

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

Xiao-wei Zhao, Yong-xin Yang*, Dong-wei Huang, Guang-long Cheng, Hui-ling Zhao

Institute of Animal Science and Veterinary Medicine, Anhui Academy of Agricultural Sciences, Hefei 230031, China

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)-1 β , 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].

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*Corresponding author: Tel: +86-551-65146065; Fax: +86-551-65146065; E-mail: yyongxin@yahoo.com

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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.

Proteomic 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 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-cholamido-propyl)-dimethylammonio]-I-propanesulfonate, 40 mmol/L Tris, 65 mmol/L dithiothreitol [DTT], and 2% immobilized pH gradient [IPG] buffer) were added, and the solution was mixed for 20 min with periodic vortexing. Subsequently, the homogenate was centrifuged at $14,000 \times g$ for 15 min at 4°C. The supernatant was then collected, and the protein concentrations were measured with a modified Bradford protein assay. The protein samples were stored at -80°C until analysis.

2-DE and image analysis

Mammary tissue proteins (800 μg) were diluted in 350 μL of

rehydration solution (8 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamido-propyl)-dimethylammonio]-I-propanesulfonate, 40 mmol/L Tris, 65 mmol/L 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 destained 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 as a fixed modification; MS/MS mode, and fragment mass tolerance, < 0.5 Da.

Label-free quantitation analysis

The powdered mammary tissues were dissolved in a solution of 8 mol/L urea and 2 mol/L thiourea. The supernatant was collected after centrifugation at $12,000 \times g$ for 30 min at 4°C. The protein concentration was measured using a modified Bradford protein assay. The protein samples (100 µg) were reduced with 10 mmol/L DTT at 37°C for 2.5 h and then alkylated with 50 mmol/L iodoacetamide at room temperature for 40 min. Next, the proteins were digested with sequencing grade-modified trypsin (Promega) using an enzyme-to-protein ratio of 1 : 50 at 37°C for 20 h.

An Ettan MDLC system (GE Healthcare, UK) was used to desalt with Zorbax 300 SB C18 columns (Agilent Technologies, USA) and separate on a reverse phase column. Mobile phase A contained 0.1% formic acid in MilliQ water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Approximately 20 µg of the trypsinized peptide mixture was loaded onto the column. Desalting and separation were initiated using 3% buffer B for 5 min, 4% to 50% buffer B for 90 min, 50% to 90% buffer B for 10 min, and 98% buffer A for 10 min at a flow rate of 200 nL/min. An electrospray ionization ion trap mass spectrometer (LTQ; Thermo Scientific, USA) was connected to the liquid chromatography apparatus to detect the eluted peptides. The MS/MS spectra were identified in the data-dependent mode. Each scan cycle included a full MS scan in profile mode and five MS/MS scans in centroid mode. Dynamic exclusion was set to a repeat count of 2, a repeat duration of 30 sec, and an exclusion duration of 90 sec. The peptides were identified using SEQUEST software (BioWorks 2.0; Thermo Electron, USA) to search the NCBI non-redundant protein database. The SEQUEST search parameters were based on Delta CN \geq 0.1 along with Xcorr values of 1.9, 2.2, and 3.75 for 1+, 2+, and 3+ charged ions, respectively. All proteins with more than one unique identification were considered for protein quantification using spectral counting. The spectral count of each protein was calculated as the number of .dta files assigned to any peptide from the protein with high SEQUEST scores that fulfilled the DTASelect filtering criteria. The fold-change of protein expression was determined using a published formula for calculating spectral counting ratios [20].

Western blot analysis

Thirty µg of mammary proteins from control and mastitic cows were separated by 12% SDS-PAGE for 1 h, and then transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet transfer apparatus (Bio-Rad Laboratories). Next, the PVDF membrane was blocked with 3% chicken serum and incubated with anti-bovine β -casein monoclonal antibodies (1 : 1,000) at 37°C for 1 h. After washing, the membrane was

incubated with horseradish peroxidase-coupled secondary antibody (1 : 5,000) for 1 h. Antibody binding was detected using diaminobenzidine as the substrate. The anti-bovine β -casein monoclonal antibodies were prepared by the Beijing Protein Institute (China).

Statistical analysis

Significance differences in staining intensities for mammary proteins from control cows and animals with severe *E. coli* mastitis according to 2-DE gel images and label-free mass spectrometry-based protein spectral counts were evaluated by t-tests using the Statistical Package of Social Science 16.0 (SPSS) program [22]. *P* values less than 0.05 were considered significant. A protein-protein interaction network of the differentially expressed proteins in mammary tissues, including the 2-DE and label-free data, was established by downloading the pathway data from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Enzyme-enzyme and protein-protein interactions were then analyzed with the KEGGSOAP package of R software. The protein-protein network was identified and graphically visualized as nodes (proteins) and edges (relationships between proteins) using Medusa software [14].

Results

2-DE and label-free-based proteomic approaches were used to compare changes in mammary tissue proteins between healthy cows and bovines with severe *E. coli* mastitis. Representative 2-DE gels of mammary tissue proteomes from the control and *E. coli* mastitic samples are presented in Fig. 1. A total of 14 proteins were downregulated while 12 proteins were

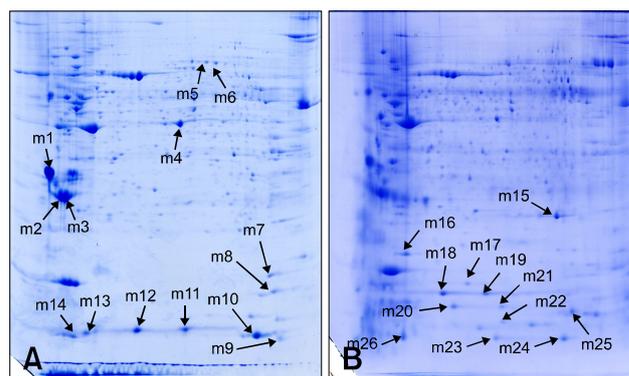


Fig. 1. Changes in the protein spots detected on two-dimensional gel electrophoresis (2-DE) gels of mammary tissues from healthy cows and animals with *Escherichia (E.) coli* mastitis. (A) 2-DE gel for mammary tissues from healthy cows. (B) 2-DE gel for mammary tissues from cows with severe *E. coli* 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 cofilin-1, α_{s1} - 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).

The label-free results revealed that the levels of 86 proteins were changed in mammary tissues from cows with *E. coli* mastitis (Table 2). Of these, 12 proteins (such as ATP synthase subunit alpha, fatty acid synthase, and isocitrate dehydrogenase) were only detected in mammary tissues of

healthy cows while 45 proteins (including cathelicidin-1, galectin-1, and S100 A9) were only detected in mammary tissues of mastitic cows. Expression of the calcium uniporter channel components, galactin, and vimentin was significantly increased whereas the levels of fatty-acid-binding protein, α_{s1} -casein, and β -casein were decreased in cows with severe *E. coli* mastitis. Our proteomics result showing the downregulation of β -casein was validated by Western blot analysis (Fig. 2).

The differentially expressed proteins in mammary tissues identified through 2-DE and label-free methods were categorized based on their molecular function, cellular components, and biological processes using gene ontology (GO) annotations. The 56 identified proteins 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	NCBI nr 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	cofilin-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	manganous 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	manganous 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
					<i>E. coli</i> cows	Healthy cows		
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	59.653	5.61	11.57	0.0	9.0	NA	NA
isocitrate dehydrogenase [NADP] cytoplasmic	gi:75056526	46.755	4.73	3.86	0.0	5.0	NA	NA
isocitrate dehydrogenase 1	gi:89573995	41.316	4.62	4.36	0.0	4.3	NA	NA
fatty acid-binding protein, heart	gi:27805809	14.770	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 lactophorin 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:28189773	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	4.38	3.7	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	5.34	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		0.000
keratin 17	gi:119936473	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

Table 2. Continued

Protein description	Accession number	Molecular weight (kDa)	Isoelectric point	Coverage (%)	Spectral counting		Fold change*	<i>p</i> value
					<i>E. coli</i> cows	Healthy cows		
annexin A2	gi:113948	38.588	4.66	9.44	1.7	0.0	NA	NA
collagen, type VI, alpha 3-like isoform 1	gi:296488811	341.389	5.04	0.54	1.7	0.0	NA	NA
collagen, type VI, alpha 3-like isoform 2	gi:296488812	319.127	5.13	0.58	1.7	0.0	NA	NA
collagen, type VI, alpha 3-like isoform 3	gi:296488813	319.731	5.14	0.57	1.7	0.0	NA	NA
collagen, type VI, alpha 3-like isoform 4	gi:296488814	276.033	5.39	0.67	1.7	0.0	NA	NA
keratin 6A-like	gi:296487894	64.683	5.13	1.89	1.7	0.0	NA	NA
keratin, type II cytoskeletal 75	gi:122132186	59.000	5.27	2.21	1.7	0.0	NA	NA
cathelicidin-2	gi:461621	20.017	8.37	9.66	2.0	0.0	NA	NA
galectin-1	gi:126174	14.734	4.95	13.33	2.0	0.0	NA	NA
Haptoglobin	gi:122137096	44.831	4.78	4.99	2.0	0.0	NA	NA
keratin 6C isoform 1	gi:296487884	60.788	5.18	1.58	2.0	0.0	NA	NA
keratin 6C isoform 2	gi:296487885	58.860	5.24	1.64	2.0	0.0	NA	NA
keratin 84	gi:296487886	64.091	5.30	1.52	2.0	0.0	NA	NA
keratin, type II cytoskeletal 59 kDa, component IV	gi:125102	18.658	5.15	4.95	2.0	0.0	NA	NA
keratin, type II cytoskeletal 68 kDa, component IA	gi:125113	18.124	7.08	4.95	2.0	0.0	NA	NA
mammary serum amyloid A3.2 precursor	gi:347300329	14.581	6.72	9.16	2.0	0.0	NA	NA
rho GDP-dissociation inhibitor 2	gi:13626951	22.779	4.58	10.50	2.0	0.0	NA	NA
serum amyloid A 3	gi:218963155	12.655	8.26	10.71	2.0	0.0	NA	NA
calcium uniporter channel component	gi:833995	2.038	4.14	100	2.3	0.0	NA	NA
cathelicidin-4	gi:417190	16.468	5.17	11.11	2.3	0.0	NA	NA
endopin 1b	gi:296475221	46.127	5.17	4.62	2.7	0.0	NA	NA
endopin 1b-like	gi:296475250	26.269	5.40	8.05	2.7	0.0	NA	NA
glyceraldehyde-3-phosphate dehydrogenase like-17 protein	gi:4877550	11.507	8.97	15.6	2.7	0.0	NA	NA
PREDICTED: serpin A3-3	gi:119903758	30.553	5.54	6.88	2.7	0.0	NA	NA
serpin A3-6	gi:296475157	46.517	4.91	4.58	2.7	0.0	NA	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	NA
Peripherin	gi:156139125	53.598	4.96	2.13	3.0	0.0	NA	NA
FGG protein	gi:74267780	49.136	4.33	5.52	3.3	0.0	NA	NA
similar to galactose-binding lectin	gi:28189829	14.734	4.95	13.33	3.3	0.0	NA	NA
alpha-1-acid glycoprotein	gi:121957959	23.168	4.76	8.91	3.7	0.0	NA	NA
cathelicidin 1-like	gi:296491743	17.617	6.58	21.94	3.7	0.0	NA	NA
cathelicidin-1	gi:416706	17.589	5.87	21.94	3.7	0.0	NA	NA
protein S100-A2	gi:134140	10.886	4.45	16.49	3.7	0.0	NA	NA
ubiquitin-S27a fusion protein	gi:3885465	17.953	9.37	10.26	3.7	0.0	NA	NA
PREDICTED: keratin, type I cytoskeletal 12	gi:119912327	56.966	4.30	1.88	4.0	0.0	NA	NA
PREDICTED: uncharacterized protein	gi:297460766	25.655	7.71	7.02	4.0	0.0	NA	NA
lipocalin 2 (oncogene 24p3)-like	gi:296482151	23.031	4.83	16.00	4.7	0.0	NA	NA
Fibrinogen alpha chain	gi:93141264	66.971	6.21	8.13	8.3	0.0	NA	NA
immunoglobulin gamma 1 heavy chain constant region	gi:91982959	35.878	4.89	4.86	12.0	0.0	NA	NA
Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine	gi:108750	50.593	4.57	3.40	13.0	0.0	NA	NA
Ig gamma-2 chain C region (clone 32.2) - bovine	gi:89611	36.020	5.46	9.79	19.3	0.0	NA	NA
IgG2a heavy chain constant region	gi:1699167	35.832	5.34	9.82	19.3	0.0	NA	NA

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

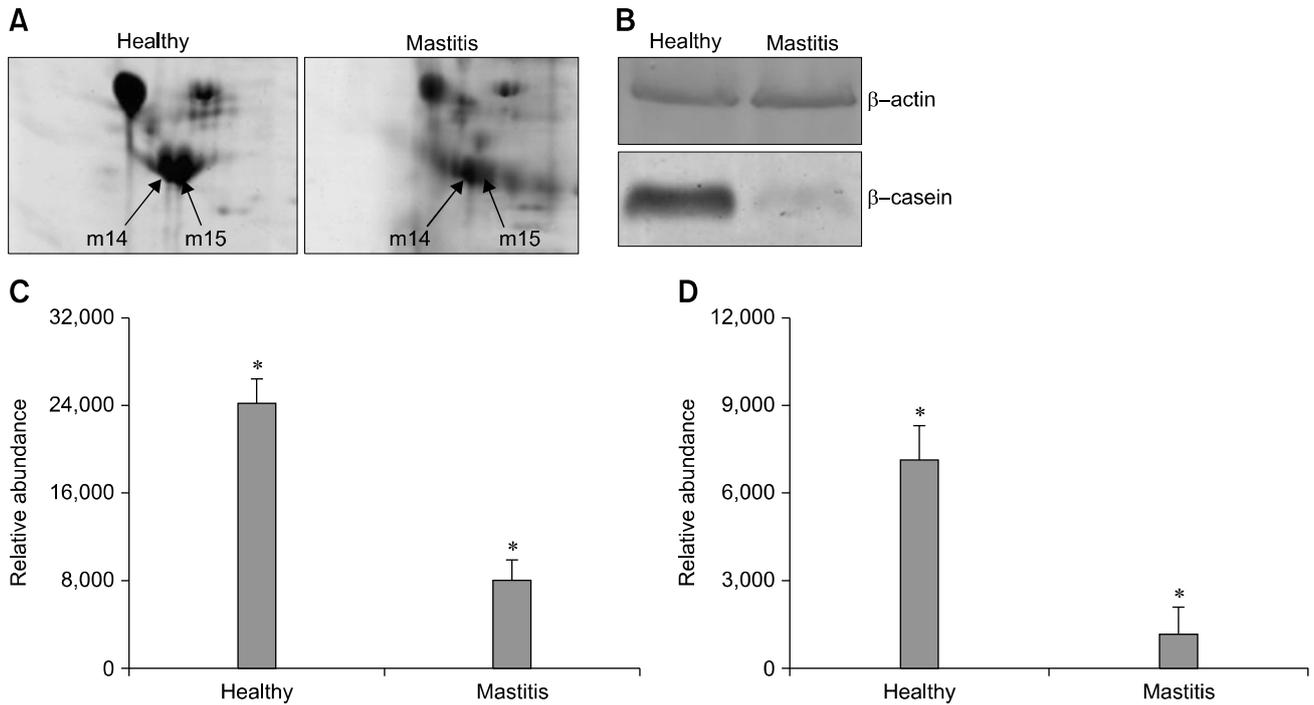


Fig. 2. Abundance of β -casein in mammary tissues from cows with severe *E. coli* mastitis and healthy animals. (A) Representative images of spots corresponding to β -casein in the 2-DE map. (B) Densitometric analysis of the β -casein spots in the 2-DE map. (C) Western blot analysis of β -casein expression. (D) Densitometric analysis of the Western blot. Values are presented as the mean \pm standard error (SE). * $p < 0.05$ ($n = 3$).

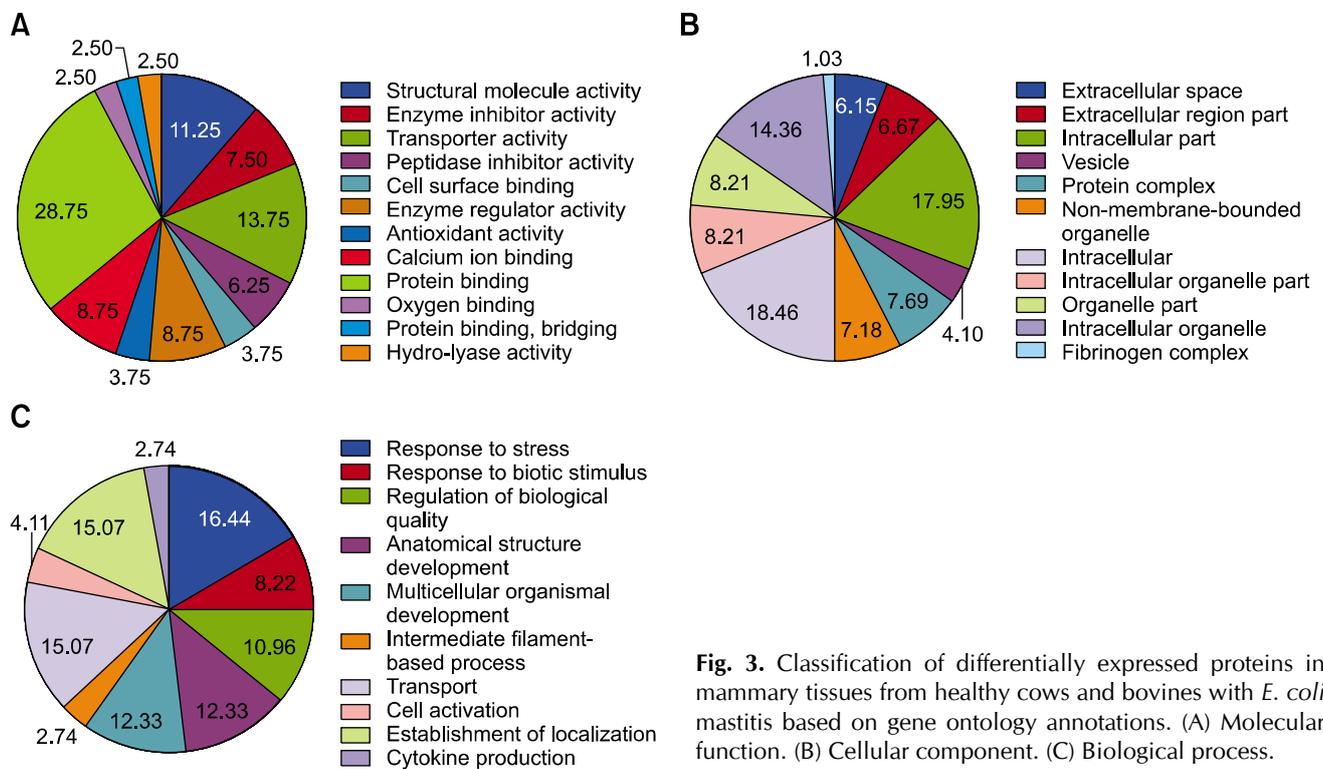


Fig. 3. Classification of differentially expressed proteins in mammary tissues from healthy cows and bovines with *E. coli* mastitis based on gene ontology annotations. (A) Molecular function. (B) Cellular component. (C) Biological process.

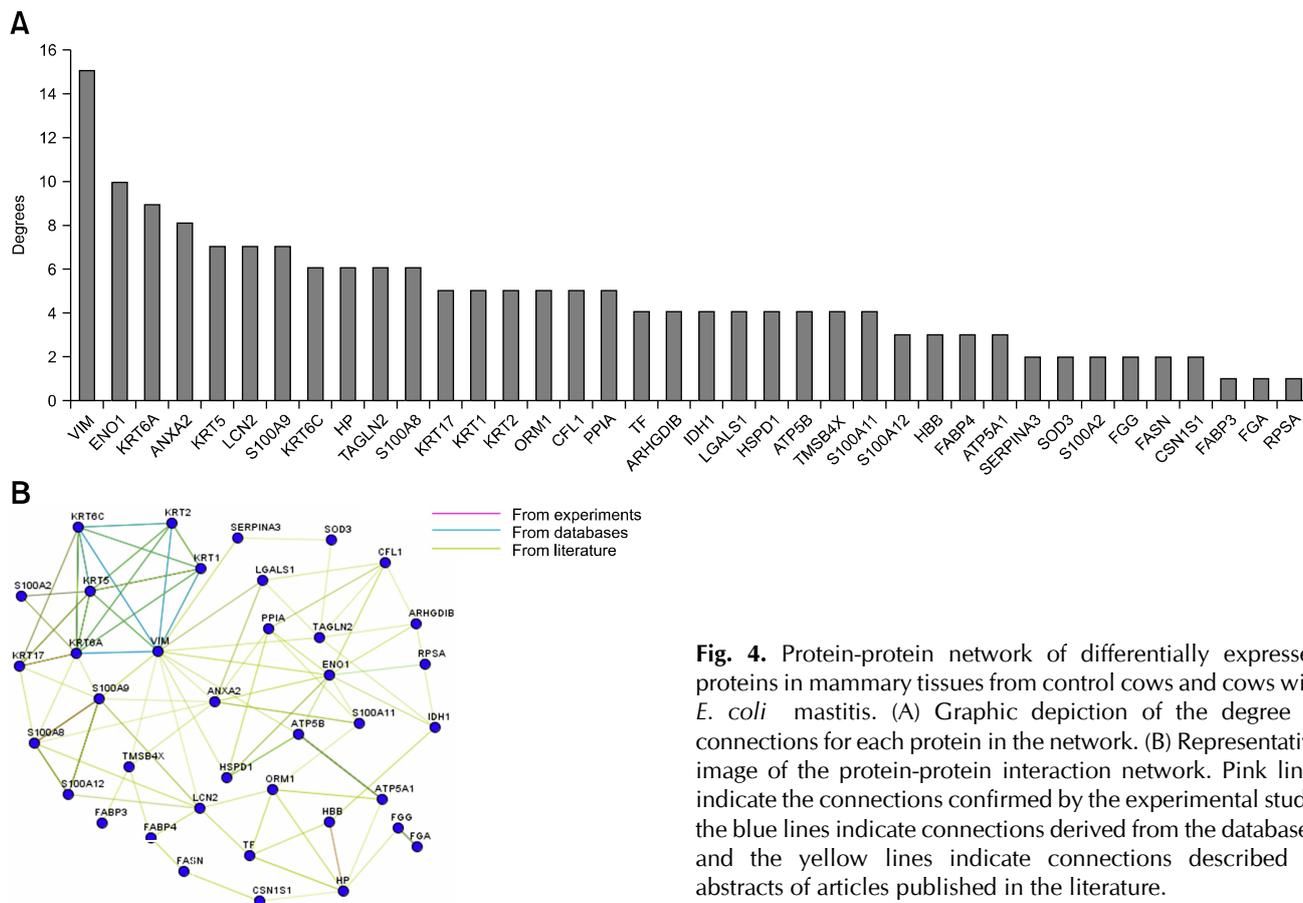


Fig. 4. Protein-protein network of differentially expressed proteins in mammary tissues from control cows and cows with *E. coli* mastitis. (A) Graphic depiction of the degree of connections for each protein in the network. (B) Representative image of the protein-protein interaction network. Pink lines 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 protein 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 (α_{s1} -, α_{s2} -, 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 α_{s1} -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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