Proteomic changes in the milk of water buffaloes (*Bubalus bubalis*) with subclinical mastitis due to intramammary infection by *Staphylococcus aureus* and by non-aureus staphylococci

Salvatore Pisanu 1, Carla Cacciotto 1,6, Daniela Puggioni 2, Sergio Uzzau 1,2, Paolo Ciaramella 3, Jacopo Guccione 3, Martina Penati 4, Claudia Pollera 4, Paolo Moroni 4,5, Valerio Bronzo 4 & Maria Filippa Addis 1,4*

Subclinical mastitis by *Staphylococcus aureus* (SAU) and by non-aureus staphylococci (NAS) is a major issue in the water buffalo. To understand its impact on milk, 6 quarter samples with >3,000,000 cells/mL (3 SAU-positive and 3 NAS-positive) and 6 culture-negative quarter samples with <50,000 cells/mL were investigated by shotgun proteomics and label-free quantitation. A total of 1530 proteins were identified, of which 152 were significantly changed. SAU was more impacting, with 162 vs 127 differential proteins and higher abundance changes (P < 0.0005). The 119 increased proteins had mostly structural (n = 43, 28.29%) or innate immune defence functions (n = 39, 25.66%) and included vimentin, cathelicidins, histones, S100 and neutrophil granule proteins, haptoglobin, and lysozyme. The 33 decreased proteins were mainly involved in lipid metabolism (n = 13, 59.10%) and included butyrophilin, xanthine dehydrogenase/oxidase, and lipid biosynthetic enzymes. The same biological processes were significantly affected also upon STRING analysis. Cathelicidins were the most increased family, as confirmed by western immunoblotting, with a stronger reactivity in SAU mastitis. S100A8 and haptoglobin were also validated by western immunoblotting. In conclusion, we generated a detailed buffalo milk protein dataset and defined the changes occurring in SAU and NAS mastitis, with potential for improving detection (ProteomeXchange identifier PXD012355).

The water buffalo (*Bubalus bubalis*) is the second most important dairy species after the cow (*Bos taurus*). Approximately 15% of the world milk production is from buffaloes and the Asian continent, with a population of about 150 million animals, is the major producer. In Europe, Italy takes the lead with a population of about 400,000 heads (95% of the European population) for 200,000 tons of milk per year. The reason for the increasing interest in buffalo breeding over recent years is the popularity of buffalo Mozzarella cheese (Protected Designation of Origin-P.D.O.) and almost lack of competition in the EU-area for this type of cheese. Mediterranean buffaloes are typically reared in central and southern Italy, and 80% of all Italian buffalo milk production originates from the Campania region. Mediterranean buffalo's milk production is ranked 4th in the Italian agricultural economy concerning sales volume in the entire country (more than 320 M€ and over 15,000 workforces). Buffalo milk is a highly valuable product, being paid at least twice the price of bovine milk, and the European Community has not defined production quotas. Italian buffaloes produce small quantities of milk; the average

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1Porto Conte Ricerche, Alghero, Italy. 2Dipartimento di Scienze Biomediche, Università degli Studi di Sassari, Sassari, Italy. 3Dipartimento di Medicina Veterinaria e Produzioni Animali, Università di Napoli Federico II, Naples, Italy. 4Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy. 5Animal Health Diagnostic Center, Cornell University, Ithaca, NY, USA. 6Present address: Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Sassari, Italy. *email: pagnozzi@portocontericerche.it; filippa.addis@unimi.it
Mastitis Test.

SDS-PAGE patterns of *Staphylococcus*-positive and healthy control milk. The SDS-PAGE analysis carried out on solubilised skim milk proteins before trypsinisation for shotgun analysis anticipated the presence of several major changes related to staphylococcal IMI (Fig. 1). The major protein bands corresponding to lactoferrin, albumin, caseins, alpha-lactalbumin and beta-lactoglobulin were clearly affected. Specifically, lactoferrin and albumin increased in staphylococcus-positive samples, while caseins, alpha-lactalbumin, and beta-lactoglobulin decreased. The appearance of other bands could also be observed, especially at low molecular weight. Alterations were generally more evident in SAU-positive milk (Fig. 1, lanes 1, 2, 3) than in NAS-positive milk (Fig. 1, lanes 4, 5, 6).

### Results

**Animals and milk samples.** To assess the changes induced on the buffalo milk proteome by high-SCC subclinical mastitis due to staphylococcal IMI, 12 quarter milk samples were subjected to comparative proteomic analysis: 6 with SCC >3,000,000 cells/mL, all culture-positive; and 6 with SCC <50,000 cells/mL, all culture-negative. SAU-positive and NAS-positive samples were collected from quarters positive for the California Mastitis Test (CMT) and classified as affected by subclinical mastitis, while all control quarters were CMT-negative and classified as healthy. The quarters belonged to 12 different animals. Sample characteristics are outlined in Table 1.

#### Table 1. Sample groups, milk samples, and their characteristics. **a**SCC: Somatic Cell Count. **b**CMT: California Mastitis Test.

| Sample group | SCCa (cells/mL × 10³) | Bacteriology |
|--------------|-----------------------|--------------|
| Positive     | Mean value 5545 (CMT⁺) | *Staphylococcus aureus* |
| 1            | 4091                  | *Staphylococcus aureus* |
| 2            | 3782                  | *Staphylococcus aureus* |
| 3            | 8440                  | *Staphylococcus aureus* |
| 4            | >10000                | Non-aureus staphylococci |
| 5            | 4035                  | Non-aureus staphylococci |
| 6            | 2924                  | Non-aureus staphylococci |
| Negative     | Mean value 23.5 (CMT⁻) | Negative |
| 7            | 46                    | Negative |
| 8            | 37                    | Negative |
| 9            | 30                    | Negative |
| 10           | 14                    | Negative |
| 11           | 8                     | Negative |
| 12           | 6                     | Negative |

Production in a standard lactation cycle (approximately 270 d) is about 2,000 kg, but milk is characterised by a higher percentage of total solids, including proteins, fat, and minerals, than cow’s milk. In view of its limited productions and high value, one of the costliest diseases is mastitis. Although buffaloes are traditionally considered less susceptible to mastitis than cattle, some researchers have reported similar mastitis frequencies for the 2 species, and the high prevalence of subclinical intramammary infections (IMI) might lead to underestimate the issue. In dairy ruminants, the somatic cell count (SCC) is typically used as an inflammatory indicator to diagnose mastitis, as a proxy of the number of neutrophils in milk. Accordingly, the current classification defines as affected by subclinical mastitis all Mediterranean buffaloes without clinical signs having a SCC >200,000 cells/mL.

The main bacterial species isolated from water buffalo milk are staphylococci. *Staphylococcus aureus* (SAU) is the most impacting intramammary pathogen, but non-aureus staphylococci (NAS) are most frequently found. In our previous study, NAS were present in 78.4% of culture-positive samples. Consequently, there is clearly a need to understand the impact of staphylococcal IMI on water buffalo milk productions and to improve its detection. Proteomic investigations are a powerful means for assessing changes in milk proteins and for uncovering novel diagnostic markers. Specifically, shotgun proteomic analysis pipelines can provide a profound characterisation of milk proteins, highlighting the alterations introduced by IMI and identifying possible markers of an inflammatory condition. However, little information is available in healthy and diseased buffalo milk. Sparse proteomic analyses, especially when compared to cow mastitis, have been performed on this species. A recent proteomic investigation provided useful information on the profile of buffalo milk with mastitis, but it was limited to one-dimensional and two-dimensional electrophoresis with one-dimensional electrophoresis with label-free quantitation to the milk of animals with subclinical mastitis due to staphylococcal IMI and of healthy animals with the following aims: to provide a vast dataset of buffalo milk proteins, to evaluate and understand the impact of subclinical staphylococcal mastitis on the buffalo milk proteome, to assess the differential impact of SAU and NAS IMI, and to identify novel markers for improving mastitis detection.

**SDS-PAGE patterns of *Staphylococcus*-positive and healthy control milk.** The SDS-PAGE analysis carried out on solubilised skim milk proteins before trypsinisation for shotgun analysis anticipated the presence of several major changes related to staphylococcal IMI (Fig. 1). The major protein bands corresponding to lactoferrin, albumin, caseins, alpha-lactalbumin and beta-lactoglobulin were clearly affected. Specifically, lactoferrin and albumin increased in staphylococcus-positive samples, while caseins, alpha-lactalbumin, and beta-lactoglobulin decreased. The appearance of other bands could also be observed, especially at low molecular weight. Alterations were generally more evident in SAU-positive milk (Fig. 1, lanes 1, 2, 3) than in NAS-positive milk (Fig. 1, lanes 4, 5, 6).

**Shotgun proteomics and differential analysis.** A shotgun proteomic analysis combining filter-aided sample preparation (FASP), high performance reverse-phase chromatography, and high resolution orbitrap mass spectrometry was performed on solubilised skim milk proteins before trypsinisation. A sequential analysis was performed to identify the presence of several major changes related to staphylococcal IMI (Fig. 1). The major protein bands corresponding to lactoferrin, albumin, caseins, alpha-lactalbumin and beta-lactoglobulin were clearly affected. Specifically, lactoferrin and albumin increased in staphylococcus-positive samples, while caseins, alpha-lactalbumin, and beta-lactoglobulin decreased. The appearance of other bands could also be observed, especially at low molecular weight. Alterations were generally more evident in SAU-positive milk (Fig. 1, lanes 1, 2, 3) than in NAS-positive milk (Fig. 1, lanes 4, 5, 6).
spectrometry was carried out to gain a more detailed picture of the alterations caused by staphylococcal infections on the water buffalo milk proteome. A total of 1530 proteins were identified, of which 1034 eligible for differential analysis (Supplementary File, Sheet 1). Mass spectrometry raw data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository18 with the dataset identifier PXD012355. Differential protein abundances were assessed in: (i) all staphylococcus-positive vs control milk; (ii) only SAU-positive vs control milk; (iii) only NAS-positive vs control milk. Results are detailed in Supplementary File 1, Sheets 2–5, and are summarised in Table 2.

When considering all staphylococcus-positive vs healthy control milk, 302 proteins showed significant changes (p ≤ 0.05) in their relative spectral count (RSC). Of these, 152 passed also the selected abundance threshold (RSC ≥ 1.5 or RSC ≤ −1.5); 119 were increased and 33 were decreased in staphylococcal mastitis (differential proteins, Table 3). Of the 119 increased differential proteins, 63 were identified in all staphylococcus-positive milk samples with at least 2 peptide spectrum matches (PSMs) and were never detected in healthy milk (Table 3, asterisk). When considering SAU-positive and NAS-positive milk separately, the number of differential proteins was higher in the former group: 162 in SAU-positive milk (128 increased and 34 decreased) and 127 in NAS-positive milk (108 increased and 19 decreased). Of these, 45 proteins were significantly changed only in SAU-positive milk (Table 3, superscript a) and 11 only in NAS-positive milk (Table 3, superscript b).

Protein abundance changes were generally in agreement (Pearson r = 0.9798) and were typically more intense in SAU-positive milk than in NAS-positive milk, as demonstrated by the Wilcoxon test (p value < 0.0005, Supplementary File, Sheet 6) and visualized by the scatter plot in Fig. 2 (slope 1.088).

**Functional characterisation of differential proteins.** The biological functions affected by staphylococcal mastitis were investigated by means of gene ontology and functional analysis. (Supplementary File 1, Sheets 7–10). Results are detailed in Table 3 and summarised in Fig. 3.

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**Table 2.** Summary of differential proteomic results. aPos: Staphylococcus-positive. bNeg: negative. cSAU: only Staphylococcus aureus-positive. dNAS: only non-aureus staphylococci-positive. *Proteins identified in at least two biological replicates and with ≥ 2 spectral counts in at least one sample of the experimental group. **p ≤ 0.05 by the beta-binomial test with FDR correction according to Benjamini-Hochberg. ***p ≤ 0.05 by the beta-binomial test with FDR correction according to Benjamini-Hochberg, and RSC ≤ −1.5 or ≥1.5.

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**Figure 1.** SDS-PAGE profiles of skim milk samples before trypsinisation for shotgun analysis. Pos: culture-positive samples. Neg: culture-negative samples. SAU: milk samples positive for Staphylococcus aureus. NAS: milk samples positive for non-aureus staphylococci. M: molecular weight markers. Sample numbers correspond to those listed in Table 1. Molecular weight references are indicated on the left. Proteins with a molecular weight corresponding to the main electrophoretic bands are indicated on the right as a reference. One microliter of skim milk was loaded in each lane.
| Accession | Description                   | RSc Pos/Neg | RSc SAU/Neg | RSc NAS/Neg | General function |
|----------|-------------------------------|------------|------------|------------|------------------|
| P48616   | Vimentin*                     | 4.78       | 4.96       | 4.58       | S                |
| A0A0A7NSG7 | Cathelicidin 4*               | 4.75       | 4.72       | 4.78       | I                |
| Q1JPB0   | Leukocyte elastase inhibitor* | 4.09       | 4.17       | 4          | I                |
| X5H0G0   | Cathelicidin 1*               | 4.04       | 4.1        | 3.98       | I                |
| C8CF42   | Prodictenecin 7*              | 3.91       | 4.02       | 3.78       | I                |
| P46193   | Annexin A1                    | 3.86       | 4.02       | 3.67       | I                |
| P52176   | Matrix metalloproteinase-9*    | 3.8        | 3.93       | 3.66       | I                |
| P54228   | Cathelicidin 6*               | 3.67       | 3.72       | 3.62       | I                |
| A0A0A7V3V9 | Cathelicidin 2*              | 3.64       | 3.74       | 3.52       | I                |
| E1AHZ7   | Protein S100A8*               | 3.52       | 3.54       | 3.51       | I                |
| Q92176   | Coronin-1A*                   | 3.51       | 3.51       | 3.51       | I                |
| A5D7D1   | Alpha-actinin-4               | 3.48       | 3.63       | 3.31       | S                |
| P62803   | Histone H4*                   | 3.47       | 3.67       | 3.24       | S                |
| Q2KID0   | Tubulin beta-5 chain          | 3.45       | 3.63       | 3.25       | S                |
| Q0VCN4   | Glycogen phosphorylase        | 3.42       | 3.43       | 3.42       | CM               |
| Q3HMM5   | Tubulin beta-4B chain         | 3.37       | 3.49       | 3.23       | S                |
| Q7T149   | Heat shock protein beta-1*    | 3.33       | 3.39       | 3.26       | PD               |
| Q7Q991   | Myosin-10*                    | 3.31       | 3.28       | 3.34       | S                |
| L0L830   | Cathelicidin 7*               | 3.26       | 3.37       | 3.15       | I                |
| P46763   | High mobility group protein B2* | 3.25    | 3.43       | 3.04       | I                |
| Q5MAR3   | Integrin beta*                | 3.22       | 3.48       | 2.9        | I                |
| Q0VCW4   | L-serine dehydratase/L-threonine deaminase* | 3.09 | 3.16 | 3.01 | AM |
| P55052   | Fatty acid-binding protein, epidermal* | 3.08 | 3.08 | 3.08 | LM |
| P2253    | Histone H1.2*                 | 3.08       | 3.06       | 3.11       | S                |
| F6MFD5   | Peptidoglycan-recognition protein | 3.08 | 3.26       | 2.86       | I                |
| P62808   | Histone H2B type 1            | 3.04       | 3.04       | 3.04       | S                |
| Q7T125   | Ras-related C3 botulinum toxin substrate 2 | 2.97 | 2.99 | 2.95 | I |
| Q7T103   | Rho GDP-dissociation inhibitor 2* | 2.97  | 3.13       | 2.79       | S                |
| Q7T126   | Transaldolase*                | 2.96       | 3.05       | 2.86       | CM               |
| V9L96    | Cathelicidin 5*               | 2.95       | 3.05       | 2.84       | I                |
| Q3H86    | Tubulin alpha-1D chain        | 2.95       | 3.11       | 2.76       | S                |
| Q5XW7    | Annexin A3*                   | 2.86       | 3.04       | 2.65       | I                |
| Q58N02   | Alpha-actinin-1               | 2.85       | 3.01       | 2.68       | S                |
| Q5E9F5   | Transgelin-2*                 | 2.85       | 2.83       | 2.86       | S                |
| Q35YV4   | Adenyl cyclase-associated protein 1 | 2.84 | 2.78 | 2.9 | S |
| Q2KJH4   | WD repeat-containing protein 1* | 2.81 | 2.63 | 2.97 | S |
| P68432   | Histone H3.1*                 | 2.79       | 2.91       | 2.66       | S                |
| A7EQQ8   | Plastin-3*                    | 2.79       | 2.86       | 2.7        | S                |
| P81948   | Tubulin alpha-4A chain        | 2.74       | 2.93       | 2.51       | S                |
| Q3HMR7   | Actin-related protein 2/3 complex subunit 2* | 2.68 | 2.61 | 2.75 | S |
| A7MB62   | Actin-related protein 2        | 2.62       | 2.6        | 2.64       | S                |
| Q5F9B1   | L-lactate dehydrogenase B chain | 2.61     | 2.69       | 2.52       | CM               |
| M1JVB9   | Haptoglobin                   | 2.59       | 2.68       | 2.49       | I                |
| A0A0C5AGQ3 | Lysozyme*                     | 2.59       | 2.64       | 2.55       | I                |
| Q6B955   | Transketolase                 | 2.59       | 2.65       | 2.54       | CM               |
| P02584   | Profilin-1                    | 2.58       | 2.6        | 2.56       | S                |
| Q5E956   | Triosephosphate isomerase     | 2.53       | 2.56       | 2.49       | S                |
| P10103   | High mobility group protein B1 | 2.52     | 2.73       | 2.28       | I                |
| A2J7N1   | Serpin A3-5                   | 2.52       | 2.67       | 2.35       | PD               |
| P3ZBD7   | Glucose-6-phosphate isomerase | 2.5        | 2.59       | 2.4        | CM               |
| P9CO59   | Histone H2A type 1            | 2.5        | 2.54       | 2.45       | S                |
| Q3T0P6   | Phosphoglycerate kinase 1     | 2.5        | 2.53       | 2.47       | CM               |
| P1958     | L-lactate dehydrogenase A chain | 2.48     | 2.43       | 2.52       | CM               |
| Q3T114   | Ribonuclase UK114*            | 2.47       | 2.4        | 2.53       | CP               |
| Q22LE5   | Isoaspartyl peptidase/L-asparaginase* | 2.46 | 2.34 | 2.58 | CP |
| P30922   | Chitinase-3-like protein 1     | 2.45       | 2.32       | 2.57       | I                |

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| Accession | Description                                                                 | Rec Pos/Neg | Rec SAU/Neg | Rec NAS/Neg | General function |
|-----------|------------------------------------------------------------------------------|-------------|-------------|-------------|-----------------|
| Q2IH60    | Heterogeneous nuclear ribonucleoproteins A2/B1*                             | 2.39        | 2.66        | 2.06        | NM              |
| A5AL2     | Histone H2B**                                                               | 2.38        | 2.59        | I           |
| P79136    | F-actin-capping protein subunit beta                                         | 2.32        | 2.41        | 2.23        | S               |
| P61157    | Actin-related protein 3                                                     | 2.31        | 2.27        | 2.35        | S               |
| Q5KR47    | Tropomyosin alpha-3 chain                                                   | 2.31        | 2.37        | 2.24        | S               |
| P99867    | Heterogeneous nuclear ribonucleoprotein A1*                                 | 2.3         | 2.45        | 2.14        | S               |
| A4IF97    | Myosin regulatory light chain 12B*                                           | 2.27        | 2.39        | 2.14        | S               |
| A3KMY5    | Ubiquitin-like modifier-activating enzyme 1*                                | 2.26        | 2.57        | 1.87        | PD              |
| Q2HJ57    | Coactosin-like protein*                                                     | 2.22        | 2.24        | 2.21        | I               |
| Q58CQ2    | Actin-related protein 2/3 complex subunit 1B*                               | 2.2         | 2.22        | 2.18        | S               |
| G3N131    | Histone H1.1*                                                               | 2.19        |             |             |                 |
| P49951    | Clathrin heavy chain 1*                                                     | 2.08        | 2.22        | 1.92        | S               |
| A5D7A0    | EF-hand domain-containing protein D2*                                        | 2.08        | 2.2         | 1.95        | S               |
| Q2TA49    | Vasodilator-stimulated phosphoprotein                                       | 2.06        | 2.42        | 1.58        | S               |
| Q2ZC84    | Cytosolic non-specific dipeptidase                                           | 2.05        | 2.18        | 1.91        | OM              |
| P10996    | Glyceraldehyde-3-phosphate dehydrogenase                                    | 2.05        | 2.14        | 1.97        | CM              |
| P60661    | Myosin light polypeptide 6                                                  | 2.02        | 2.15        | 1.87        | S               |
| Q3T0D0    | Heterogeneous nuclear ribonucleoprotein K*                                  | 2.01        | 2.08        | 1.94        | NM              |
| P33433    | Histidine-rich glycoprotein*                                                 | 2.01        | 2.52        |             |                 |
| Q4US35    | Proteasome activator complex subunit 1                                      | 2.01        | 2.07        | 1.96        | PD              |
| Q32P15    | Serine/threonine-protein phosphatase 2A 65kDa regulatory subunit A alpha isoform | 1.98        | 2.29        | 1.59        | S               |
| Q56Z9     | Glia maturation factor gamma*                                                | 1.97        | 2.02        | 1.92        | S               |
| Q6VCH1    | Complement C1s subcomponent*                                                | 1.96        | 2.24        | 1.62        | I               |
| Q3S2H7    | Leukotriene A-4 hydrolase*                                                  | 1.96        | 1.91        | 2.02        | I               |
| P31081    | 60kDa heat shock protein, mitochondrial*                                     | 1.95        | 2.12        | 1.76        | I               |
| Q2L2P9    | Fermitin family homolog 3*                                                  | 1.94        | 2.15        | 1.69        | I               |
| D2U6Q0    | Serum amyloid A protein*                                                     | 1.93        | 1.86        | 2           | I               |
| P62328    | Thymosin beta-4*                                                            | 1.91        | 2.03        | 1.78        | I               |
| P13752    | BOLA class I histocompatibility antigen, alpha chain BL3-6*                  | 1.9         | 2.08        | 1.7         | I               |
| Q2K4I3    | Protein FAM49B*                                                             | 1.89        | 1.87        | 1.92        | I               |
| Q76255    | Resistin*                                                                   | 1.89        | 1.92        | 1.85        | LM              |
| Q76LV2    | Heat shock protein HSP 90-alpha                                              | 1.88        | 2.06        | 1.68        | PD              |
| Q3SZ54    | Eukaryotic initiation factor 4A-1                                            | 1.85        | 1.82        | 1.88        | GE              |
| Q3ML4     | Adenosylhomocysteinase                                                       | 1.83        | 1.82        | 1.84        | AM              |
| P80724    | Brain acid soluble protein 1*                                               | 1.82        | 1.61        | 2           | GE              |
| P60712    | Actin, cytoplasmic 1                                                        | 1.8         | 2.01        | 1.57        | S               |
| Q9Q6V1    | Glutathione peroxidase*                                                      | 1.8         | 2.05        | 1.5         | OM              |
| P5859     | Purine nucleoside phosphorylase*                                            | 1.78        | 1.51        | 2           | NM              |
| Q9X5J4    | Alpha-enolase                                                               | 1.77        | 1.8         | 1.75        | CM              |
| Q27970    | Calpain-1 catalytic subunit*                                                | 1.77        | 2.13        |             | PD              |
| Q865V6    | Macrophage-capping protein*                                                 | 1.76        | 1.65        | 1.86        | S               |
| Q99MB8    | Serine- tRNA ligase, cytoplasmic*                                           | 1.74        | 2.02        |             | AM              |
| Q9BH1     | Peroxiredoxin-5, mitochondrial                                              | 1.73        | 1.92        | 1.52        | OM              |
| P51122    | Acidic leucine-rich nuclear phosphoprotein 32 family member A*               | 1.7         | 1.72        |             | CT              |
| Q14816    | Actin-related protein 2/3 complex subunit 4*                                | 1.7         | 1.71        | 1.69        | S               |
| A4FU08    | F-actin-capping protein subunit alpha-1*                                     | 1.68        | 1.63        | 1.73        | S               |
| P08686    | Plasminogen                                                                 | 1.68        | 1.77        | 1.58        | C               |
| P07589    | Fibronectin                                                                 | 1.67        |             |             | I               |
| P13753    | BOLA class I histocompatibility antigen, alpha chain BL3-7**                 | 1.65        | 1.96        |             | I               |
| Q46522    | Cytochrome b-245 heavy chain*                                               | 1.64        | 2           |             | I               |
| Q3SZI4    | 14-3-3 protein theta                                                        | 1.62        | 1.69        | 1.55        | GE              |
| A6H742    | Plastin-1*                                                                   | 1.62        | 1.54        | 1.69        | S               |
| D2U6Z5    | Ceruloplasmin*                                                              | 1.61        | 1.75        |             | IM              |
| Q3T035    | Actin-related protein 2/3 complex subunit 3**                               | 1.57        | 1.71        |             | S               |
| P20000    | Aldehyde dehydrogenase, mitochondrial**                                     | 1.57        | 1.71        |             | CM              |

Continued
| Accession  | Description                                                                 | Rsc Pos/Neg | Rsc SAU/Neg | Rsc NAS/Neg | General function |
|------------|------------------------------------------------------------------------------|-------------|-------------|-------------|------------------|
| P01030     | Complement C4b                                                                | 1.57        | --          | 1.72        | 1                |
| P0C054     | Histone H2A.Z                                                                | 1.56        | 1.66        | --          | S                |
| Q87996     | Lysozyme C, tracheal isozyme                                                  | 1.56        | 1.51        | 1.61        | I                |
| Q5E98      | Chloride intracellular channel protein 1                                      | 1.55        | 1.52        | 1.57        | C                |
| P68138     | Actin, alpha skeletal muscle                                                   | 1.54        | 1.72        | --          | S                |
| X2I2Z01    | Signal transducer and activator of transcription                            | 1.54        | 1.59        | --          | I                |
| Q2ZBT1     | Transitional endoplasmic reticulum ATPase                                      | 1.54        | 1.68        | --          | CT               |
| Q2H1G5     | Vacular protein sorting-associated protein 35                                 | 1.54        | 1.84        | --          | I                |
| Q97680     | Thoreodoxin                                                                  | 1.53        | 1.79        | --          | OM               |
| P19483     | ATP synthase subunit alpha, mitochondrial                                      | 1.52        | --          | --          | CT               |
| P02676     | Fibrinogen beta chain                                                         | 1.79        | --          | --          | C                |
| P02672     | Fibrinogen alpha chain                                                        | 1.83        | --          | --          | I                |
| Q5F9R3     | EH domain-containing protein 1                                               | 1.78        | --          | --          | CaM              |
| P08829     | ATP synthase subunit beta, mitochondrial                                       | 1.66        | --          | --          | OM               |
| P81644     | Apolipoprotein A-II                                                           | 1.64        | --          | --          | LM               |
| Q1B7M5     | LIM and SH3 domain protein 1                                                  | 1.61        | --          | --          | S                |
| A6H7G2     | Drebrin-like protein                                                          | 1.58        | --          | --          | I                |
| Q2ZBH0     | T-complex protein 1 subunit beta                                              | 1.57        | --          | --          | PD               |
| Q3MHL7     | T-complex protein 1 subunit zeta                                               | 1.52        | --          | --          | PD               |
| Q7SH1      | Alpha 2-macroglobulin                                                         | 1.52        | --          | --          | I                |
| P27214     | Annexin A14                                                                  | 1.52        | --          | --          | S                |
| Q3SZV7     | Hemopexin                                                                    | 1.5          | --          | --          | IM               |
| P11116     | Galectin-1                                                                   | 1.61        | --          | --          | I                |
| P35466     | Protein S100-A4                                                               | 1.6          | --          | --          | I                |
| Q02675     | Dehydroxymidase-related protein 2                                            | 1.51        | --          | --          | S                |
| Q3T054     | GTP-binding nuclear protein Ran                                                | 1.61        | --          | --          | CT               |
| Q3T0D7     | GTP-binding protein SAR1a                                                     | -1.51       | --          | --          | CT               |
| Q716P7     | Fatty acid synthase                                                           | -1.52       | -1.8        | --          | LM               |
| P02638     | Protein S100-B                                                               | -1.53       | --          | --          | I                |
| P10790     | Fatty acid-binding protein, heart                                             | -1.53       | --          | --          | LM               |
| M9WP41     | BTN1A1                                                                      | -1.57       | --          | --          | S                |
| Q17Q5E5    | Calcium and integrin-binding protein 1                                       | -1.56       | -1.85       | --          | I                |
| Q9N0T1     | Stanniocalcin-1                                                               | -1.57       | -1.9        | --          | CaM              |
| A0A0C5GDW2 | Myostatin                                                                    | -1.59       | --          | --          | S                |
| Q6R5U5     | Butyrophilin                                                                 | -1.6         | -1.75       | --          | LM               |
| G1AQP5     | Xanthine dehydrogenase/oxidase                                               | -1.61       | -1.81       | --          | LM               |
| E9NV81     | ATP-binding cassette sub-family G (WHITE) member 2                             | -1.62       | -1.95       | --          | LM               |
| Q05927     | 5’-nucleotidase                                                              | -1.65       | -1.88       | --          | NM               |
| M4G36      | Insulin induced protein 1                                                     | -1.65       | -1.88       | --          | S                |
| G7788      | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1                            | -1.68       | -2.19       | --          | S                |
| A5FK13     | Volume-regulated anion channel subunit LRRC8C                                 | -1.68       | --          | --          | LM               |
| Q2KIY5     | Putative phospholipase B-like 2                                               | -1.69       | -2.24       | --          | LM               |
| P81127     | Gamma-soluble NSF attachment protein                                         | -1.71       | --          | --          | CT               |
| Q58C6Y6    | Prostamide/prostaglandin F synthase                                          | -1.73       | -1.83       | --          | LM               |
| Q68065     | Tyrosine-protein kinase receptor Tie-1                                        | -1.73       | --          | --          | A                |
| P37980     | Inorganic pyrophosphatase                                                     | -1.74       | -1.99       | -1.53       | LM               |
| Q3T0Q2     | Transmembrane protein 59                                                      | -1.75       | -1.87       | -1.63       | CT               |
| Q3SYV1     | Transmembrane protein 263                                                     | -1.77       | --          | --          | CT               |
| Q3SY61     | Calciumurin B homologous protein                                              | -1.81       | -1.87       | -1.75       | CaM              |
| Q58DD4     | Synecean-2                                                                   | -1.81       | -2.13       | --          | S                |
| D3K5B6     | Plasma membrane calcium-transporting ATPase                                   | -1.93       | -2.04       | -1.82       | CaM              |
| C1KGU3     | Solute carrier family 11 member 2                                             | -1.93       | -2.02       | -1.85       | CT               |
| Q5G77      | Glycoprotein-3-phosphate acyltransferase 1, mitochondrial                    | -2.03       | -2.13       | -1.91       | LM               |
| A0A08FF5E5 | Acyl-CoA synthetase short-chain family member 2                               | -2.08       | -2.19       | -1.97       | LM               |
| P08239     | Guanine nucleotide-binding protein G(x) subunit alpha                          | -2.14       | -2.25       | -2.03       | CT               |

Continued
**Table 3.** Significantly differential proteins in Staphylococcus-positive milk with $R_{SC} \geq 1.5$ or $R_{SC} \leq -1.5$. The table reports the differential proteins obtained when considering all staphylococcus-positive milk samples (Pos/Neg), only SAU-positive milk samples (SAU/Neg) or only NAS-positive milk samples (NAS/Neg). The general functional classification is indicated as follows: A, angiogenesis; AM: aminoacid metabolism; CaM, calcium metabolism; CM, carbohydrate metabolism; CP: catabolic process; CT, cellular transport; C, coagulation; GE: gene expression; I, immunity; IM, iron metabolism; LM: lipid metabolism; NM: nucleotide metabolism; OM: oxidative metabolism; PD, protein degradation; S, structure. Gene ontology results are detailed in Supplementary File. aSignificantly changed only in SAU-positive milk. bSignificantly changed only in NAS-positive milk. cSignificantly changed only in NAS-positive milk.

**Increased proteins.** Results are summarised in Fig. 3A. When considering proteins increased in all staphylococcus-positive milk samples ($n = 152$), the highest number of proteins had structural functions ($n = 43$, 28.29%), including actin and actin-binding proteins, tubulins, and other cytoskeletal proteins. Histones were comprised in this ontology class because of their structural function in nucleosomes; nevertheless, histones also play a significant role in innate immunity of the mammary gland within Neutrophil Extracellular Traps (NETs)\(^\text{16,26}\). Immunity was the function with the second higher number of proteins ($n = 39$, 25.66%). Cathelicidins were the most significantly increased protein family, with high $R_{SC}$ values: cathelicidin 4 ($R_{SC}$ 4.75), cathelicidin 1 ($R_{SC}$ 4.04), probactenecin 7 ($R_{SC}$ 3.91), cathelicidin 6 ($R_{SC}$ 3.67), cathelicidin 2 ($R_{SC}$ 3.64), cathelicidin 7 ($R_{SC}$ 3.26), and cathelicidin 5 ($R_{SC}$ 2.95). This class also included antimicrobial proteins and neutrophil granule proteins such as S100 proteins, leukocyte elastase inhibitor, matrix metalloproteinase, and two lysozyme proteoforms ($R_{SC}$ 2.59 and $R_{SC}$ 1.56, respectively). The acute phase proteins haptoglobin ($R_{SC}$ 2.59) and serum amyloid ($R_{SC}$ 1.93) were also significantly increased. Other proteins of interest were high mobility group protein B2 ($R_{SC}$ 3.25), epidermal fatty-acid binding protein ($R_{SC}$ 3.08), peptidoglycan-recognition protein ($R_{SC}$ 3.08), and complement fragments. In line with its antimicrobial function, Histone H2B ($R_{SC}$ 2.38) was included in this ontology class. Other significantly increased proteins belonged to carbohydrate metabolism ($n = 10$) followed by protein degradation ($n = 6$), and oxidative metabolism ($n = 4$). Aminoacid metabolism ($n = 3$), gene expression and nucleotide metabolism ensued ($n = 3$). Proteins involved in catabolic process, lipid metabolism, coagulation ($n = 2$), cellular transport, and iron metabolism ($n = 1$) were also represented. When considering only SAU-positive milk, some functions were represented by a higher number of significant proteins: structure (44 vs 43 proteins), immunity (40 vs 39), protein degradation (8 vs 6), oxidative metabolism (5 vs 4), lipid metabolism, coagulation (3 vs 2), and cellular transport (2 vs 1). Calcium metabolism was also highlighted ($n = 1$). On the other hand, when considering only NAS-positive milk, less significant proteins were generally observed in most classes.

**Increased networks.** Based on STRING analysis, the biological process involving most increased proteins was Immunity, with a total of 50 significant term descriptions, ranging from defence response (24 gene counts, $FDR < 0.000000005$) and response to external stimulus (23 gene counts, $FDR < 0.000000005$) to lymphocyte activation (3 gene counts, $FDR < 0.05$). The second process was Structure, with a total of 22 significant term descriptions, ranging from cytoskeleton organization (27 gene counts, $FDR < 0.000000005$) and actin-filament-based process (22 gene counts, $FDR < 0.000000005$) to actin filament-based movement ($FDR < 0.05$). Other significant biological processes were Catabolic process, Gene expression, Protein degradation, Carbohydrate metabolism, Oxidative metabolism, Nucleotide metabolism, Aminoacid metabolism, and Coagulation (with 12, 4, 4, 5, 3, 2, 2, and 1 significant term descriptions, respectively). Details are reported in Supplementary File, Sheet 8. Several significant Reactome terms were also obtained for increased proteins. Of note, Neutrophil Degranulation was the most significant ($FDR < 0.000000005$) with 17 gene counts, followed by Innate Immune System ($FDR < 0.000000005$) with 21 gene counts. Immune System, Regulation of actin dynamics for phagocytic cup formation, Apoptosis, and Antimicrobial peptides were other significantly relevant terms (Supplementary
Material, Sheet 9). Figure 4 illustrates the Reactome network generated by STRING when investigating the interactions among proteins increased in milk upon staphylococcal mastitis.

Decreased proteins. Results are summarised in Fig. 3B. When considering all staphylococcus-positive samples, most of the 22 differential proteins (59.10%) belonged to lipid metabolism (n = 13), followed by cellular transport (n = 6), immunity, structure, and calcium metabolism (n = 3). Other decreased proteins had functions ranging from angiogenesis to cellular response and nucleotide, iron, and carbohydrate metabolism (n = 1). Once again, most protein functions were more represented in SAU-positive samples.

Decreased networks. Based on STRING analysis, the biological process involving most decreased proteins was Structure, with a total of 6 significant term descriptions, ranging from anatomical structure morphogenesis (FDR 0.00051) with 7 observed gene counts, to membrane organization (FDR 0.0243) with 3 observed gene counts. Structure was followed by Cellular transport and by Immunity, Lipid metabolism, Cellular homeostasis, Cellular response, Angiogenesis and Nucleotide metabolism. Details are reported in Supplementary File, Sheet 10.
Western immunoblotting validation. According to label-free quantitation, cathelicidins were the most increased protein family upon staphylococcal mastitis, with similar increase in both SAU-positive and NAS-positive milk (Table 3). Other proteins of interest were S100 proteins and acute phase proteins, including haptoglobin, also in view of the previous results generated by proteomic studies carried out on milk from sheep12,13 and cows11,21 with mastitis. Therefore, these were further investigated by Western Immunoblotting (Fig. 5). Concerning cathelicidin, the abundance of all proteoforms in terms of NSAF values was generally higher in SAU-positive milk than NAS-positive milk, while none was detectable in culture-negative milk (Fig. 5A). Western immunoblotting with anti-pan-cathelicidin antibodies22,23 confirmed the shotgun proteomic results; all staphylococcus-positive milk samples were positive for cathelicidins and all healthy milk samples were negative. In addition, a stronger cathelicidin signal was observed in SAU-positive milk (Fig. 5D and Supplementary Fig. 1). S100A8 was also among the top 10 increased proteins in both SAU and NAS IMI, with similar increases in the two milk sample groups (Fig. 5B). Western immunoblotting produced matched results with similar band intensities, with slightly stronger signals in samples with higher NSAF values (Fig. 5E). Haptoglobin was also increased in both sample groups (Table 3), and western immunoblotting confirmed the shotgun proteomics findings. However, some differences in signal intensity were observed, not related to the IMI agent (Fig. 5F). Although
few peptides were detected in 4 out of 6 samples by shotgun proteomics, haptoglobin was not detected by western immunoblotting in bacteriologically negative, low SCC quarters (Fig. S5C).

Discussion

This was the first differential proteomic study investigating the changes induced by infectious mastitis in water buffalo milk. The application of proteomics in this field presents some challenges, since the knowledge regarding buffalo udder health is less well defined when compared to dairy cows and information on sequence and function databases is less complete. Despite these limitations, shotgun proteomics enabled a profound characterisation of buffalo milk proteins, defining the changes that occur in staphylococcal mastitis, and provided indications on their differences in mastitis due to SAU or NAS IMI. Staphylococcal IMI, both by SAU and NAS, induced significant changes even in subclinical conditions; in SAU-positive buffaloes, as expected, these changes were more intense. This could be already appreciated by examining the SDS-PAGE profile; the typical milk pattern was maintained, but the main bands changed in abundance and other bands appeared in the lower molecular weight region, with more intense alterations appearing in SAU-positive milk.

Based on shotgun proteomics, the most significantly increased proteins were of structural origin, followed by immunity, and STRING analysis highlighted Immunity as the most relevant biological process influenced by staphylococcal IMI, followed by structure. This was consistent with the extensive cytoskeletal rearrangements occurring in the mammary epithelium as a result of inflammation, as well as of neutrophil degranulation, chemotaxis, and extravasation; accordingly, Neutrophil Degranulation was the most significantly increased reaction in the Reactome database.

When considering individual proteins, the highest increase in staphylococcal mastitis was observed for vimentin. Vimentin is the most abundant intermediate filament protein with a critical role in stabilisation of cellular architecture\(^{24}\). However, recent studies highlighted its involvement in the innate immune response to bacterial pathogens as a ligand for pattern recognition receptors\(^{25}\) and as an inhibitor with NLRP3 for regulation of inflammasome activity\(^{26}\). Interestingly, in a recent study on response of bovines to intramammary infection by *Streptococcus uberis*, vimentin was one of the top 15 up-regulated proteins at 57, 81, and 312 hours after intramammary challenge\(^{11}\). In view of these results, it will be interesting to further investigate on the role of vimentin in mastitis, as already done in other inflammatory conditions\(^{27}\).

When considering protein families, cathelicidins showed the highest increase in staphylococcal mastitis. Seven cathelicidin members were identified, all of them with high R\(_{SC}\). Cathelicidins are a family of proteins involved in antimicrobial defence and regulation of immunity that have undergone gene duplication and divergence in ruminants, leading to a family of proteins with similar functions\(^{28,29}\). Their significant increase in milk following mammary gland infection has already been reported for cows and ewes, both in natural and experimental infections\(^{30–33}\). Cathelicidins are released by epithelial cells upon microbial sensing, by degranulation of neutrophils that enter the mammary gland as a result of an inflammatory stimulus, as well as together with other granule contents within NETs\(^{34,35}\). The presence of NETs and their role in the antimicrobial defence of the water buffalo mammary gland is supported also by the significant increase in histones, the basic component of the nucleoproteic web released during NET formation. Based on gene ontology analysis, histones were classified as structural proteins in consideration of their key role in the nucleosome, but their function in mastitis might be more related to immune response. Once again, this contributes to the indication of immunity as the most relevant biological process according to STRING analysis. Together with cathelicidins, other neutrophil granule proteins were significantly increased, including neutrophil elastase and myeloperoxidase, further supporting the extensive contribution of the neutrophil influx into the mammary gland to the changes observed in the milk proteome. In line with this, integrin was also one of the top increased proteins: integrin is crucial for neutrophil extravasation and entrance in the mammary alveolus\(^{36}\). Another important group of significantly increased proteins were acute phase proteins (APP), including haptoglobin, serum amyloid protein A, and ceruloplasmin. The APP increase in
milk has been reported and is well known in cows and in sheep\(^ {31-33,35}\). Another protein of interest was epidermal fatty-acid binding protein (FABP5). Among numerous other biological roles, FABPs are involved in inflammation processes regulated by fatty acids through their interaction with peroxisome proliferator-activated receptors (PPARs), and FABP5, adding to keratinocytes in skin epidermis, is widely expressed in immune cells where it regulates immunological functions\(^ {36-38}\). Of note, numerous increased proteins carried out defence functions and were involved in innate immunity. Another increased class was proteolysis, both due to increased protein turnover following inflammation as well as to the release in milk of numerous host and pathogen proteases.

On the other hand, over half of the proteins that decreased in staphylococcal mastitis were involved in lipid metabolism. Numerous biosynthetic enzymes were affected, including fatty acid synthase, glycerol-3-phosphate acyltransferase, acyl-Co-A synthetase, sterol-4-alpha-carboxylate 3-dehydrogenase, deoxycholating, long-chain fatty acid-CoA ligase, lanosterol synthase, and others. Of note was also the combined decrease of butyrophilin and xanthine dehydrogenase/oxidase (XD/XO), two of the most important structural components of the milk fat globule (MFG)\(^ {39-41}\). MFGs are secreted by the epithelial cells of the mammary gland starting from intracellular precursors, the secretory granules\(^ {42}\). These are transported to the cell surface and are pinched off the cell membrane in a process controlled by the interactions between plasma membrane butyrophilin and butyrophilin in the lipid droplet phospholipid monolayer\(^ {43}\). XD/XO enables a more efficient secretion of MFGs, and plays a crucial role in stabilising the MFG through its interactions with butyrophilin\(^ {44,45}\). Combined with the decrease in cellular transport proteins, the second in order of abundance, this suggests that cellular secretion functions, including milk fat globule release, are impaired. Accordingly, STRING analysis confirmed an involvement of the biological processes related to anatomical structure morphogenesis, membrane organization, and lipid metabolism. Interestingly, another FABP isoform, fatty-acid binding protein, heart (FABP3), was decreased in staphylococcal mastitis. Although its role in buffaloes still requires investigation\(^ {46}\), FABP3 has been reported as positively related to sheep, goat and cow milk quality, being involved in lipid droplet synthesis and accumulation\(^ {47-49}\). Therefore, the proteomic changes induced by staphylococcal mastitis can potentially affect relevant quantitative, qualitative and structural aspects of water buffalo milk that impact sensorial and textural features of the derived dairy products, including the highly valued “mozzarella di bufala”. It will be of interest to further investigate on this aspect with a combined proteomic and lipidomic approach.

Interesting perspectives for mastitis diagnosis and monitoring are also opened by this study. An efficient detection of mastitis episodes in the herd is crucial for controlling intramammary infections and reducing antibiotic use, and therefore markers and methods providing better diagnostic performances are needed. Several differential proteins have potential as mastitis markers, as already assessed in cows and sheep. Of these, cationicolins, S100 proteins and haptoglobin have shown to possess diagnostic value when implemented in the ELISA format\(^ {50-52}\). The western immunoblotting validation of proteomic results encourages their application also in the water buffalo. Other proteins detected in this study have been implemented in ELISAs for mastitis detection in cows, including milk amyloid\(^ {53,54}\), and might also be worth investigating for their diagnostic potential in buffalo.

Most of the changes induced by staphylococcal mastitis were more intense in SAU IMI than NAS IMI, although the mean SCC value was similar in the two groups. This emerged in all the experiments carried out in this study. By SDS-PAGE analysis, the banding profile was more altered (Fig. 1); shotgun proteomics indicated a higher number of differential proteins (Tables 2 and 3) as well as a slightly stronger impact on RSC values. Finally, western immunoblotting showed more intense cationicolin-positive bands (Fig. 4). All these results point to a stronger ability of SAU to alter the buffalo milk proteome when compared to NAS, most likely due to its higher virulence. Other known issues of SAU infections are the contagious nature and therefore ability to spread in the herd, not to mention the adverse consequences of toxins that can contaminate dairy products and cause food poisoning in the human consumer. Therefore, SAU should be eliminated from the herd and adequate biosecurity measures should be applied for preventing its entry and spread in the farm. Nevertheless, the results of this study further highlight the relevant impact of mastitis due to NAS IMI on the buffalo milk proteome as well. Further studies will be needed to investigate on the ability of different NAS species to cause milk alterations in this dairy species. Another aspect that will need to be elucidated is the impact on the milk proteome of staphylococcal colonisation without detectable changes in somatic cell counts, also when considering the recent findings on the mammary gland microbiota\(^ {53,54}\).

In conclusion, this study generated an extensive dataset of buffalo milk proteins, identified the changes induced by staphylococcal mastitis providing novel information on affected functions and proteins, and revealed differences in the intensity of such alterations according to the pathogen, opening novel perspectives for the development of immunoassay-based systems aimed at improving udder health monitoring in this large ruminant.

Methods

Animals and milk samples. The study was carried out on 12 quarter milk samples collected from a water buffalo herd in the context of a survey on mammary gland health in Campania (Italy) receiving an institutional approval by the Ethical Animal Care and Use Committee of the University of Naples “Federico II” (No. 2016/0052967”). All procedures were carried out conforming to the relevant rules and regulations on animal welfare. Before sampling, all animals enrolled were submitted to a clinical examination. The clinical udder health status was characterised according to our previous study\(^ {9}\), and CMT was performed on milk samples of each quarter. Teats were carefully cleaned and disinfected with disposable towels embedded with chlorhexidine, and the first streams of milk were discarded. Then, approximately 50 mL of milk was collected aseptically from each teat into sterile vials. Samples were brought to the laboratory and stored at 4°C for a maximum of 24 h until bacteriological assays and SCC enumeration were performed.

Bacteriological analysis and somatic cell count. Bacteriological analysis was performed according to the National Mastitis Council standards (2017). Ten μL of each milk sample was spread onto blood agar plates (5%
defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 h. Colonies were provisionally identified based on Gram stain, morphology, and haemolysis patterns, and the number of each colony type was recorded. Only samples with at least five colonies with the same characteristics were considered positive. Representative colonies were then sub-cultured on blood agar plates and incubated again at 37°C for 24 h to obtain pure cultures. Gram-positive cocci were tested for catalase and coagulase production. Those showing positive reaction to both tests were identified as SAU. Those showing positive reaction for catalase and negative reaction for coagulase tests were classified as NAS. Somatic cell count was determined using an automated counter (Bentley Somacount 150; Bentley Instruments, Chaska, MN).

**Milk sample preparation for proteomic analysis.** Milk was thawed at room temperature and centrifuged at 800 × g at 4°C for 10 min. Fat was removed and the pellet formed by cells and caseins was resuspended. Skim milk was diluted 1:1 with lysis buffer (2% SDS, 0.4% Tween-20, 130 mM DTT, 500 mM Tris-HCl pH 8.8, and plus protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO), incubated at 95°C for 10 min and then sonicated in a refrigerated water bath for 10 min. The suspension was centrifuged at 10,000 × g for 10 min at 4°C. The extract was checked for quality by SDS-PAGE as described below. For shotgun proteomic analysis, 7 μl of extract were subjected to filter-aided sample preparation (FASP) as described previously. Briefly, protein samples were subjected to reduction, alkylation, and trypsin digestion on Amicon Ultra-0.5 centrifugal filter units with Ultrafree-10 membrane (Millipore, Billerica, MA, USA). Peptide concentration of samples was determined by measuring absorbance at 280 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, San Jose, CA, USA) using MassPREP E. coli Digest Standard (Waters, Milford, MA, USA) to create a calibration curve.

**SDS-PAGE and western immunoblotting.** SDS-PAGE and Western immunoblotting were carried out on a Criterion Cell with AnyKD™ Criterion TGX™ precast gels and with a Trans Blot® Turbo™ Blotting System (Bio-Rad Laboratories, Hercules, CA, USA) according to the user manual, as detailed previously with minor modifications. Briefly, 2 μl of the above extract, containing proteins from 1 μl of skimmed milk, were mixed with loading buffer, reduced and denatured, loaded into the wells, and subjected to electrophoretic separation. After the run, gels were stained with Coomassie SafeStain™ (Bio-Rad) for protein visualisation or transferred onto nitrocellulose with the Trans Blot® Turbo™. The nitrocellulose was then blocked, incubated with either monoclonal anti-cathelicidin antibodies as previously described, rabbit polyclonal anti-S100A8 prestige antibodies (Sigma-Aldrich), or sheep polyclonal anti-haptoglobin antibodies (Invitrogen, Carlsbad, CA, USA), followed by the appropriate secondary antibodies, developed with a chemiluminescent substrate, and digitalised with a VersaDocMP 4000 System (Bio-Rad), as detailed previously.

**Tandem mass spectrometry analysis of peptides.** All peptide mixtures were analysed on a Q-Exactive interfaced with an UltiMate 3000 RSLCnanoLC system (Thermo Scientific, San Jose, CA, USA), as described previously. A total of 4 μg of each peptide mixture were concentrated and washed onto a trapping pre-column (Acclaim PepMap C18, 75 μm × 2 cm nanoViper, 3 μm, 100 Å, Thermo Scientific) and fractionated on a C18 RP column (Acclaim PepMap RSLC C18, 75 μm × 50 cm nanoViper, 2 μm, 100 Å, Thermo Scientific) at flow rate of 250 nL/min using a linear gradient of 245 minutes from 5 to 37.5% eluent B (0.1% formic acid in 80% acetonitrile) in eluent A (0.1% formic acid). Fragmentation occurred by Higher Energy Collisional Dissociation (HCD) and nitrogen as the collision gas. Proteome Discoverer (version 1.4; Thermo Scientific) was used for protein identification using Sequest-HT as search engine. Each MS/MS spectrum was analysed as follows. Database: database custom obtained by merging B. taurus and Bubalus bubalis databases downloaded from Swiss-Prot and TrEMBL (release 2017_05 and 2016_11, respectively; enzyme: trypsin, with two missed cleavages allowed; precursor mass tolerance: 10 ppm; MS/MS tolerance: 0.02 Da; charge states: +2, +3, and +4; cysteine carbamidomethylation as static modification and methionine oxidation as dynamic modifications. The percolator algorithm was used for protein significance and for peptide validation (false discovery rate, FDR, <0.01%). Peptide and protein grouping according to the Proteome Discoverer’s algorithm were allowed, applying the strict maximum parsimony principle.

**Proteomic data analysis.** Protein abundance changes were assessed by the spectral counting (SpC) approach. For proteins having more than one entry, only those with the highest number of unique peptides and SpCs were selected for downstream analyses. Differential analysis was performed on proteins identified in at least two biological replicates and SpC ≥ 2 (expressed as Peptide Spectrum Matches, PSMs, in Supplementary File) in at least one sample of the experimental group. The normalised spectral abundance factor (NSAF) and the Rsc (that is, the log2 of the protein abundance ratio) were calculated in order to evaluate the relative abundance of single proteins in all samples and the abundance changes of proteins between groups, respectively. Statistical significance was assessed by the beta-binomial test with FDR correction according to Benjamini-Hochberg. Only proteins with Rsc ≥ 1.5 or ≤ −1.5 in mastitis and a p-value ≤ 0.05 were considered for downstream analyses. Gene ontology (GO) analysis on differentially expressed proteins was carried out based on the biological processes and molecular functions reported by UniProtKB database and integrated with a manual curation of the protein list. The same approach was applied to evaluate protein-protein interaction network using the STRING database (Version 11, http://string-db.org/), after replacing Bubalus bubalis UniProt IDs with the corresponding Bos taurus UniProt IDs, by sequence alignment of identified peptides using Basic Local Alignment Search Tool (BLAST). In this analysis, only functional interactions with high confidence (combined score >0.7) were evaluated. The Wilcoxon test was performed to demonstrate a statistically significant differences between SAU Neg and NAS/Neg Rsc, by using the MedCalc Statistical Software version 18.9 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018).
Data availability
The data have been deposited to the ProteomeXchange with identifier PXD012355.

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Author contributions
Proteomic analysis experiments: S.P. and C.C. Clinical procedures and sampling: P.C. and J.G. Microbial culture: S.U., P.C., P.M., V.B. and M.F.A. Western blot validation: G.M.G.P. Study design: S.P., M.F.A. Manuscript drafting: S.P. and M.F.A. Critical revision, editing and approval of the final manuscript: all authors.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to D.P. or M.F.A. 
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