Comparative proteomic analysis of proteins expression changes in the mammary tissue of cows infected with Escherichia coli mastitis

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Cows infected with Escherichia (E.) coli usually experience severe clinical symptoms, including damage to mammary tissues, reduced milk yield, and altered milk composition. In order to investigate the host response to E. coli infection and discover novel markers for mastitis treatment, mammary tissue samples were collected from healthy cows and bovines with naturally occurring severe E. coli mastitis. Changes of mammary tissue proteins were examined using two-dimensional gel electrophoresis and label-free proteomic approaches. A total of 95 differentially expressed proteins were identified. Of these, 56 proteins were categorized according to molecular function, cellular component, and biological processes. The most frequent biological processes influenced by the proteins were response to stress, transport, and establishment of localization. Furthermore, a network analysis of the proteins with altered expression in mammary tissues demonstrated that these factors are predominantly involved with binding and structural molecule activities. Vimentin and α-enolase were central “functional hubs” in the network. Based on results from the present study, disease-induced alterations of protein expression in mammary glands and potential markers for the effective treatment of E. coli mastitis were identified. These data have also helped elucidate defense mechanisms that protect the mammary glands and promote the pathogenesis of E. coli mastitis.

Keywords: dairy cows, Escherichia coli, mammary tissue, mastitis, proteome

Introduction

Escherichia (E.) coli mastitis is a common disease in dairy cattle that is most frequently observed during early lactation, and infected animals exhibit a wide range of disease severity [8]. After intramammary inflammation, the milk composition changes, resulting in poor milk quality, increased whey protein, and decreased casein levels associated with elevated proteolytic activity [5,13,31]. Additionally, the levels of various components of the host defense system including inflammatory cytokines, such as interleukin (IL)-1β, interferon-γ (INF-γ), IL-12, and transforming growth factor-α (TGF-α), along with anti-bacterial proteins, including cathelicidin 1, lactoperoxidase, and lactoferrin, increase in milk [1,3,31]. These changes are mainly caused by intramammary inflammation and damage to the mammary gland.

In previous studies, altered mRNA and protein levels in mammary tissues of cows infected with mastitis have been reported. At the transcriptional level, individual gene and global gene-expression analyses have been conducted to study the effects of intramammary inflammation [7,12]. In particular, transcriptional analyses of uninfected mammary gland quarters, neighboring uninfected quarters, and quarters infected with the mastitis pathogens E. coli and Staphylococcus (S.) aureus have been performed. The results demonstrated that changes in transcription are principally associated with immune response functions [7,12,15]. When evaluating proteins as chief actors within the cell, some groups have focused on examining the changes of mammary proteins from cows infected with mastitis. For example, psoriasin, a skin-resident anti-bacterial chemotactic protein belonging to the S100 family, was detected in the teat cistern of E. coli-infected cows with immunoblotting and immunohistochemistry [26]. Granulocyte chemotactic protein-2 and IL-8 were found to be expressed in mammary epithelial cell lines and neutrophils in response to exposure to lipopolysaccharides [38]. In particular, proteomics has been used to investigate mammary cancer cases [10,35], liver health, and changes in the mammary gland during lactation [25].
Previously, we compared changes in mammary proteins between healthy and mastitic cows using a two-dimensional gel electrophoresis (2-DE) approach [37]. However, a proteomic analysis of mammary proteins has not been performed to evaluate host defense responses.

Proteome approaches allow us to determine the whole-protein profiles of mammary tissue from cows infected with mastitis and detect any proteins affected by intramammary inflammation. We hypothesized that comparison of mammary proteins from cows with naturally occurring *E. coli* mastitis and healthy cows using a proteomics-based analysis would provide novel information. Thus, the objective of this investigation was to evaluate changes in mammary tissue from cows with severe mastitis compared to healthy bovines using 2-DE-based and label-free quantitative proteomics approaches. The results may provide promising information that will allow us to better understand the host defense mechanisms and discover molecular markers for treating mastitis.

**Materials and Methods**

**Sample collection and preparation**

Mammary tissue samples were obtained from the cistern tissues of slaughtered cows. Two cm or more of the mammary epithelium were collected from three healthy and mastitic cows from the Anhui area (China). The cows with *E. coli* mastitis and healthy cows were diagnosed according to visibly abnormal milk (e.g., clots, flakes, or watery texture) accompanied by swelling in the affected mammary quarter [13]. This diagnosis was made based on bacteriological culture of the milk on blood agar for all organisms along with McConkey and EMB agar for *E. coli*. The samples were also subjected to Gram staining and microscopy evaluation [29]. The healthy cows were identified based on somatic cell counts less than 200,000 cells/mL in milk measured using a Fossomatic 5000 instrument (Foss, Denmark). All samples were washed with PBS to remove the milk and debris, and then stored in liquid nitrogen.

The mammary tissues were manually homogenized with a mortar and pestle in liquid nitrogen. The resulting powder was transferred into a tube, three volumes of lysis solution (8 mol/L urea, 2 mol/L thiourea, 4% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 40 mmol/L Tris, 65 mmol/L dithiothreitol [DTT], and 2% IPG buffer). Next, IPG strips (pH 3−10) 17 cm in length were used to separate the proteins in the first dimension using a Protean IEF cell (Bio-Rad Laboratories, USA) according to the manufacturer’s instructions. After focusing, the IPG strips were placed in an equilibration solution (6 mol/L urea, 2% SDS, 20% glycerol, 50 mmol/L Tris-HCl, and 0.01% bromophenol blue) containing 2% DTT and 2.5% iodoacetamide for 12 min with shaking. For the second dimension, the strips were transferred to 12% SDS-PAGE gels and attached to the gel surface with 0.5% low-melting point agarose. The proteins were separated in a Protean II xi instrument (Bio-Rad Laboratories) at 50 V for 30 min and then at 200 V for 5 h.

The gels were incubated in a solution with 40% ethanol and 10% acetic acid for at least 3 h, and then stained with colloidal Coomassie blue G-250 solution [9]. Triplicate gels were run for each sample. The stained gels were scanned using a GS800 calibrated densitometer (Bio-Rad Laboratories) with 256 grayscale and 600-dpi parameters. Spot detection was performed using PDQuest 8.0.1 software (Bio-Rad Laboratories). To calculate the corrected protein intensities, the background values were removed and resulting intensities were then plotted as a function of the protein concentration. Protein spots that exhibited at least a two-fold decrease or increase in staining intensity were selected for further evaluation.

**In-gel digestion**

The selected protein spots were excised, transferred to tubes, and washed twice with MilliQ water for 15 min. Subsequently, the gel pieces were dehydrated with 50% acetonitrile in 0.1 mol/L ammonium bicarbonate for 15 min at room temperature and dehydrated in acetonitrile. The dried gel pieces were then rehydrated in 0.01% sequence-grade trypsin (Promega, USA) and incubated at 37°C for 16 h. The digestion was terminated by the addition of 1% trifluoroacetic acid.

**Mass spectrometry identification and database search**

The extracted peptides were loaded onto an AnchorChip plate for mass spectrometry analysis. Peptide mass spectra were obtained using an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Germany). The most prominent tryptic peptides were subjected to MS/MS analysis. The spectra were processed using FlexAnalysis 2.2 software tools (Bruker Daltonics). Protein identification was performed using Mascot software to search the National Center for Biotechnology Information (NCBI, USA) non-redundant protein database (http://www.ncbi.nlm.nih.gov). Critical parameters included the following: monoisotopic mass accuracy, < 100 ppm; missed cleavages, 1; minimum signal-to-noise (S/N) ratio, 15; carbamidomethylation of cysteine, and no modifications on other amino acids.
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Fig. 1. Changes in the protein spots detected on two-dimensional gel electrophoresis (2-DE) gels of mammary tissues from healthy cows and animals with Escherichia (E.) coli mastitis. (A) 2-DE gel for mammary tissues from healthy cows. (B) 2-DE gel for mammary tissues from cows with severe E. coli mastitis. The labels and arrows indicating spots corresponding to proteins with altered expression levels.
upregulated in mammary tissues from cows with severe *E. coli* mastitis. Spots corresponding to these proteins were then subjected to mass spectrometry for identification. The levels of cofflin-1, α1,- and β-casein, serotransferrin, and fatty acid-binding protein were decreased whereas expression of inflammatory mediators and host defense proteins, including manganous superoxide dismutase, cathelicidin-1, S100 calcium-binding protein A11 (S100A11), S100 calcium-binding protein A12 (S100A12), and S100 calcium-binding protein A8 (S100A8), were increased in cows with mastitis (Table 1). Of these, 12 proteins (such as ATP synthase, protein A12 (S100 A12), and S100 calcium-binding protein A11 (S100A11), S100 calcium-binding protein A12, S100 calcium-binding protein A8 (S100A8), were increased in cows with mastitis (Table 1). Of these, 12 proteins (such as ATP synthase, protein A12 (S100 A12), and S100 calcium-binding protein A11 (S100A11), S100 calcium-binding protein A12, S100 calcium-binding protein A8 (S100A8), were increased in cows with mastitis (Table 1).

The label-free results revealed that the levels of 86 proteins were changed in mammary tissues from healthy cows versus cows with severe mastitis. Spots corresponding to these proteins were then subjected to mass spectrometry for identification. The levels of 86 proteins were identified through 2-DE and label-free methods were assigned to each category as listed in Fig. 3. When molecular functions

### Table 1. Spots corresponding to proteins with altered expression in mammary tissues from healthy cows versus cows with severe *E. coli* mastitis as identified by MALDI TOF/TOF MS

| Spot | Protein name | NCBInr accession number | Molecular weight (kDa) | Isoelectric point | Protein score | Change fold in intensity | p value |
|------|--------------|--------------------------|------------------------|------------------|---------------|------------------------|---------|
| m1   | alpha S1 casein | gi:159793197             | 23.473                 | 4.90             | 369           | -1.9                  | 0.046   |
| m2   | casein beta   | gi: 223906                | 23.559                 | 5.13             | 196           | -1.9                  | 0.037   |
| m3   | casein beta   | gi: 223906                | 23.559                 | 5.13             | 121           | -3.6                  | 0.041   |
| m4   | isocitrate dehydrogenase [NADP] cytoplasmic | gi: 75832090           | 47.072                 | 6.13             | 487           | -2.4                  | 0.017   |
| m5   | serotransferrin precursor | gi: 114326282          | 79.856                 | 6.75             | 304           | -1.9                  | 0.023   |
| m6   | serotransferrin precursor | gi: 114326282          | 79.856                 | 6.75             | 427           | -2.1                  | 0.039   |
| m7   | cofflin-1     | gi: 5031635               | 18.719                 | 8.22             | 273           | -2.0                  | 0.007   |
| m8   | peptidyl-prolyl cis-trans isomerase A | gi: 47523764        | 18.086                 | 8.34             | 244           | -2.2                  | 0.015   |
| m9   | hemoglobin subunit alpha | gi: 116812902         | 15.175                 | 8.07             | 374           | D                     | NA      |
| m10  | hemoglobin subunit beta | gi: 27819608          | 14.704                 | 7.01             | 323           | D                     | NA      |
| m11  | fatty acid-binding protein | gi: 4758328            | 14.906                 | 6.29             | 305           | D                     | NA      |
| m12  | fatty acid binding protein | gi: 458862             | 13.343                 | 7.03             | 306           | D                     | NA      |
| m13  | fatty acid binding protein | gi: 296480594         | 14.751                 | 5.52             | 340           | D                     | NA      |
| m14  | fatty acid binding protein | gi: 296480594         | 14.751                 | 5.52             | 86            | D                     | NA      |
| m15  | manganese superoxide dismutase | gi: 7555818          | 27.746                 | 8.70             | 302           | +2.0                  | 0.010   |
| m16  | annexin A2 | gi: 27807289             | 38.873                 | 6.92             | 366           | U                     | NA      |
| m17  | manganese superoxide dismutase | gi: 7555818          | 27.746                 | 8.70             | 216           | U                     | NA      |
| m18  | cathelicidin-1 precursor | gi: 27807341          | 17.931                 | 7.57             | 321           | U                     | NA      |
| m19  | cathelicidin-1 precursor | gi: 27807341          | 17.931                 | 7.57             | 320           | U                     | NA      |
| m20  | fatty acid-binding protein | gi: 27805809         | 14.827                 | 6.73             | 323           | U                     | NA      |
| m21  | fatty acid-binding protein | gi: 27805809         | 14.827                 | 6.73             | 244           | U                     | NA      |
| m22  | S100 calcium binding protein A11 | gi: 296489536       | 11.361                 | 6.08             | 227           | U                     | NA      |
| m23  | protein S100-A12 | gi: 27807183          | 10.679                 | 5.92             | 322           | U                     | NA      |
| m24  | beta-casein A2 variant | gi: 248147             | 5.112                  | 9.52             | 82            | U                     | NA      |
| m25  | hemoglobin subunit beta | gi: 27819608         | 14.704                 | 7.01             | 307           | U                     | NA      |
| m26  | protein S100-A8 | gi: 165973998           | 10.567                 | 5.15             | 287           | U                     | NA      |

*Negative values indicate decreased expression in cows with *E. coli* mastitis, positive values correspond to increased expression in animals with *E. coli* mastitis, “D” indicates that the protein was detected only in healthy cows, and “U” means that the protein was detected only in animals with *E. coli* mastitis. NA, not available.
Table 2. Differentially expressed proteins in mammary tissue from healthy cows and cows with *E. coli* mastitis as identified by LC-MS/MS

| Protein description | Accession number | Molecular weight (kDa) | Isoelectric point | Coverage (%) | Spectral counting | Fold change* | p value |
|---------------------|------------------|------------------------|-------------------|--------------|------------------|-------------|---------|
| ATP synthase subunit beta, mitochondrial precursor | gi:28461221 | 56.249 | 4.63 | 15.34 | 0.0 | 15.3 | NA | NA |
| fatty acid-binding protein, adipocyte | gi:227993 | 14.581 | 4.95 | 13.74 | 0.0 | 10.0 | NA | NA |
| ATP synthase subunit alpha, mitochondrial precursor | gi:296473661 | 69.563 | 5.61 | 11.57 | 0.0 | 9.0 | NA | NA |
| isostrate dehydrogenase [NADP] cytoplasmic | gi:75056526 | 46.755 | 4.73 | 3.86 | 0.0 | 5.0 | NA | NA |
| isocitrate dehydrogenase 1 | gi:69573995 | 41.316 | 4.62 | 4.36 | 0.0 | 4.3 | NA | NA |
| fatty acid-binding protein, heart | gi:27805809 | 14.377 | 5.65 | 14.39 | 0.0 | 4.0 | NA | NA |
| ATP synthase alpha subunit precursor, partial | gi:162719 | 38.852 | 7.76 | 9.75 | 0.0 | 3.7 | NA | NA |
| fatty acid synthase | gi:296476108 | 274.380 | 5.26 | 1.15 | 0.0 | 3.3 | NA | NA |
| 60S acidic ribosomal protein P1 | gi:296483645 | 11.507 | 4.16 | 14.04 | 0.0 | 2.7 | NA | NA |
| smooth muscle protein SM22 homolog - bovine (fragments) | gi:543113 | 19.332 | 5.06 | 7.60 | 0.0 | 1.3 | NA | NA |
| 60 kDa heat shock protein, mitochondrial | gi:262205483 | 60.939 | 5.18 | 2.09 | 0.0 | 2.3 | NA | NA |
| 27 kDa lactofrin precursor | gi:450463 | 17.141 | 5.96 | 7.19 | 0.0 | 2.0 | NA | NA |
| beta-casein | gi:162805 | 25.131 | 4.90 | 15.63 | 1.3 | 11.0 | -2.5 | 0.005 |
| beta-casein A3 | gi:459292 | 25.082 | 4.74 | 15.63 | 1.3 | 11.0 | -2.5 | 0.005 |
| Beta-lactoglobulin | gi:125910 | 19.870 | 4.59 | 15.73 | 1.3 | 10.0 | -2.4 | 0.008 |
| beta-lactoglobulin variant B precursor | gi:669061 | 19.870 | 4.59 | 15.73 | 1.3 | 10.0 | -2.4 | 0.008 |
| beta-lactoglobulin, partial | gi:162748 | 17.156 | 4.44 | 18.54 | 1.3 | 10.0 | -2.4 | 0.008 |
| fatty-acid-binding protein | gi:1683169 | 5.066 | 6.23 | 19.15 | 0.7 | 5.0 | -2.1 | 0.038 |
| alpha-s2-like casein precursor | gi:162929 | 26.002 | 4.95 | 9.91 | 2.0 | 10.0 | -1.9 | 0.039 |
| inhibitor,angiotensin converting enzyme | gi:223434 | 1.384 | 6.00 | 100 | 3.3 | 14.3 | -1.8 | 0.024 |
| alpha-S1-casein | gi:115646 | 24.513 | 4.37 | 32.24 | 9.3 | 36.3 | -1.8 | 0.031 |
| alpha S1 casein, partial | gi:159793191 | 23.598 | 4.31 | 33.50 | 9.7 | 36.3 | -1.7 | 0.030 |
| similar to 40S ribosomal protein SA (P40) | gi:28189793 | 18.598 | 6.96 | 9.36 | 1.3 | 5.7 | -1.6 | 0.010 |
| alpha S1-casein B | gi:6015490 | 24.513 | 4.37 | 32.24 | 9.3 | 33.0 | -1.6 | 0.041 |
| Vimentin | gi:109659186 | 53.695 | 4.68 | 39.06 | 27.3 | 7.7 | +1.9 | 0.024 |
| unnamed protein product | gi:298545133 | 47.247 | 5.11 | 4.83 | 0.7 | +2.0 | 0.033 |
| alpha-enolase | gi:109940077 | 47.296 | 5.05 | 4.38 | 4.0 | 0.7 | +2.1 | 0.050 |
| thymosin, beta 4-like | gi:296482436 | 5.049 | 3.34 | 29.55 | 3.3 | 0.3 | +2.3 | 0.003 |
| keratin 13 | gi:296476325 | 48.969 | 4.48 | 2.43 | 5.7 | 0.7 | +2.5 | 0.000 |
| keratin 13-like | gi:296476419 | 47.183 | 4.51 | 2.53 | 5.7 | 0.7 | +2.5 | 0.000 |
| keratin, type I cytoskeletal 14 | gi:125079 | 10.715 | 5.30 | 11.83 | 5.7 | 0.7 | 0.0 | 0.000 |
| keratin 17 | gi:129996473 | 48.682 | 4.57 | 4.76 | 7.7 | 0.7 | +2.9 | 0.034 |
| thymosin beta 4 X-linked | gi:125658150 | 5.050 | 5.02 | 61.36 | 4.7 | 0.3 | +2.8 | 0.025 |
| thymosin beta-4 | gi:85700159 | 5.050 | 5.02 | 61.36 | 4.7 | 0.3 | +2.8 | 0.025 |
| immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3a | gi:343197010 | 11.307 | 5.19 | 47.17 | 10.3 | 0.7 | +3.3 | 0.021 |
| Unknown (protein for MGC:159378) | gi:151556360 | 24.717 | 4.42 | 21.55 | 10.0 | 0.7 | +3.3 | 0.033 |
| Unknown (protein for MGC:159411) | gi:148878143 | 24.531 | 4.53 | 21.28 | 10.0 | 0.7 | +3.3 | 0.033 |
| Unknown (protein for MGC:159455) | gi:148744128 | 24.790 | 4.61 | 21.19 | 10.0 | 0.7 | +3.3 | 0.033 |
| immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d | gi:343197030 | 11.293 | 5.16 | 32.08 | 8.7 | 0.7 | +3.1 | 0.004 |
| IGL@ protein | gi:148744106 | 24.577 | 4.46 | 21.37 | 11.7 | 0.7 | +3.5 | 0.003 |
| immunoglobulin lambda-like polypeptide 1 | gi:134025924 | 24.748 | 4.23 | 21.28 | 10.3 | 0.3 | +3.8 | 0.019 |
| SERPINA3-1 | gi:117916661 | 46.208 | 4.96 | 3.65 | 1.3 | 0.0 | NA | NA |
| SERPINA3-2 | gi:121531626 | 46.208 | 4.96 | 3.65 | 1.3 | 0.0 | NA | NA |
| S100 calcium-binding protein A9 | gi:109939714 | 17.103 | 6.01 | 12.18 | 1.0 | 0.0 | NA | NA |

*Fold change* indicates the relative change in protein expression between healthy cows and cows with *E. coli* mastitis. The *p* value is calculated using a Student's t-test.
were evaluated, the highest enrichment was found for enzyme regulatory activities that included ones involving structural molecules, enzyme inhibitors, transporters, peptidase inhibitors, antioxidants, and hydro-lyases. Another major

Table 2. Continued

| Protein description                  | Accession number | Molecular weight (kDa) | Isoelectric point | Coverage (%) | Spectral counting | Fold change* | p value |
|--------------------------------------|------------------|------------------------|-------------------|--------------|------------------|--------------|---------|
| annexin A2                           | gi:113948        | 38.588                 | 4.66              | 9.44         | 1.7              | 0.0          | NA      |
| collagen, type VI, alpha 3-like isoform 1 | gi:296488811    | 341.389               | 5.04              | 0.54         | 1.7              | 0.0          | NA      |
| collagen, type VI, alpha 3-like isoform 2 | gi:296488812    | 319.127               | 5.13              | 0.58         | 1.7              | 0.0          | NA      |
| collagen, type VI, alpha 3-like isoform 3 | gi:296488813    | 319.731               | 5.14              | 0.57         | 1.7              | 0.0          | NA      |
| collagen, type VI, alpha 3-like isoform 4 | gi:296488814    | 276.033               | 5.39              | 0.67         | 1.7              | 0.0          | NA      |
| keratin 6A-like                       | gi:296478794     | 64.683                | 5.13              | 1.89         | 1.7              | 0.0          | NA      |
| keratin, type II cytoskeletal 75     | gi:122132186     | 59.000                | 5.27              | 2.21         | 1.7              | 0.0          | NA      |
| cathelicidin-2                       | gi:461621        | 20.017                | 8.37              | 9.66         | 2.0              | 0.0          | NA      |
| galectin-1                           | gi:126174        | 14.734                | 4.95              | 13.33        | 2.0              | 0.0          | NA      |
| Haptoglobin                          | gi:122137096     | 44.831                | 4.78              | 4.99         | 2.0              | 0.0          | NA      |
| keratin 6C isoform 1                 | gi:296478784     | 60.788                | 5.18              | 1.58         | 2.0              | 0.0          | NA      |
| keratin 6C isoform 2                 | gi:296478785     | 58.860                | 5.24              | 1.64         | 2.0              | 0.0          | NA      |
| keratin 84                           | gi:29647886      | 64.091                | 5.30              | 1.52         | 2.0              | 0.0          | NA      |
| keratin, type II cytoskeletal 59 kDa, component IV | gi:125102       | 18.658                | 5.15              | 4.95         | 2.0              | 0.0          | NA      |
| keratin, type II cytoskeletal 68 kDa, component 1A | gi:125113     | 18.124                | 7.08              | 4.95         | 2.0              | 0.0          | NA      |
| mammary serum amyloid A3.2 precursor | gi:347300329     | 14.581                | 6.72              | 9.16         | 2.0              | 0.0          | NA      |
| rho GDP-dissociation inhibitor 2     | gi:13626951      | 22.779                | 4.58              | 10.50        | 2.0              | 0.0          | NA      |
| serum amyloid A 3                    | gi:218963155     | 12.655                | 8.26              | 10.71        | 2.0              | 0.0          | NA      |
| calcium uniporter channel component  | gi:833995        | 2.038                 | 4.14              | 100          | 2.3              | 0.0          | NA      |
| cathelicidin-4                       | gi:417190        | 16.468                | 5.17              | 11.11        | 2.3              | 0.0          | NA      |
| endopin 1b                           | gi:296475221     | 46.127                | 5.17              | 4.62         | 2.7              | 0.0          | NA      |
| endopin 1b-like                      | gi:296475250     | 26.269                | 5.40              | 8.05         | 2.7              | 0.0          | NA      |
| glyceroldehyde-3-phosphate dehydrogenase like-17 protein | gi:4877550     | 11.507                | 8.97              | 15.6         | 2.7              | 0.0          | NA      |
| PREDICTED: serpin A3-3               | gi:119903758     | 30.553                | 5.54              | 6.88         | 2.7              | 0.0          | NA      |
| serpin A3-6                          | gi:296475157     | 46.317                | 4.91              | 4.38         | 2.7              | 0.0          | NA      |
| serpin peptidase inhibitor, clade A (alpha-1 antiprotease, antitrypsin), member 3 | gi:296475228     | 46.315                | 5.24              | 4.61         | 2.7              | 0.0          | NA      |
| Peripherin                           | gi:156139125     | 53.598                | 4.96              | 2.13         | 3.0              | 0.0          | NA      |
| FGG protein                          | gi:74267780      | 49.136                | 4.33              | 5.52         | 3.3              | 0.0          | NA      |
| similar to galactose-binding lectin  | gi:28189829      | 14.734                | 4.95              | 13.33        | 3.3              | 0.0          | NA      |
| alpha-1-acid glycoprotein            | gi:121957959     | 23.168                | 4.76              | 8.91         | 3.7              | 0.0          | NA      |
| cathelicidin-1-like                  | gi:296491743     | 17.617                | 6.58              | 21.94        | 3.7              | 0.0          | NA      |
| cathelicidin-1                       | gi:416706        | 17.589                | 5.87              | 21.94        | 3.7              | 0.0          | NA      |
| protein S100-A2                      | gi:134140        | 10.886                | 4.45              | 16.49        | 3.7              | 0.0          | NA      |
| ubiquitin-527a fusion protein        | gi:3885465       | 17.953                | 9.37              | 10.26        | 3.7              | 0.0          | NA      |
| PREDICTED: keratin, type I cytoskeletal 12 | gi:119912327    | 56.966                | 4.30              | 1.88         | 4.0              | 0.0          | NA      |
| PREDICTED: uncharacterized protein   | gi:297406766     | 25.655                | 7.71              | 7.02         | 4.0              | 0.0          | NA      |
| lipocalin 2 (lncogene 24p3)-like     | gi:296482151     | 23.031                | 4.83              | 16.00        | 4.7              | 0.0          | NA      |
| Fibrinogen alpha chain               | gi:93141264      | 66.971                | 6.21              | 8.13         | 8.3              | 0.0          | NA      |
| immunoglobin gamma 1 heavy chain constant region | gi:91982959    | 35.878                | 4.89              | 4.86         | 12.0             | 0.0          | NA      |
| Ig heavy chain precursor (B/MT.4A.17.HS.A5) - bovine | gi:108750       | 50.593                | 4.57              | 3.40         | 13.0             | 0.0          | NA      |
| Ig gamma-2 chain C region (clone 32.2)-bovine | gi:89611       | 36.020                | 5.46              | 9.79         | 19.3             | 0.0          | NA      |
| IgG2a heavy chain constant region    | gi:1699167       | 35.832                | 5.34              | 9.82         | 19.3             | 0.0          | NA      |
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Fig. 2. Abundance of β-casein in mammary tissues from cows with severe *E. coli* mastitis and healthy animals. (A) Representative images of spots corresponding to β-casein in the 2-DE map. (B) Densitometric analysis of the β-casein spots in the 2-DE map. (C) Western blot analysis of β-casein expression. (D) Densitometric analysis of the Western blot. Values are presented as the mean ± standard error (SE). *p < 0.05 (n = 3).

Fig. 3. Classification of differentially expressed proteins in mammary tissues from healthy cows and bovines with *E. coli* mastitis based on gene ontology annotations. (A) Molecular function. (B) Cellular component. (C) Biological process.
Fig. 4. Protein-protein network of differentially expressed proteins in mammary tissues from control cows and cows with *E. coli* mastitis. (A) Graphic depiction of the degree of connections for each protein in the network. (B) Representative image of the protein-protein interaction network. Pink lines indicate the connections confirmed by the experimental study, the blue lines indicate connections derived from the databases, and the yellow lines indicate connections described in abstracts of articles published in the literature.

functional category was binding, which included binding to calcium ions, proteins, oxygen, and the cell surface. Analysis of the cellular component categories revealed that the highest enrichment was in the intracellular, intracellular organelle, and intracellular categories. Identification of biological processes influenced by the proteins showed that the most common processes were response to stress, transport, and establishment of localization.

To investigate the relationship between the differentially expressed proteins, a network analysis was performed using the KEGGSOAP package. Known interactions obtained from the KEGG database were visualized as nodes and edges. The number of relationships for each protein was calculated. The network map and degree of relationship for each identified proteins are depicted in Fig. 4. Interestingly, most of the proteins in the network were involved in binding and structural molecule activity. Vimentin and α-enolase were central “functional hubs” in the map (i.e., these factors were involved in more relationships than the other proteins).

**Discussion**

In the current study, differentially expressed proteins in mammary tissues of healthy cows and cows with severe *E. coli* mastitis were identified using 2-DE and label-free proteomics approaches. The proteomics results for β-casein were confirmed by Western blot analyses. In addition, a protein interaction network of the differentially expressed proteins was constructed, and demonstrated that vimentin and α-enolase act as central “functional hubs” in the network.

In our investigation, the levels of caseins (αs1-, αs2-, and β-casein) were decreased in mammary tissues of cows with severe *E. coli* mastitis. This result is due to reduced protein synthesis caused by DNA-remethylation around a signal transducer and activator of transcription 5 (STAT5)-binding enhancer in the αs1-casein promoter as was observed in a previous study [34]. Additionally, numerous studies have shown that the concentration of caseins is decreased in milk from cows infected with mastitis [6,13,33].

Our results revealed that the levels of several families of antimicrobial proteins were elevated in the mammary tissue of cows with severe *E. coli* mastitis. For instance, several members of the cathelicidin family of proteins (cathelicidin 1, 2, and 4) that were increased in cows with severe mastitis form a family of cationic antimicrobial peptides that perform a wide spectrum of antimicrobial activities [39]. Cathelicidins were
also found to be upregulated in milk samples from mastitic cows in previous studies [27,30]. These results suggest that cathelicidins can eliminate mastitis pathogens and protect the mammary glands.

Our data showed that S100 A11, S100 A2, S100 A9, S100 A12, and S100 A8 were upregulated in the mammary gland of cows with mastitis. These proteins are Ca²⁺-binding proteins belonging to the S100 multigenic family that have been shown to have antimicrobial activities associated with innate immunity, and are predominantly produced by neutrophils, monocytes, and activated macrophages [21]. A recent study demonstrated that S100 A12 expression is significantly increased in milk from cows subjected to intra-mammary challenge with E. coli [5]. This increase is the result of enhanced S100 A12 expression in bovine mammary tissue in response to the challenge [17]. According to our results, the increased levels of antimicrobial proteins corresponded to a reduction in the relative abundance of caseins in the mammary gland of cows infected with mastitis, indicating that a defense response to infections is mounted in the mammary gland of cows.

More notably, the levels of cytoskeletal-associated proteins, such as thymosin β-4, peripherin, vimentin, cytokeratin-14, and keratin 13, were significantly increased in cows with severe mastitis. Thymosin β-4, a multi-functional regenerative peptide, is specifically produced and released by the thymic gland, and possesses hormonal activities that modulate the immune response [18], regulate intracellular signal transduction, and affect the cytoskeletal structure [28]. In addition, thymosin β-4 plays a vital role in wound repair as well as the regeneration of injured cells and tissues by decreasing the number of myofibroblasts in wounds to reduce scar formation and fibrosis [11,23]. In the current study, thymosin β-4 levels were elevated in cows with severe E. coli mastitis. This protein has been used to protect cells and tissues from damage while reducing apoptosis, inflammation, and microbial growth [24]. Peripherin is a type III intermediate filament protein that is widely expressed in the cell body and axons of neurons in the peripheral nervous system. Numerous studies have demonstrated that peripherin expression is upregulated during neuronal injury and mediates neuronal repair [19,36]. This factor serves as a highly specialized cytoskeletal stress protein that promotes cellular organization and homeostasis [32]. In the current study, some cytoskeleton-associated proteins that exhibited increased expression in cows with severe E. coli mastitis have cytoprotective functions, including the inhibition of apoptosis, organelle homeostasis, and scaffolding.

We further determined that vimentin and α-enolase are central “functional hubs” in the protein-protein interaction network we generated because these factors were involved in more relationships than the other proteins. Vimentin is the intermediate filament protein normally expressed in mammary epithelial cells and has been used to evaluate primary cultures of mammary epithelial cells [2]. Little information is available about the relationship between intramammary infection and vimentin. In addition, α-enolase, a non-classical plasminogen-binding protein and a blood coagulation factor, has been found in bovine neutrophils and bacteria [4,16]. The levels of both vimentin and α-enolase were increased in the mammary glands of cows with severe E. coli mastitis in the present study, and may serve as effector proteins for the immune defense or facilitate pathogen invasion. These results require further investigation.

In summary, changes of mammary proteins in healthy cows compared to cows with E. coli mastitis were investigated using proteomic approaches. Results of a protein-protein network analysis indicated that the differentially expressed proteins are associated with binding and structural molecule activities in cows infected with severe E. coli mastitis. Additionally, vimentin and α-enolase act as central “functional hubs” in this network. Based on our findings, potential effective markers may be identified for the treatment of mastitis and foster a better understanding of mechanisms underlying the host defense system activated in response to E. coli mastitis.

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Conflict of Interest

There is no conflict of interest.

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