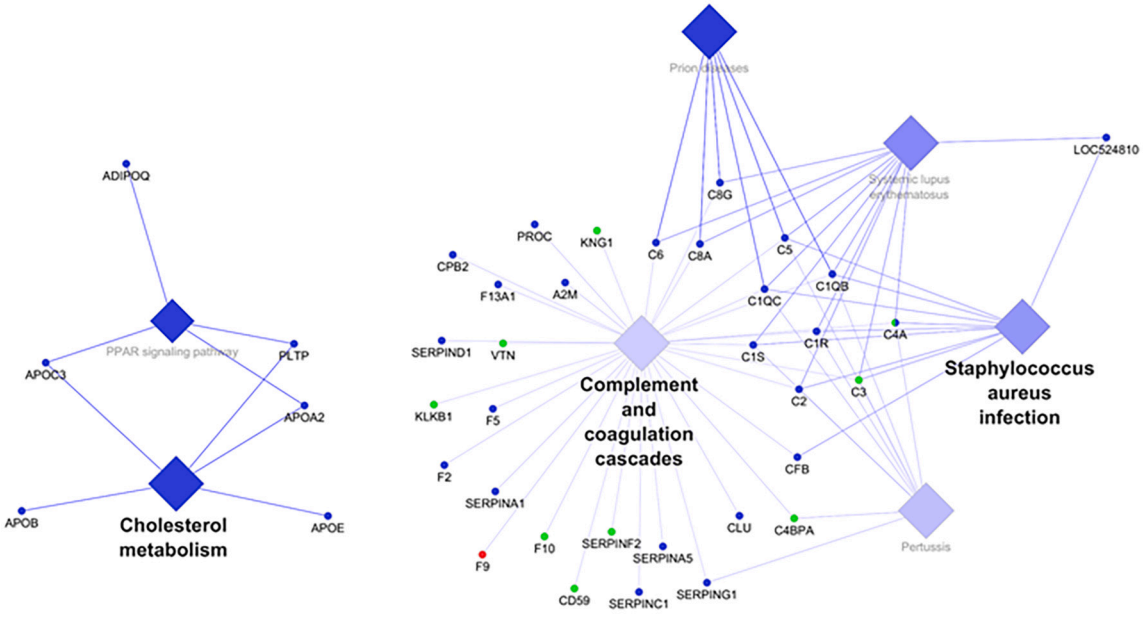

Fig. 4. Interactions of serum proteins with their enriched pathways.

mean concentration was not significantly higher in milk from cows with clinical mastitis. Potentially the production of these proteins reaches a plateau which is not increased in the clinical disease, either by reaching a limit of synthesis or they are removed from the milk at a rate that is equivalent to synthesis during the clinical stage of the disease. The mean abundance of other milk proteins such as A2M (variant 18), PZP, ACTR2, CATHL3, and CNN2 showed a further increase in clinical mastitis, compared to their abundance in the subclinical group. These proteins showed an association with the disease severity which may be of value in monitoring their concentration as an indicator of a subclinical case approaching the clinical level of disease. A third category of proteins increasing with mastitis, were those that were not increased by subclinical mastitis but were elevated in cases with the clinical disease. Among these was A2M (variant 19) and CATHL-1 both of which had

Table 5
Proteins changing in milk and serum (groups abbreviation: H – control group; SC – subclinical mastitis; C – clinical mastitis). NS = npt significant.

Accession Number	Gene	Description	Sample	log2 fold change		
				SC vs H	C vs SC	C vs H
Proteins increased in both milk and serum (n = 11)						
74267962	ALB	Serum albumin	milk	NS	1.081	1.066
1351907			serum	0.442	NS	0.52
528979082	C4BPA	Complement component 4 binding protein, alpha chain	milk	NS	0.587	0.682
146231832			serum	0.312	−0.147	0.164
2144490	HP	Haptoglobin, bovine	milk	1.484	NS	2.128
2144490			serum	NS	1.853	2.445
509264436	IGHD	Immunoglobulin delta heavy chain variable region	milk	NS	NS	0.751
509264513			serum	0.667	0.195	0.861
1150079692	IGHG	Immunoglobulin gamma heavy chain	milk	0.371	1.145	1.516
509264677			serum	0.491	NS	0.693
15088675	IGLL	Immunoglobulin lambda light chain	milk	0.252	NS	0.452
1276609			serum	0.604	−0.246	0.359
164450481	KNG1	Kininogen-2	milk	NS	0.782	0.968
164450481			serum	0.367	−0.168	0.198
159895416	ORM1	Alpha-1-acid glycoprotein	milk	NS	0.818	0.624
121957959			serum	0.724	NS	0.78
296471870	SAA1	Serum amyloid A1	milk	1.236	NS	2.069
296471870			serum	1.029	0.636	1.665
1387253120	SAA3	Serum amyloid A3	milk	1.236	NS	2.069
620597439			serum	1.012	0.645	1.657
113911795	TF	Transferrin	milk	NS	0.766	0.855
602117			serum	0.418	NS	0.349
Proteins decreased in both milk and serum (n = 8)						
312893	APOE	Apolipoprotein E	milk	NS	−1.328	−0.99
1387217499			serum	−0.515	NS	−0.574
73586954	AZGP1	Alpha-2-glycoprotein 1, zinc-binding	milk	NS	−0.476	−0.526
73586954			serum	−0.308	−0.262	−0.57
550544591	B2M	Beta-2-microglobulin	milk	NS	−0.65	−0.689
1387193714			serum	−0.464	0.315	−0.149
29,487133	C1S	Complement C1s subcomponent	milk	NS	−0.663	−0.498
296487133			serum	−0.372	NS	−0.326
163115	CLU	Glycoprotein III precursor, clusterin	milk	NS	−1.096	−1.067
163115			serum	−0.31	−0.324	−0.634
982928498	CSN1S1	Alpha-S1-casein	milk	−0.355	−1.076	−1.43
1387271774			serum	−0.589	NS	−0.481
1391916210	CSN2	Beta-caesin	milk	NS	−0.942	−1.007
119388700			serum	−0.846	NS	−0.426
2323398	IGL	Immunoglobulin light chain variable region	milk	−0.483	NS	−0.777
1220359011			serum	−0.359	−0.271	−0.63
Proteins increased in milk and decreased in serum (n = 17)						
408689601	A2M	Alpha-2-macroglobulin variant 19	milk	NS	1.628	1.95
408689581			serum	−0.251	NS	−0.215
27806743	AMBP	Alpha-1-microglobulin/bikunin precursor (AMBP) protein	milk	−0.146	0.737	0.591
74354219			serum	0.138	−0.262	−0.124
61554,88	AOC3	Copper amine oxidase, liver isozyme	milk	NS	1.085	1.12
61680008			serum	−0.281	NS	−0.312
114052298	APOA2	Apolipoprotein A-II	milk	NS	1.123	1.471
114052298			serum	−0.292	−0.128	−0.421
86438511	APOF	Apolipoprotein F	milk	NS	0.962	0.94
528949675			serum	−0.29	−0.508	−0.798
146345391	CFB	Complement factor B	milk	0.263	NS	0.505
146345391			serum	−0.187	NS	−0.26
81294264	CP	Ceruloplasmin	milk	NS	0.764	0.91
81294264			serum	−0.308	NS	−0.232
13358874	HRG	Histidine-rich glycoprotein	milk	NS	0.828	0.836
13358874			serum	−0.196	−0.217	−0.413
146186952	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	milk	NS	0.827	0.78
146186952			serum	−0.221	NS	−0.247
8979739	LDHB	Lactate dehydrogenase B	milk	0.664	NS	0.809
28189883			serum	NS	−0.143	−0.253
201067368	PGLYRP1	Peptidoglycan recognition protein 1	milk	0.631	0.682	1.313
296486107			serum	−0.494	−0.15	−0.644
415585	RBP4	Retinol binding protein	milk	NS	0.502	0.556
157831280			serum	NS	−0.279	−0.422
520785	SAO	Serum amine oxidase	milk	NS	0.857	0.895
520785			serum	−0.273	NS	−0.307
226373739	SERPINA3–6	Serpin A3–6	milk	NS	1.254	1.306
226373739			serum	−0.351	0.238	NS
157280001	SERPIND1	Heparin cofactor 2	milk	NS	0.599	0.685
528989611			serum	−0.281	−0.096	−0.377
138,222720	SERPINF2	Alpha-2-antiplasmin	milk	NS	0.737	0.669

(*continued on next page*)

Table 5 (*continued*)

Accession Number	Gene	Description	Sample	log2 fold change		
				SC vs H	C vs SC	C vs H
1387222723			serum	−0.517	0.097	−0.42
4680177	VL1X	Immunoglobin light chain VJ region	milk	0.573	NS	1.034
975864			serum	−1.709	NS	−1.542
Proteins decreased in milk and increased in serum (n = 2)						
79157011	CD59	CD59 molecule, complement regulatory protein	milk	0.144	−0.46	−0.316
79157011			serum	0.948	−0.348	0.6
34538,98	IGH	immunoglobulin heavy chain constant region	milk	NS	−0.418	−0.347
1000346			serum	0.621	NS	0.495

isoforms in the previous category (increase in subclinical, further increase in clinical) which may mean that immunoassays would not be able to differentiate these types of response. CNN2 along with apolipoproteins APOA1 and APOA2 were also proteins which were not raised in subclinical but were elevated in milk from cows with clinical mastitis. The presence of these proteins only in milk from cows with clinical mastitis, is presumably linked to their origin, whether it is by release from mammary epithelium and the somatic cells, transfer from the blood through the mammary blood barrier or breakdown of tissue during the inflammatory reaction of the innate immune response to the invading bacteria.

Although proteins that increase in concentration in milk during mastitis have had more attention, especially as possible biomarkers of the disease, there were significant decreases in abundance of a number of milk proteins. It is well established that the major proteins of milk decrease in concentration during mastitis [52] and it was observed here that variants of β-lactoglobulin (PAEP) were decreased in milk from cows with mastitis, where a fall was found in both subclinical and clinical or only in the clinical mastitis group. Other proteins that were reduced in milk from the cows with mastitis included MUC15 and MUC1 indicative of a problem with the mammary epithelia which would be a normal location for their production and BTN1A1, a component of the milk fat globule membrane, showing a further consequence of abnormal milk production.

The changes in the milk proteome found in this investigation are consistent with the alterations in milk described previously [46,53,54]. Alterations in protein abundance seen when comparing subclinical to clinical mastitis have been investigated previously [20,48] and are also consistent with the results shown here although there are some differences in the proteins identified. In comparison to the findings of Maity et al. [20] these differences are likely to be related to sample sources, sample preparation and the MS used for the investigations. However, the main findings of increased changes in milk proteome going from healthy milk to subclinical to clinical are similar. The finding that some proteins are raised to similar levels in subclinical and clinical groups while other proteins show a further increase in clinical while a third group of proteins are only raised in clinical mastitis is a valuable contribution to the pathophysiology of the mastitis proteome and may indicate that measuring different proteins as potential biomarkers of the disease may provide insights into the progression and severity of the disease.

Pathway enrichment analysis has shown that the mastitis has affected diverse pathways of the milk proteome including PPAR signalling pathway, cholesterol metabolism and complement and coagulation cascades. These pathways are in concordance with pathogenesis of mastitis requiring appropriate coordination of multiple signalling pathways triggered by innate immune recognition of pathogen molecules, initiating an inflammatory reaction and host defence response. The disparate source of the proteins used to determine pathway analysis, as the proteins may be secreted from mammary epithelia, somatic cells in the milk, deriving from blood neutrophils and from leakage of serum proteins through the mammary blood barrier, complicates the interpretation of pathway analysis but it is valuable to know that the host response reactions have been activated by the disease.

The immunoassay by SPARCL of HP in milk showed that there was an increase in the amount of HP from the low, virtually undetectable levels in milk from healthy cows, increasing in cows with mastitis [55–57]. The highest levels were found in the milk from cows with clinical mastitis, however this was not a significant difference to that in subclinical mastitis according to the TMT proteomic results but was significantly different by immunoassay. Immunoassay using SPARCL for M-SAA3, showed increases in the concentration from healthy to subclinical and clinical mastitis and, while proteomics showed significant difference between healthy and clinical mastitis groups, immunoassay determination also revealed significant difference between subclinical and healthy group. Significant difference in abundance for ORM1 was detected only by proteomics. These results along with the correlations between results of the abundance of the proteins determined by TMT proteomics and by SPARCL immunoassay provide validity for the proteomics analysis of milk.

4.3. *Changes in the serum proteome during mastitis*

A lower number of serum proteins showed differential abundance compared to proteins in milk, which would be expected with the local site of infection being in the mammary gland. Serum proteins of Type 1, which increased in the subclinical group, but did not show further increases in the clinical group, were generally host defence proteins, being involved in coagulation, antibody and acute phase responses, examples being coagulation factor X (F10), Ig heavy chain variable region (IGH) and ORM1. Only a few proteins showed an increase in subclinical and then a further increase in clinical and were either SAA isoforms (SAA1, SAA3) or Ig heavy chain related (IGHA, IGHD). Acute phase proteins were prominent among those serum proteins that were not elevated in subclinical but were significantly increased in samples from the clinical cases, notably haptoglobin (HP), liposaccharide binding protein (LBP) and a further form of SAA (SAA3). These changes are consistent with observations of serum proteome during mastitis that have previously been described [49,58].

Serum proteins that decreased in the subclinical group but showed no further change in the clinical group included fibronectin (FN1), transport proteins such as transthyretin (TTR) and phospholipid transfer protein (PLTP) as well as some host defence proteins such as IL-1 rector (IL1RAP) and Ig-λ (VL1X). The milk protein, β-casein (CSN2) was found in this group, suggesting that in serum from healthy cows it is present in serum probably by a low level leakage from the mammary gland. Host defence protein were also in the group that decreased in subclinical and with a further decrease in clinical mastitis, such as coagulation factor XIII (F13A1), complement component 1 (C1QB) and peptidoglycan recognition protein L (PGLYRP2). Among these proteins are also members of serpin (serine protease inhibitors) family such as antithrombin III, inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1) and inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) with functions to prevent excess activation of proteases and limit the potential injurious actions of protease activation on endothelial and epithelial tissues [59]. Their gradual decrease with severity of mastitis and subsequent increase of inflammation suggest a possibility of transfer across the mammary

blood barrier or other form of consumption in responding to the mastitis causing pathogens.

Proteins of host response, including complement C3 and C6 (C3, C6) and Ig Δ heavy chain (IGHD) were found to decrease in clinical mastitis in serum but not significantly in subclinical mastitis. Gelsolin, the actin-scavenging protein, also showed lower abundance only in clinical mastitis group. Actins are released into the systemic circulation after disruption of the cell membrane as a result of necrosis and were found in milk of subclinical mastitis group in higher abundance compared to healthy animals. During the scavenging process, actin–gelsolin complexes are formed and subsequently cleared by the reticuloendothelial system, resulting in consumption of gelsolin [60]. In different diseases, decline of gelsolin precedes, and therefore might predict, tissue and organ injury, as well as being a predictor of complications associated with actin scavenging and anti-inflammatory features of gelsolin [61].

Pathway analysis from the differentially abundant serum proteins identified the most altered pathways due to mastitis, such as complement and coagulation cascade, PPAR signalling pathway and cholesterol metabolism. Inflammation and haemostasis are tightly interrelated pathophysiologic processes that may affect each other considerably, as evidenced in this study by many of the proteins belonging to coagulation, fibrinolysis and complement pathways. Pathway interaction analysis of serum proteome has affected similar diverse pathways as in the milk proteome indicating that the host immune reaction and defence is reflected both locally, in the mammary gland, and systemically.

The findings on serum proteome changes during mastitis and the effect of increasing the severity of the condition from subclinical to clinical are valuable findings of this investigation. While it has been established that there are changes in the serum proteins during this condition, especially in the APP [8,62] the range of proteins and the modulation in their abundance patterns is providing further insights into the pathophysiology of mastitis. While the increase in the abundance of host defence protein in serum APP such as HP, SAA and ORM1, was to be expected as there would be a cytokine driven innate immune reaction to the presence of pathogen or induced by stress, one of major findings was that the abundance of a number of proteins decreased in serum during mastitis. This could be due to transfer to the mammary gland where the front line of defence against infection is taking place. Whether this would be by diffusion through weakened tight junction, which also allows the transfer of neutrophils from blood to the mammary gland, or whether there is a facilitated transfer would need to be examined in the future. Additionally, increased utilisation without replacement by synthesis during systematic inflammatory response could also contribute to decreased abundance of some serum proteins.

The immunoassay by SPARCL of HP in serum showed that there was an increase in the amount of HP in the clinical mastitis group compared to subclinical group, however this was not a significant difference according to the proteomics. This would be expected with the SPARCL assay, being of greater accuracy than the TMT quantitative proteomics used here. Immunoassay using SPARCL for M-SAA3, showed increases in the concentration from healthy to subclinical to clinical, while the TMT proteomics only showed significant difference between clinical and subclinical group and between clinical mastitis and healthy group. These results along with the correlations between results of the abundance of the proteins determined by TMT proteomics and by SPARCL immunoassay provide validity for the proteomics analysis of milk.

4.4. Relation of changes in the milk proteome and serum proteome during mastitis

There have been few previous studies that have simultaneously examined change in serum and milk proteins during mastitis. Early investigations of the changes in the proteomes of these biofluids in mastitis have used 2-dimension electrophoresis and have found similar results in the changes in the high abundant proteins as found in this investigation [58]. The use here of TMT quantitative proteomics enabled a deeper view of change in the lower as well high abundant proteins allowing valuable insights into the host reactions to mastitis. Among the proteins ($n = 11$), that were increased in abundance in both milk and serum most had host defence functions including APP such as HP, SAA, and ORM1, along with transferrin (TF), Ig Δ and γ heavy chain (IGHD, IGHG) and albumin (ALB). Many of the proteins ($n = 8$) that were reduced in abundance in both milk and serum included Ig light chain (IGL) and β-2 microglobulin (B2M) can also be related to host responses and their reduction in abundance may be related to their being used for functional roles in the host defence and not being replaced by synthesis at a high enough rate. The milk proteins β-casein (CSN2) and a-s1 casein (CSN1S1) were also reduced in abundance in serum as well as in milk, which would be explained by the reduction of their synthesis in milk during mastitis and a concomitant decrease in the serum as less would be transferred from the mammary gland to blood which has been shown here to occur in serum from healthy cows.

Of great interest was the finding that proteins ($n = 17$) were found that had an increased abundance in milk but a reduced abundance in serum. Among these were proteins involved in host defence such as α_2-macroglobulin (A2M), α_1-microglobulin/bikunin protein (AMBP), proteins involved in transport such as apolipoproteins AII and F (APOA2, APOF) and retinol binding protein (RBP4). An explanation for the disparity in abundance change that occurred with the development of mastitis is that these proteins are required in the mammary gland in the host response to the pathogens causing the mastitis and they are transferred from the blood, thereby increasing the abundance in the milk but reducing their abundance in serum with their systemic synthesis rates not being sufficient to maintain normal levels in the blood. Changes in the abundance levels of these proteins and modulation of their abundance patterns could act as biomarkers of the host response to predict infection of mammary gland. If these proteins have a value in combatting the mastitis causing pathogens, their further investigation could open up potential therapies based on their action which could be used without resorting to antimicrobials known to develop resistance.

5. Conclusions

The infection of the mammary gland that causes bovine mastitis leads to significant changes in the proteome, both of milk and of the serum of the affected dairy cow. Identification of protein profiles in the bovine milk and serum is a comprehensive and valuable strategy for discovery of potential biomarkers to be used in diagnostics of mastitis, disease monitoring and therapeutic possibilities. Alteration of abundance patterns of milk and serum proteins, together with pathway analysis revealing multiple interactions related to the proteins affected by mastitis, provide a valuable contribution to understanding of mastitis pathophysiology and serve as a valuable tool in the development of new diagnostic methods.

Ethics

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Zagreb, Croatia (Reference number 25161-01/139-20-26).

Author contributions

Conceptualization and study design: R.T., P.D.E, V.M.; investigation: R.T., J.K., A.H., C.C. methodology: R.T., J.K., A.H., A.Ga, A.Ge., C.C., B. B.; formal analysis: A.Ge, J.K., A.H.; data curation: A.H., P.D.E.; validation: A.C, B.B., C.C.; visualization: A.Ge, A.Ga., J.K.; supervision; P.D. E., R.T., V.M., funding acquisition and resources: R.T., P.D.E., V.M.; writing - original draft: J.K., A.H., B.B., C.C., A.Ge. P.D.E.; writing - review & editing: J.K., A.H., A.Ge, A.Ga., C.C., V.M., P.D.E.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest, except for C.C.C. of Life Diagnostics Inc., the manufacturer of the SPARCL protein immunoassays.

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Supplementary data

The LC-MS/MS spectra from the healthy and bovine mastitis groups of milk and serum have been archived by submitting to PRIDE archive via ProteomeXchange with the dataset identifier PXD022595. Additionally, the full list of proteins identified in the bovine mastitis milk and serum samples are provided in the supplementary data, Tables S1 and S2 respectively. Table S3 lists Significantly enriched KEGG pathways, Table S4 shoes Gene Ontology for the differentially abundant proteins and Table S5 gives the HP, SAA and AGP (ORM1) abundance by TMT proteomics and SPARCL immunoassay in milk and serum. Supplementary figures show in Fig. S1: Changes in the milk proteome in subclinical and clinical mastitis compared to healthy milk. Fig. S2 Changes in the serum proteome in subclinical and clinical mastitis compared to the serum proteome of healthy cows. Fig. S3: Significantly enriched KEGG pathways from the differentially abundant proteins among three groups mastitis from milk and serum samples Fig. S5: HP, SAA and AGP (ORM1) abundance by TMT proteomics and SPARCL immunoassay in milk and serum. Fig. S5 Interactions of milk and serum proteins with their enriched pathways; Fig. S6 Venn diagram of significantly increased and decreased proteins in milk and serum. Fig. S7 Boxplots between H, SC and C for haptoglobin (HP), serum amyloid A (SAA1) and 1 acid glycoprotein (ORM1) abundance for proteomics and SPARCL immunoassay. Fig. S8. Correlations of all samples between proteomics and SPARCL immunoassay for HP, SAA1 and ORM1.

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