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Anti-inflammatory mechanism of ginsenoside Rg1: Proteomic analysis of milk from goats with mastitis induced with lipopolysaccharide

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ABSTRACT

Previous investigation showed that intravenous injection of ginsenoside Rg1 had a therapeutic effect on *Escherichia coli* lipopolysaccharide-induced mastitis in lactating goats and it protected animals from lipopolysaccharide challenge via toll-like receptor 4 signaling pathway. The present study was to use proteomic approach to explore the anti-inflammatory mechanisms of Rg1. Nine dairy goats were randomly divided into three groups with 3 animals in each: groups 1 and 2 received intra-mammary infusion of lipopolysaccharide and then intravenously injected with saline or Rg1 solution; animals in group 3 were first intramammarily and then intravenously administered saline solution, and served as a control group. Milk whey at 6 h post lipopolysaccharide challenge was prepared for tandem mass tags based quantitative proteomic analysis. The results showed that 791 proteins were totally identified from the whey. Of them, 98 proteins between groups 1 (lipopolysaccharide + Saline) and 3 (Saline + Saline), and 34 proteins between groups 2 (lipopolysaccharide + Rg1) and 1 were significantly different. Group 1 than group 3 had significantly more inflammatory factors such as interleukin 6, acute phase proteins, blood coagulation factors, complement proteins, and oxidative stress markers while these factors were reduced in group 2 treated with Rg1. In addition, proteins in group 2 associated with peroxisome-proliferator-activated receptor γ activation and recovery of milk fat and production were upregulated compared to group 1. Therefore, Rg1 may exert its anti-inflammatory effect on lipopolysaccharide-induced mastitis in goats via modulating expression of proteins relating to peroxisome-proliferator-activated receptor γ and toll-like receptor 4 signaling pathway.

1. Introduction

Escherichia coli is one of the main aetiological agents causing mastitis in dairy ruminants. *E. coli* releases bacterial cell wall components, such as lipopolysaccharide (LPS), which induce markedly increased local inflammatory mediators with a strong systemic acute phase response [1]. An increasing amount of evidence has demonstrated that LPS can trigger the most potent microbial initiators of inflammatory responses which decrease milk production through mammary epithelial apoptosis [2–4] and increase milk somatic cell count (SCC) [4,5]. In addition to the hyper-inflammatory response in mastitis, prolonged or excessive inflammation induces apoptosis of endothelial cells [6] which is pathogenic and can lead to tissue damage and possibly death [7]. Toll-like receptor 4 (TLR4) is most significant during *E. coli* mastitis and is primarily activated in response to LPS. Activation of TLR4-dependent signaling pathways by LPS results in either production of pro-inflammatory factors or initiation of endothelial apoptosis [8,9]. Blocking the activation of TLR4 may inhibit the inflammatory response caused by LPS [10,11].

Ginsenoside Rg1 (Rg1) is one of the constituents extracted from *Panax ginseng* C. A. May and is reported to have anti-inflammatory, antioxidant and anti-apoptosis effects [12–14]. Rg1 has been found to compete with LPS binding to TLR4 and reduce LPS-stimulated production of inflammatory mediators such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, exhibiting anti-inflammatory effect and protecting animals against LPS-induced lethality [15,16]. Intra-mammary infusion of LPS has been used to induce mastitis model in dairy animals. The local and systemic signs in LPS-induced mastitis are similar to those observed in an acute *E. coli* induced intra-mammary infusion [17–19], such as the increasing milk SCC and dilation of blood

Abbreviations: SCC, somatic cell count; TLR4, toll-like receptor 4; Rg1, ginsenoside Rg1; IV, intravenous; TMT, tandem mass tags; HMT, Hangzhou mastitis test; IMM, intra-mammary; BW, bodyweight; PLC, post LPS challenge; APPs, acute phase proteins; Apos, apolipoproteins; NF-κB, nuclear factor-κB; PPAR, peroxisome-proliferator-activated receptor

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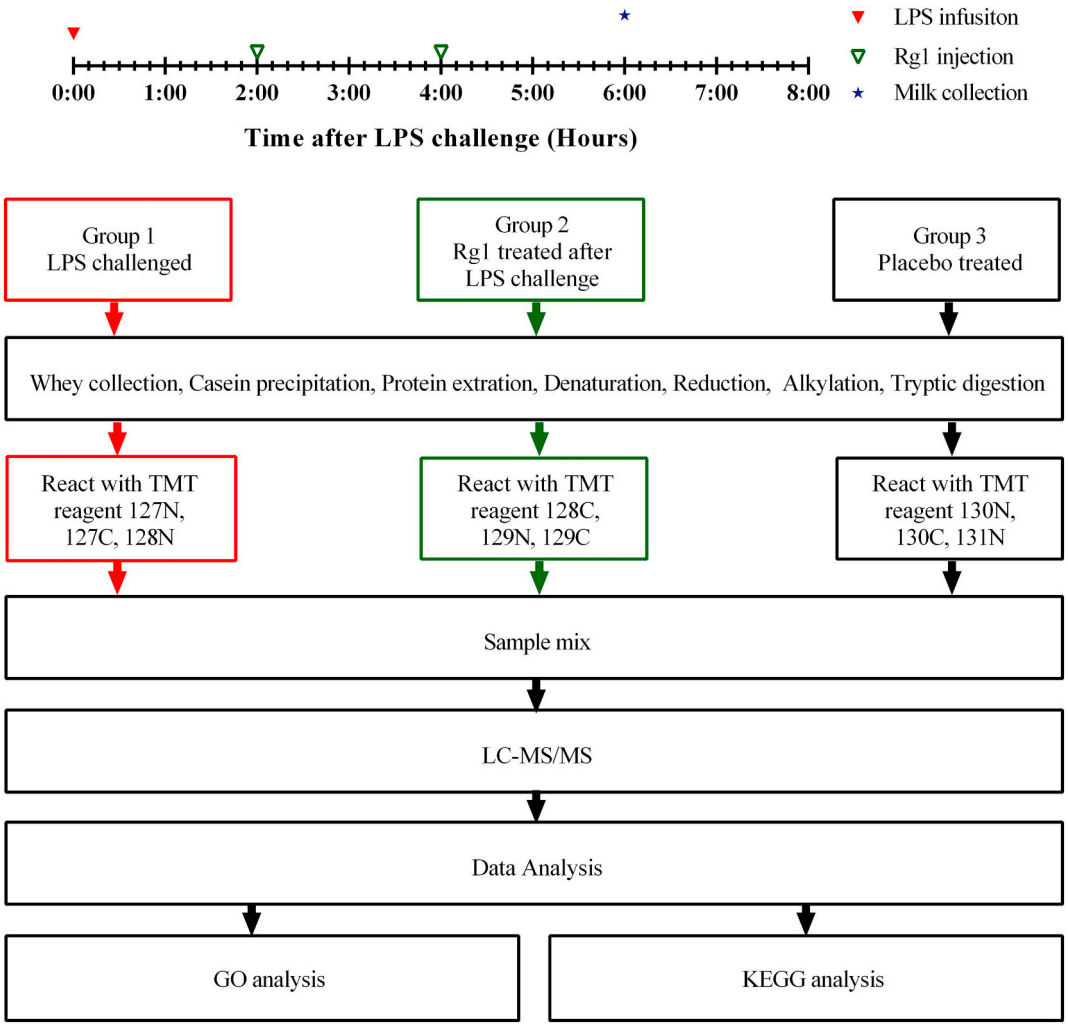

Fig. 1. Schematic diagram of experimental design.

vessels [20]. We observed the same results after intra-mammary infusion of LPS in goats. We also found that intravenous (IV) injection of Rg1 significantly diminished clinical manifestation and signs in LPS-induced caprine mastitis [19]. This study was to use Tandem Mass Tags (TMT) proteomic approach to analyze milk whey from the goats intravenously injected with Rg1 after induction of mastitis by intra-mammary infusion of LPS and aimed to detect changed proteins after Rg1 treatment and molecular pathways that are associated with the therapeutic effect of Rg1.

2. Materials and methods

2.1. Chemicals

Ginsenoside Rg1 extracted from the root of *Panax ginseng* C.A. Meyer was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chendu, China). The purity of Rg1 was > 99.7% as determined by the high-performance liquid chromatography method. Lipopolysaccharide (LPS, *Escherichia coli* O111:B4) was purchased from Sigma Chemical Co. (MO, USA).

2.2. Animals and treatments

Nine clinically healthy Saanen goats (22 to 23 months old) in their first lactation, approximately 192 to 215 days in milk and in body-weight at 49.8 ± 1.8 kg, were used. They had milk with negative bacteriological examination and Hangzhou mastitis test (HMT, an indirect test for estimating milk SCC) scores at the beginning of this study. The goats were randomly divided into three groups. The left udder half of each goat received intra-mammary (IMM) infusion of LPS (50 μg/kg bodyweight, BW) in groups 1 and 2 or saline solution in group 3 at time 0. The dose of LPS was chosen based on previous reports [21–23], and our preliminary experiment where mastitis was successfully induced with the same dose of LPS in a goat. After 2 h post LPS challenge (PLC), each goat received twice IV injections of saline solution containing Rg1 (2.5 mg/kg BW) (groups 2) or without Rg1 (groups 1 and 3) at a 2-hour interval (Fig. 1). The animals were purchased from Hangzhou Caiyang Animal Husbandry Limited Company, Hangzhou, China. All experiments related to animal procedure were performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by Zhejiang University Institutional Animal Care and Use Committee.

2.3. Collection of milk and preparation of milk whey

Teats were scrubbed with a pad of absorbent cotton soaked in 70% alcohol and dried; the udder was massaged, and the first three streams of milk from the teat was discarded before sampling. Two days before experiment, the udder half milk samples were aseptically collected for bacteriological examination and HMT analysis. For HMT analysis, mastitic paddle wells were used to receive milk from individual quarters. The procedures and interpretation were as described by Hu et al.

[24]. For bacteriological examination, milk sample was incubated on a blood agar and incubated for 24 to 48 h at 37 °C [25]. The goats with milk of HMT score 0 and bacteriological examination negative were used in this study.

Each treatment was performed in triplicate. For preparation of milk whey, 10 mL of raw milk was collected from the left udder half of each goat at 6 h PLC. The milk samples were centrifuged at 4000 ×*g* for 15 min to remove the cream layer. The skim milk was acidified at pH 4.6 by addition of 10% acetic acid to precipitate casein [26]. Then the whey protein was precipitated from supernatant by trichloroacetic acid (final concentration 11%) and dissolved by ST buffer (2% SDS, 10 mM Tris, pH 6.8) after washed with chilled acetone for further TMT analysis.

2.4. TMT analysis

TMT analysis was performed according to the manufacturer's instructions of TMT reagent (Thermo Fisher Scientific, USA). Basically, the proteins of the whey fractions were determined using a BCA Protein Assay Kit (Bio-Rad, USA). 200 μg proteins were put into the 10 kda filter (Pall, USA), and low-molecular-weight components were removed by repeated ultrafiltration. Reduced cysteine residues was blocked and the protein suspensions were digested with 5 μg trypsin. The resulting peptides were collected as a filtrate. After estimated by NanoDrop, peptides were dried by Savant (Thermo Fisher Scientific, USA). 100 μg peptide mixture of each sample was labeled using TMT reagent (Fig. 1). TMT-labeled digested samples were fractionated into 10 fractions by an increasing acetonitrile step-gradient elution according to instructions.

Each fraction was injected for LC-MS/MS analysis performed on Easy-nLC 1000 and coupled to LTQ Obitrap ETD (Thermo Fisher Scientific, USA) [27]. The peptide mixture was loaded onto Acclaim PepMap100 C18 trap column connected to the C18-reversed phase analytical column (Thermo Fisher Scientific, USA) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (100% acetonitrile) at a flow rate of 300 nL/min: 4–7% of buffer B in 2 min, 7–25% of buffer B in 98 min, 25–35% of buffer B in 4 min, 35–90% of buffer B in 2 min, 90% of buffer B for 18 min, 90–4% of buffer B in 2 min, 4% of buffer B for 6 min.

MS data was acquired in positive ion mode using a data-dependent top10 method. Dynamically choosing the most abundant precursor ions from the survey scan (350–1600 *m/z*) for higher energy collisional dissociation (HCD) fragmentation. Survey scans were acquired at a resolution of 60,000 at *m/z* 200 and resolution for HCD spectra was set to 30,000 at *m/z* 200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%.

2.5. Statistical analysis

2.5.1. MS data and mascot search

MS/MS spectra were searched using Maxquant 1.6.0.16 (Max-Planck-Institute of Biochemistry, Martinsried, Germany) against the *Ruminantia* database (UniProt Proteome ID: 9845) [28,29], and were imported into the MaxQuant-integrated Andromeda search engine. The searching parameters were set to digestion with trypsin with two missed cleavages. Carbamidomethylation of cysteine was set as a static modification, and TMT 10plex of N terminus and methionine oxidation were set as dynamic modifications. Precursor mass tolerance was set to 20 ppm, with the fragment mass tolerance to 0.02 Da.

2.5.2. Data analyzed by Perseus

Perseus 1.4.1.3 (Max-Planck-Institute of Biochemistry, Martinsried, Germany), a free software, was used to perform the statistical analysis. The protein intensities after MaxQuant analysis were imported and log2-transformed. Then the data were performed by two-sample Student's *t*-test using Perseus [30]. Proteins with a fold-change of at least 2, false discovery rate (FDR) -adjusted (p-values < 0.05), > 2 peptides and 20 scores were supposed to be differently abundant between the different groups. The analysis parameters were listed in Supplementary Table 1.

2.5.3. Enrichment statistics analysis

All milk proteins identified were assigned their gene symbol via the Uniprot Knowledgebase (http://www.uniprot.org/). Gene ontology (GO) analyses of the identified proteins was performed using WEB-based Gene Set Analysis Toolkit 2017 (http://www.webgestalt.org/option.php; [28,31]). Briefly, using gene ontology enrichment analysis, *Bos taurus* as background, 0.05 significance level, and minimum no. of genes in a category was set at 5. The enrichment analysis was run adjusting FDR using the Benjamini-Hochberg procedure to obtain the results independently for the three set of proteins. Proteins were classified using the GO functional annotations for biological process, molecular function and cellular component.

Analyses Pathway analyses of the identified proteins was conducted using the STRING software (https://string-db.org; [32]). Fisher's exact test were performed in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis, considering the whole quantified protein annotations as background dataset. Benjamini-Hochberg correction for multiple testing was used to adjust derived p-values further, and only functional categories and pathways with $p < 0.05$ were considered as significant.

3. Results

3.1. Summary of quantitative proteomic analysis

We used LS-MS/MS technology to identify differentially expressed proteins in whey of milk collected from the goats receiving IMM infusion of LPS or saline, and then IV injection of Rg1 or saline solution. A total of 791 proteins detected from the whey were found to match 3838 unique spectra (Supplementary Table 2). Of them, 34 proteins in groups 2 vs 1, 53 proteins in groups 2 vs 3, and 98 proteins in groups 1 vs 3 were significantly different (Table 1). Some of differentially expressed proteins were listed in Table 2.

3.2. Distribution of biological processes, molecular functions and cellular components

Based on GO (Supplementary Table 3), the most abundant biological process was related to biological regulation, and the major molecular functional category related protein binding. Of 98 changed proteins in groups 1 vs 3, 53 in groups 2 vs 3 and 34 in groups 1 vs 2, the annotated proteins in GOTM were 85, 46 and 34, respectively. Many biological modulatory proteins were in response to stimulus, which accounted for 44 proteins in groups 1 vs 3 and 20 in groups 2 vs 3, and 11 in groups 1 vs 2 (Fig. 2). In the changed proteins, extracellular proteins were 40 in groups 1 vs 3, 19 in groups 2 vs 3, and 7 in groups 1 vs 2.

3.3. Changed proteins in groups 1 vs 3

Of 98 changed proteins in groups 1 vs 3, 54 were upregulated and 44 were downregulated with a ratio ranging from 0.16- to 29.34-fold changes (Fig. 3). Many of the upregulated proteins in milk whey are related to the classic acute phase response, such as acute phase proteins (plasma proteins, APPs), complement factors and proteins involved in blood coagulation, which are all well known to take part in the host's immediate response to infections and physical trauma. Other proteins included apolipoproteins (Apos), inflammatory mediators. Particularly, proteins associated with inflammation and defense against pathogens, i.e. complement C3, complement C5 (C5), serpin A1 (SERPINA1), fibrinogen γ chain (FGG) protein, fibrinogen alpha chain (FGA) protein,

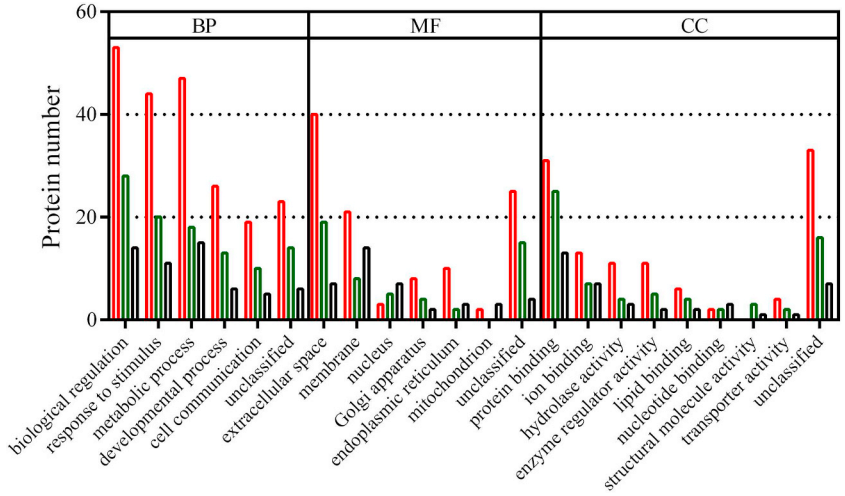

Fig. 2. Groups of goat whey proteins according their biological process (BP), molecular function (MF) and cellular component (CC). Significantly changed proteins were in groups 1 vs 3 (black bars), groups 2 vs 3 (light gray bars) and group 2 vs 1 (gray bars).

IL-6 and serotransferrin (TF) were higher in group 1 than 3. APPs such as serum amyloid A (SAA), α-2-macroglobulin (A2M), albumin (ALB), ceruloplasmin (CP), transthyretin (TTR) and pentaxin (CRP) increased after LPS infusion. Furthermore, of 44 downregulated proteins, milk proteins such as α-lactalbumin (Supplementary Table 4) and β-lactoglobulin are considered to be important constituents of milk. Other proteins with low-abundance were also detected and they were α-antiproteinase, fatty acid transporter (CD36), trefoil factor 2 (TFF2), β-1,4-galactosyltransferase-I (B4GALT1), tumor necrosis factor-inducible gene 6 protein (TSG-6) and vanin 1 (VNN1) (Fig. 4).

3.4. Changed proteins in groups 2 vs 1

Of 34 changed proteins in groups 2 vs 1, 25 were upregulated and 9 were downregulated with a ratio ranging from 0.26- to 4.17-fold changes (Fig. 3). Proteins associated with the oxidative stress and inflammatory response were lower in group 2 than in group 1. They were FGA, ALB, selenoprotein P1, TTR, complement 8 β chain (C8B), complement 6 (C6), C5, IL-6 and amine oxidase (AOC). Proteins associated with protection of mammary gland and recovery of lactose were upregulated, including eukaryotic translation initiation factor 4B (EIF4B), Parkinsonism associated deglycase (PARK7) and B4GALT1. Proteins related to inflammatory response such as APPs, complement factors and Apos showed decreased tendency. Specifically, they were CRP (0.29-fold lower than group 1), complement C5, C6, C7, C8A, C8B and complement factor D, H, I. Furthermore, proteins involved in proteolytic (GO:0006508) activity of milk were downregulated, these proteins included CFD, glycosylphosphatidylinositol specific phospholipase D1, kallikrein B1 (KLKB1), plasminogen activator (PLAT), plasminogen (PLG) and protein C (PROC). Meanwhile, proteins related to the regulation of peroxisome-proliferator-activated receptor (PPAR)-γ were upregulated, including CD36, fatty acid-binding protein 5 (FABP5), TFF2, transaldolase 1 (Taldo1), myristoylated alanine-rich C-kinase substrate (MARCKS), tissue inhibitors of metalloproteinase 2 (TIMP2) (Fig. 4).

3.5. Changed proteins in groups 2 vs 3

Of 53 changed proteins in groups 2 vs 3, 51 were upregulated and 2 were downregulated with a ratio ranging from 0.34- to 9.70-fold changes (Fig. 3). In 10 proteins, i.e., apolipoprotein C-III (ApoC3), apolipoprotein A1 (ApoA1), CRP, ALB, FGA, A2M, C4b-binding protein alpha chain and TF with 10.66- to 29.34-fold higher than in group 1 than group 3, 9 proteins remained found in group 2 but only 2.97- to 7.57-fold higher than in group 3 (Figs. 5, 6). Interestingly, proteins TFF2 and TSG-6 were 9.70- and 5.54-fold higher in groups 2 while only 2.25- and 2.40-fold higher in group 1 than in group 3 (Fig. 4).

3.6. Correlation analysis of proteins

By KEGG pathway analysis, 24 of 98 proteins in groups 1 vs 3 were assigned to 3 pathways, while 15 of 53 proteins in groups 2 vs 3 and only 4 of 34 proteins in groups 1 vs 2, were assigned to 4 and 1 pathways, respectively (Supplementary Table 2). The major KEGG pathways were complement and coagulation cascades, with 20 proteins in groups 1 vs 3 and 10 proteins in groups 2 vs 3 while 4 proteins in groups 2 vs 1 involved (Figs. 7, 8).

4. Discussion

In previous study, we demonstrated that IV injection of Rg1, a ginseng saponin, extracted from *Panax ginseng* C.A. May was therapeutically effective on LPS-induced mastitis in goats. The present study identified 791 proteins in milk whey of goats by means of proteomic approach. Among these proteins, 98 significantly changed 6 h PLC and 34 changed after IV injection of Rg1 in the goats with IMM infusion of LPS. These changes in proteins might be caused by the anti-inflammatory effect of Rg1 on mastitis in goats.

As LPS originates from cell walls of gram negative bacteria and is a strong initiator of inflammation, intra-mammary inflammation induced by IMM infusion of LPS has usually been used as *E. coli* mastitis model. Once LPS is infused into the mammary gland, TLR4/nuclear factor-κB (NF-κB) signaling pathway is activated to produce pro-inflammatory factors and APPs, and cause mastitis [9,33]. IL-6 signaling pathway is important in ruminant mastitis. After exposure to LPS, mammary cells produce pro-inflammatory components including IL-6. APPs (i.e. A2M, CRP, TTR, ALB) are expressed following upregulation of IL-6 [34]. Analysis of proteins in whey from cows with coliform mastitis using proteomics has been previously reported. Boehmer et al. [35,36] observed proteins that stand out as logical candidates for further analyses include various APPs and vascular-derived proteins such as C3, C4, TF, ALB, TTR, FGA, ITIH4 in mastitic whey of *E. coli* infected cows by 2-DE and label-free methods, respectively. Danielsen et al. [37] and Hinz et al. [38] also discovered ApoA1, SAA, SERPINA1, A2M, AOC, C4, TF, ALB and FGA in whey after IMM infusion of LPS in cows. They found that the different expressed proteins were important to balance the immune responses, indicated more than one activated complement pathway and showed hydrolysis of casein during LPS-induced mastitis.

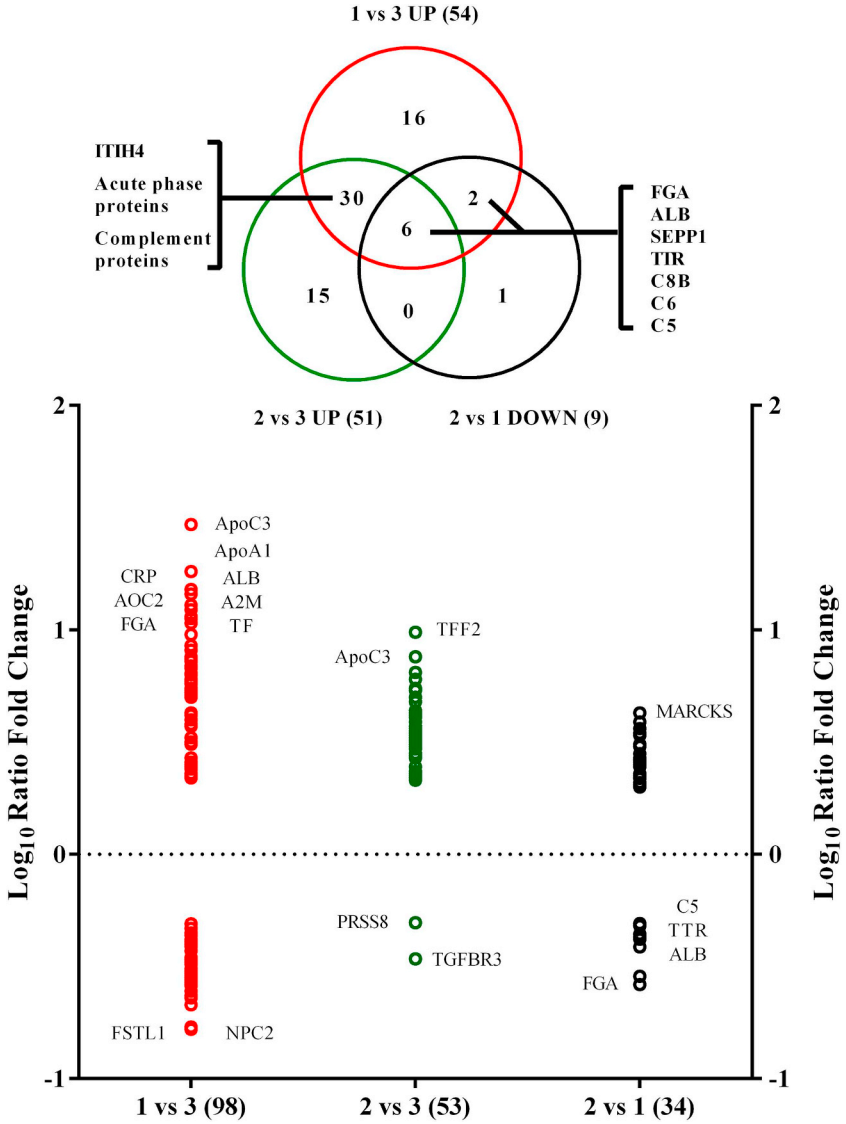

Fig. 3. Significantly up- and down-regulated proteins in whey from goats of LPS IMM + saline IV (1), LPS IMM + Rg1 IV (2) and control group (3).

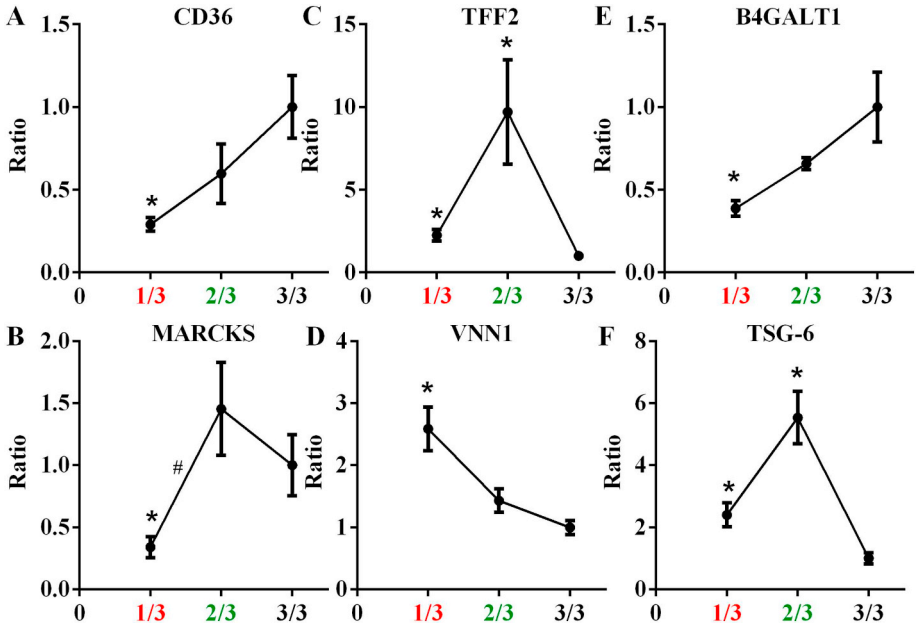

Fig. 4. Differentially expressed proteins fatty acid transporter (CD36) (A), myristoylated alanine-rich C kinase substrate protein (MARCKS) (B), trefoil factor 2 (TFF2) (C), vanin 1 (VNN1) (D), beta-1,4-galacto-syltransferase I (B4GALT1) (E) and tumor necrosis factor alpha-induced protein 6 (TSG-6) (F) in whey. Data were expressed as means ± SE; *, statistical difference between groups; #, statistical difference between groups 1 and 2.

Table 1
Proteins identified in whey based on Uniport database for ruminants.

Database	Total proteins	Groups 1 vs 3		Groups 2 vs 3		Groups 2 vs 1	
Uniport "*Ruminantia*"	791	98	54 up 44 down	53	51 up 2 down	34	25 up 9 down

Apart of that, Olumee-Shabon et al. [39] only found few proteins in caprine whey at 18 h PLC, including ApoA1 and SAA by 2-DE method. The collapse of the blood-milk barrier was not observed as has been found in dairy cattle. With high dose of LPS in present study, we observed upregulated proteins such as IL-6, A2M, CRP, CP, SERPINA1, ITIH4, SAA, TTR, TSG-6, TF, ALB, FGA, Apos and complement factors in whey 6 h PLC in goats. These proteins have been reported to be involved in acute phase response signaling pathway in cows [34], and cause fever and blood-milk barrier damage in mammary glands [40–42].

The breakdown of blood-milk barrier may due to the decreased expression of occludin and zonula occludens-1 which have been found to cause a significant increase in tight junction permeability and epithelial barrier damage after LPS treatment [40]. For cows, Zhao and Lacasse [4], and Wellnitz et al. [20] observed that LPS caused extracellular fluid components to enter the gland and mix with the milk. For example, the increased serum albumin in milk has been suggested as a marker for infection and for estimating transfer of proteins implicated in the udder defense [43,44]. Meanwhile, blood polymorphonuclear leukocytes are attracted into the udders where they release oxidants

Table 2
Some proteins differentially expressed in whey based on Uniprot database.

Uniprot ID	Proteins	Gene symbols	Sequence cover. [%]	FC[a] ratio (groups)		
				1/3	2/1	2/3
Acute-phase protein						
W5NSA6	Alpha-2-macroglobulin	A2M	31.5	11.53*	0.26	2.97*
B3VHM9	Serum albumin	ALB	80.6	12.90*	0.39*	4.99*
W5P4S5	Ceruloplasmin	CP	34.9	8.59*	0.41	3.52*
W5PD71	Pentaxin	CRP	11.6	15.08*	0.29	4.35*
W5NRG7	Inter-alpha-trypsin inhibitor heavy chain family member 4	ITIH4	20.0	5.10*	0.42	2.15
W5PJR0	Serum amyloid A protein	SAA	21.5	6.42*	0.38	2.41
I1WXR3	Alpha-1-antitrypsin transcript variant 1	SERPINA1	23.6	5.83*	0.53	3.10*
P12303	Transthyretin	TTR	46.9	5.49*	0.43*	2.35*
Apolipoproteins						
W5NX51	Apolipoprotein A1	ApoA1	71.8	18.26*	0.36	6.49*
W5NWM2	Apolipoprotein A4	ApoA4	33.2	7.41*	0.13	0.97
V6F9A3	Apolipoprotein C-III	ApoC3	35.4	29.34*	0.26	7.57*
W5QGP4	Apolipoprotein D	ApoD	18.3	6.00*	0.26	1.58
Complement system						
W5P6F4	Complement C5	C5	16.3	7.61*	0.49*	3.73*
W5PGT6	Complement C6	C6	10.9	5.35*	0.48*	2.57
W5PDR7	Complement C8 alpha chain	C8A	20.1	3.11*	0.53	1.64
W5PE53	Complement C8 beta chain	C8B	15.7	5.26*	0.44*	2.32*
W5PJ66	Complement factor D	CFD	13.9	2.43*	0.52	1.27
A0A212CRV5	Complement factor H	CFH	23.0	5.38*	0.43	2.32
W5P5I0	Complement factor I	CFI	20.9	3.06*	0.59	1.80
Blood coagulation						
W5Q5H8	Fibrinogen alpha chain	FGA	47.5	12.18*	0.29*	3.48*
W5Q5A6	Fibrinogen gamma chain	FGG	35.7	9.45*	0.33	3.12*
W5NYU0	Plasminogen activator	PLAT	5.0	2.49*	0.80	1.98
W5P3R3	Plasminogen	PLG	39.2	6.70*	0.45	3.00*
Others						
E9NRZ3	Beta-1,4-galactosyltransferase I	B4GALT1	23.1	0.39*	1.70	0.66
E3UT57	Interleukin 6	IL-6	8.2	7.07*	0.24*	1.69
W5Q3K6	Protein C	PROC	4.3	4.15*	0.46	1.92
W5PLZ4	Trefoil factor 2	TFF2	34.4	2.25*	4.31	9.70*
W5PGW9	TNF alpha induced protein 6	TSG-6	15.7	2.40*	2.31	5.54*
W5PYG2	Vanin 1	VNN1	19.0	2.59*	0.55	1.43
W5P1J8	Amine oxidase	AOC	15.3	14.60*	0.44	6.38*
P02756	Beta-lactoglobulin	β-LG	72.8	0.27*	2.21	0.59
H6WVW6	Selenoprotein P1	SEPP1	11.3	5.51*	0.42*	2.30
Q3MHP6	Eukaryotic translation initiation factor 4B	EIF4B	4.8	0.74	3.45*	2.57
F8U3U7	Fatty acid transporter	CD36	11.0	0.29*	2.05	0.49
F1N2N5	Myristoylated alanine-rich C-kinase substrate	MARCKS	10.2	0.34*	4.25*	1.45
A0A0E3UT00	TIMP metallopeptidase inhibitor 2	TIMP2	28.4	0.25*	2.27*	0.57
W5PK66	Parkinsonism associated deglycase	PARK7	24.9	0.64	2.44*	1.55
W5Q750	Protein S	PROS	4.2	3.80*	0.44	1.68
W5PF65	Transferrin	TF	52.4	10.66*	0.38	4.09*

Numbers: 1, LPS IMM + saline IV, 2, LPS IMM + Rg1 IV and 3, control group;
* Statistical difference between groups.
[a] Fold change.

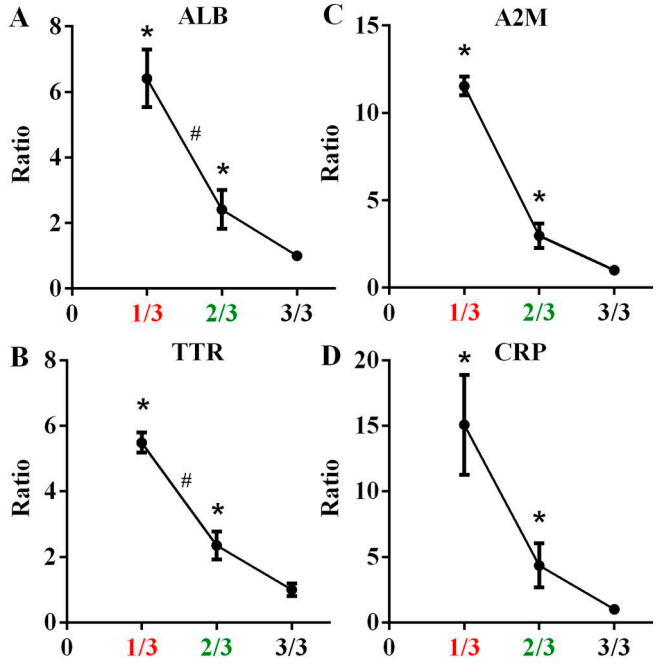

Fig. 5. Differentially expressed acute phase proteins, including serum albumin (ALB) (A), transthyretin (TTR) (B), alpha-2-macroglobulin (A2M) (C) and pentaxin (CRP) (D) in whey. Data were expressed as means ± SE; *, statistical difference between groups; #, statistical difference between groups 1 and 2.

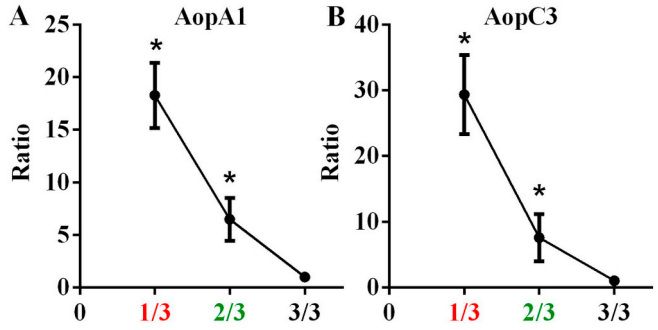

Fig. 6. Differentially expressed Apolipoproteins A1 (ApoA1) (A) and C3 (ApoC3) (B) in whey. Data were expressed as means ± SE; *, statistical difference between groups; #, statistical difference between groups 1 and 2.

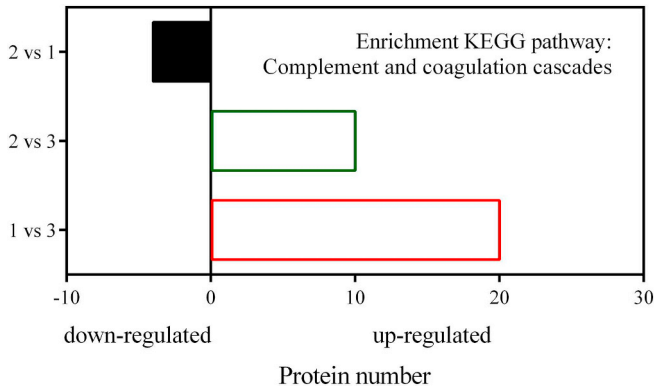

Fig. 7. Proteins expressed in goat whey according to analysis of the most enriched KEGG pathway-complement and coagulation cascades.

and proteases [45,46], destroying the epithelial cells and causing dramatically decreased milk production.

APPs, including SAA, ALB, CP, A2M, SERPINA1, CRP, TTR, ITIH4, were detected in milk 6 h PLC (Fig. 5). The same changes have been previously reported [47]. APPs are produced in response to a variety of stimulations by pro-inflammatory cytokines. A2M is a serum pan-protease inhibitor and is markedly higher in mastitic whey than normal whey [48]. SAA proteins belong to a polymorphic family of apolipoproteins associated with high-density lipoproteins [49] and the increase in milk SAA has been previously found after challenge [50]. ALB is produced in the liver and thought to leak into the mammary gland from blood circulation. As previously found, decreased ALB in serum may be due to damage of blood-milk barrier induced by LPS [19]. Other proteins such as fibrinogen transferred from blood to milk were observed in whey of LPS-challenged goats. Fibrinogen is a biomarker of vascular rupture that can promote hemostasis, thrombosis, coagulation, fibrosis, and prevent infection and even death. Fibrinogen-monocyte and neutrophil interactions may increase inflammation in the early stages of the disease and exacerbate tissue damage when it progresses to chronic disease [51].

Apos are typically associated with high density lipoproteins, and apolipoproteins during inflammation have been investigated [52,53]. Although several apolipoproteins were previously identified in bovine milk [35–37,54], the specific role of the apolipoproteins during mastitis remains unclear. In LPS-mediated inflammation in cows, highly upregulated Apos are observed [37]. Like ApoA1, overexpression of ApoA4 reduced secretion of proinflammatory cytokines in LPS-infused mice [55]. Apos is not only transported from blood to the site of inflammation, but also may be synthesized by the mammary glands in response to LPS [37,56]. The increase of ApoC3 was observed in the mastitic milk and can lead to hyperlipidemia, which causes immunosuppression, aggravates inflammation, and is detrimental to restoring liver function and maintaining energy. It may relate to the inhibition of PPAR.

Other important proteins related with the integrity of blood-milk barrier are complement components. The mammary gland of dairy animals, which is prone to infection by various bacteria, mobilizes local and systemic immune defenses to cope with pathogens. The complement system plays an important part in the innate immunity against microorganisms through its bactericidal, opsonic, and phlogistic functions, and consists of a collection of proteins present in serum and milk. When inflammation develops, the component fragments induce histamine release from mast cells, increase vascular permeability and the recruitment of phagocytes at sites of inflammation [57]. Moreover, it has become apparent that proteins of the complement system can influence the immune response and constitute a bridge between innate and acquired immunity [58]. These functions of the complement have proven to be of broader consequence for host defense than is the complement-mediated lysis [59]. The proteins that comprise the complement system are synthesized mainly by hepatocytes, but other cellular sources include monocytes and tissue macrophages. Thus, the amount of the complement in the milk of healthy glands is low. The researchers suggested that, the physiological barrier between blood and milk is locally broken by the inflammatory response or the lesions developed during infection, and exudation of plasma proteins can take place. When sizeable inflammation develops, larger amounts of the complement are mobilized from the blood, and the complement can play its normal part within the frame of innate and specific immune responses. Exudation of the complement proteins is most likely to account for most of the enhancement of the complement activities in mastitic milk during the acute phase [60].

After injection of Rg1 in group 2, IL-6 and proteins involved in acute phase response signaling pathway including IL-6, CP, TTR, ALB, FGA and complement factors were downregulated. Meanwhile, proteins related to the regulation of PPARγ or assigned with protection of mammary gland were upregulated. These proteins were CD36, FABP5, TFF2, Taldo1, MARCKS and TIMP2. PPARγ is a transcription factor that acts as an influential pleiotropic regulator of anti-inflammation, anti-oxidant, phagocyte mediated cleanup processes and lipid synthesis [34,61,62]. The activation of PPARγ is inhibited by binding to NF-κB

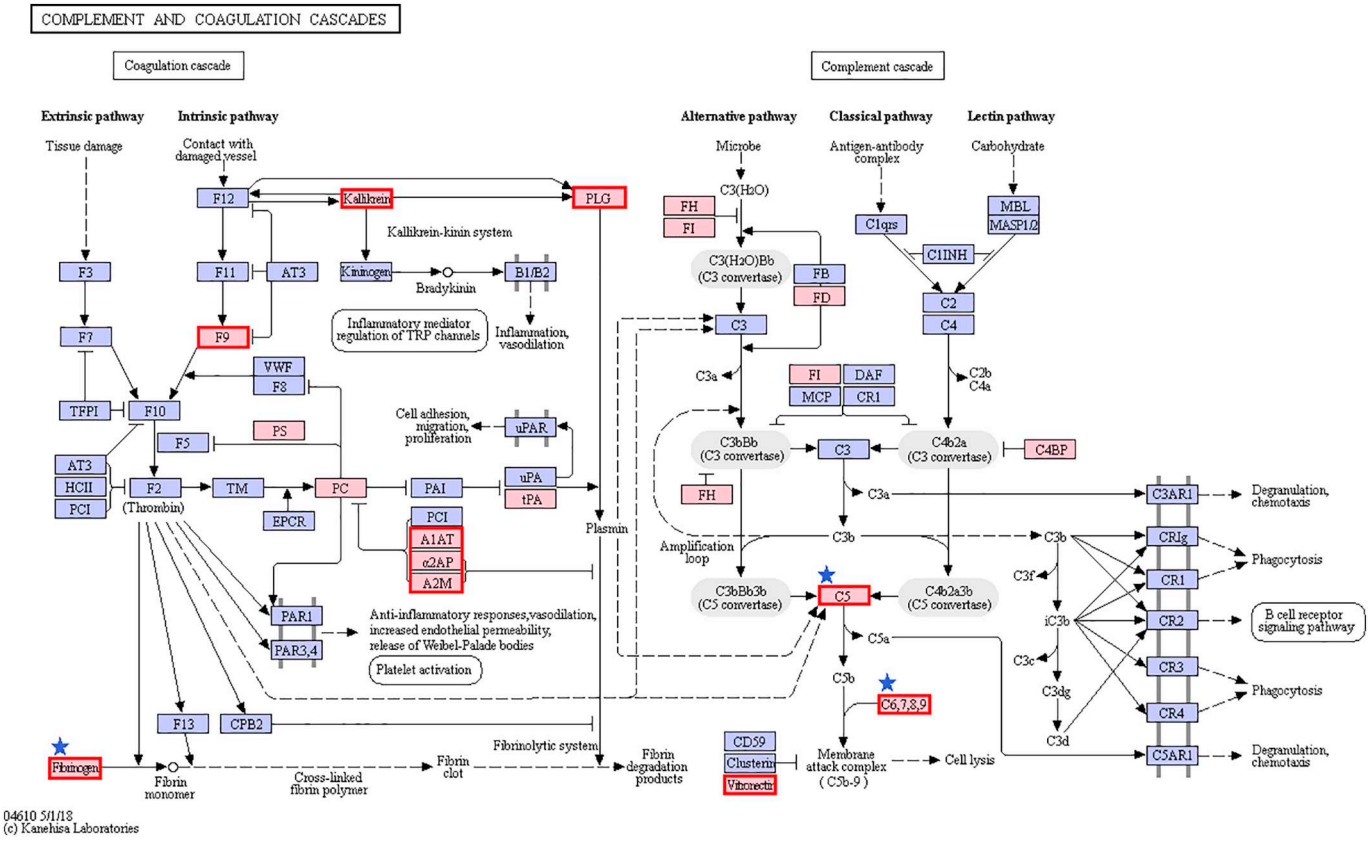

Fig. 8. Significantly different proteins involved in complement and coagulation cascades. Red blocks: groups 1 vs 3; red lines: groups 2 vs 3; blue stars: groups 2 vs 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

p65 in the nucleus after activation of NF-κB pathway which can cause the downregulation of genes involved in fatty acid import, de novo fatty acid synthesis and triacylglycerol synthesis [34]. Li et al. [63] and Zhang et al. [64] previously found that Rg1 can normalize the expression of PPARγ via decreasing NF-κB expression in rats. Furthermore, the recovery of milk fat and decrease of milk free fatty acid had been observed after Rg1 treatment (Supplementary Fig. 1).

One of the important proteins for mammary protection was TFF2 which can be induced by PPARγ pathway [65,66]. Later in 2010, Zhang et al. [67] found TFF2 can promote wound healing by stimulating cell migration via mitogen activated protein kinase pathway and preventing cell apoptosis. Besides, we also detected decreased VNN1, an oxidative stress sensor in epithelial cells [68,69], which might indicate that Rg1 treatment inhibited oxidative stress and upregulated concentration of an identified anti-oxidant protein (PARK7), a protein believed to be involved in negative regulation of hydrogen peroxide-induced cell death. Another relevant finding from the present investigation was that EIF4B was dramatically upregulated after Rg1 treatment in goats with IMM infusion of LPS. EIF4B silencing was found to promote caspase-dependent apoptosis, and mammalian EIF4B is suggested to be required for cell proliferation and survival [70]. Thus, induction of PARK7, EIF4B and TFF2 by Rg1 may help recovery of milk production and decrease of vascular-derived proteins in milk by promoting the survival of mammary epithelial cells. These changes may be attributed to the anti-inflammatory effect of Rg1 reported by Su et al. [15]. They found that Rg1 competed binding with LPS to TLR4 and inhibited IκB phosphorylation.

In conclusion, IMM infusion of LPS activated NF-κB via TLR4 pathway, increased IL-1 and TNF-α, upregulated IL-6 and inhibited PPARγ pathway. Overexpression of IL-6 lead to upregulation of the proteins such as A2M, CRP, CP, ITIH4, FGA, FGG, C4, C5 and KLKB1 [34]. Apart from this, the inhibition of PPARγ pathway (i.e. CD36, FABP5, MARCKS, TIMP2) was related to the deregulation of lipid metabolism during mastitis at the early stage. Rg1 treatment recovered milk fat, downregulated proteins including IL-6, APPs and complement factors and increased proteins related to PPARγ (i.e. CD36, FABP5, TFF2, Taldo1, MARCKS, TIMP2). These might be attributed to the competitive effect of Rg1 on LPS to bind TLR4, and normalizing PPARγ pathway to inhibit the overexpression of NF-κB and pro-inflammatory factors during LPS challenge. LPS is the major bacterial toxin during *E. coli* mastitis. This study showed that intramammary infusion of LPS increased inflammatory factors such as C3, C5, SERPINA1, FGA, FGG, IL-6 and TF followed by tissue damages evidenced by elevated agents such as FGA, FGG, ALB, CP, TTR and CRP. Therefore, it may stimulate the mammary gland to recovery if the agents such as Rg1 are administered to block the action of LPS when antibiotics are used to treat *E. coli* mastitis.

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Conflict of interest

The authors declare that they have no competing interests.

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