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Preliminary study on gene regulation and its pathways in Chinese Holstein cows with clinical mastitis caused by *Staphylococcus aureus*

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Abstract

Introduction: Clinical mastitis (CM) is one of the most common diseases of dairy cows globally, has a complex aetiology and recurs easily. *Staphylococcus aureus* is a frequently isolated pathogen responsible for bovine mastitis and remains difficult to eradicate. **Material and Methods:** To characterise the transcriptional profiles of dairy cows infected by *S. aureus*, we performed an RNA-seq analysis of peripheral blood leukocytes in lactating Chinese Holstein dairy cows with CM and did the same with healthy cows' samples as controls. **Results:** A total of 4,286 genes were detected in the CM cases infected with *S. aureus* which were differentially expressed compared to the controls, 3,085 of which were upregulated, the remainder being downregulated. Notably, we observed that some differentially expressed genes (DEGs) had strong protein–protein interaction. Of these, six downregulated DEGs (*AKR1C4*, *PTGS2*, *HNMT*, *EPHX2*, *CMBL*, and *IDH1*) were involved in the metabolic pathway, while eight upregulated DEGs (*VWF*, *GP9*, *MYLK*, *GP6*, *F2RL3*, *ITGB3*, *GP5*, and *PRKG1*) were associated with the platelet activation pathway. **Conclusion:** The transcriptome dataset of CM cases would be a valuable resource for clinical guidance on anti-inflammatory medication and for deeper understanding of the biological processes of CM response to *S. aureus* infection, and it would enable us to identify specific genes for diagnostic markers and possibly for targeted therapy.

Keywords: clinical mastitis, cattle, *Staphylococcus aureus*, inflammatory response, RNA-seq.

Introduction

Bovine mastitis is an inflammatory response of the udder tissue due to physical trauma or microorganism infections, most significant among these being bacterial infections (10). Despite considerable efforts to control bovine mastitis and explain its causes, it remains the most costly and common disease of dairy cattle worldwide, having a negative economic effect on the dairy industry due to reduced milk production, increased treatment costs, reduced fertility, and increased culling of affected animals. Mastitis can be classified as clinical or subclinical in form according to the degree of inflammation (1). The incidence rate of clinical mastitis (CM) ranges from 13%–40% yearly, varying by country and type of cattle housing, and the average cost of CM can amount to $744 for treatment per case (17). The disease can be easily diagnosed based upon visible symptoms, which are udder inflammation showing redness in the affected part or complete udder, warmth, swelling, pain upon touch, milk clots, discoloration, and changes in the consistency of milk (27). Severe cases of CM can be fatal. More importantly, the recurrent nature, a frustrating aspect of CM, brings challenges in the therapeutic and management approaches to bovine CM (17).

Staphylococcus aureus, a common mastitis pathogen widespread in the natural environment of dairy farms, is a frequently isolated bacterium responsible for bovine mastitis (5). This bacterial disease causes a substantial reduction in both milk production and quality, and consequent considerable economic losses in the dairy industry. The bacterium can propagate and persevere in various ways once it adheres to host tissues

or prosthetic materials, and expresses many virulence proteins involved in evading the host defences, hence facilitating microbial colonisation of the mammary glands of animals (35). Because of the resistance of *S. aureus* to antibiotics, infection with this pathogen has remained recalcitrant to eradication. *Staphylococcus aureus* remains a predominant pathogen causing the most virulent forms of bovine mastitis and poses the greatest challenge to dairy production worldwide.

Sequencing of RNA (RNA-seq) has become a powerful, effective and well-known tool to characterise transcriptome profiles in different tissues or organs. It has also been widely used for exploring differentially expressed genes (DEGs) associated with complex traits and diseases. Published studies on CM have investigated bovine mammary gland tissue (29) or bovine mammary epithelial cells (32) challenged with *S. aureus*, and have also identified a set of genes in dairy cows with a subclinical mastitis response to *S. aureus* infection (31). Although the immune response and inflammatory processes are known as key mechanisms for bovine mastitis, limited studies have been carried out on the functional genes and pathways underlying clinical mastitis with *S. aureus* infection. In this regard, this study aims to gain a deeper knowledge of the host's transcriptional response to *S. aureus* infection in developed CM using RNA-seq. The present study will also provide insight into CM pathogenesis with *S. aureus* infection by identifying some novel functionally relevant genes and biological processes as specific key actors defining the course of the disease.

Material and Methods

***Staphylococcus aureus* isolation and identification.** A total of 20 lactating Chinese Holstein dairy cows kept on the private Yajie dairy farm located in Luoyang, China, were recruited to the present study. To detect pathogenic bacteria in cattle with mastitis, milk samples were collected from each cow and examined. Considering *Escherichia coli*, *Streptococcus agalactiae* and *S. aureus* as the most common bacteria associated with mastitis, the three corresponding indexes were used for subsequent detection in this study. In brief, agar with the addition of 10% bovine blood was used for the isolation of pathogens. The samples were inoculated onto plates and incubated at 37°C for 24 h. The grown colonies were identified by morphological (size and colour) and physiological properties (Gram staining and haemolysis). Suspected colonies were then transferred into semisolid slope agar to be identified and sub-cultured on eosin-methylene blue medium, Group B Streptococcus medium or Baird-Parker agar, as appropriate for the particular colony. Then, triple sugar iron, indole, methyl-red, Voges-Proskauer and citrate utilisation tests were performed to identify *E. coli*. Catalase, hippurate hydrolysis, Christie-Atkins-Munch-Peterson and sorbitol tests were carried out to identify *Streptococcus agalactiae*. The identification of *S. aureus* was carried out based on growth characteristics and biochemical tests according to Hogan *et al.* (15) and PCR amplification for the *S. aureus* protein A gene (*spa*) (3). To investigate whether *S. aureus* infection affects clinical mastitis, only the milk samples identified as positive for *S. aureus* exclusively were selected for subsequent research.

Sample collection. Seven dairy cows were selected based on the method described above. Three of them were the mastitic group (M, n = 3), because they had the clinical features of mastitis, such as flakes and clots in milk, udder inflammation (hyperaemia, pain and udder enlargement). Four healthy cows formed the non-mastitic control group (NM, n = 4), because of these cows' somatic cell counts (SCC) below 200,000 cells/mL at the monthly check-up and freedom from any clinical signs of mastitis.

RNA isolation, cDNA library construction, and RNA sequencing. A 10 mL whole blood sample was collected by venepuncture from each animal. Peripheral blood leukocyte (PBL) enrichment was achieved by depleting the red blood cells and centrifuging for 15 min at 1,000 × *g*. The leukocytes were mixed with 1 mL of Trizol (TransGen Biotech, Beijing, China), and the total RNA was extracted from the mixture according to the manufacturer's protocol. RNA quality and quantity were determined by the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) in 1% agarose gel. A total of 4 μg purified total RNA for each sample was used to construct RNA-seq libraries. Overall, the mRNA for each library was enriched using magnetic beads with Oligo (dT) and then fragmented into short segments. Then, these short segments were utilised for synthesising the cDNA with the addition of deoxyribonucleotide triphosphate buffer and DNA polymerase I. The obtained fragments underwent size selection and PCR amplification enrichment, and were subsequently used to construct the library for each sample. Sequencing of each library was performed using the HiSeq 2500 platform (Illumina, Santa Clara, CA, USA).

Sequencing data analysis. For the raw data, Trimmomatic v0.40 software was used to remove the low quality reads, reads containing poly-N sequences, and adaptor sequences (6). After filtering, the clean reads of each library were aligned to the bovine reference genome (*ARS-UCD1.2*) using HISAT v2.2.1 software (19). The count matrix and transcripts per million values for each gene or transcript were analysed using StringTie v2.1.3 software (20) and the edgeR R package (24), respectively. Differentially expressed gene analysis between the M and NM groups was performed using the DESeq2 software (22). A false discovery rate (FDR) corrected P value ≤ 0.05 and fold change > 2 were defined as the selection threshold for the DEGs. Gene ontology (GO) enrichment analysis (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed for the upregulated and downregulated DEGs using KEGG Orthology-Based

Annotation System (KOBAS) software with an adjusted cutoff value of $P < 0.01$. The STRING database (https://string-db.org) was used to explore the protein–protein interactions (PPI) among the DEGs. In the PPI analysis, the responses of metabolic pathways and platelet activation pathways to CM were considered important and the DEGs located on these pathways were investigated. The PPI network of the DEGs was visualised using Cytoscape v3.8.2 software.

Verification of DEGs by quantitative real-time PCR. Seven randomly selected DEGs (*VWF*, *ITGB3*, *F2RL3*, *PRKG1*, *GDA*, *HNMT*, and *GPT2*) of biological interest were investigated by quantitative real-time PCR (qRT-PCR) to validate the accuracy of the RNA-seq results. The *β-actin* gene was set as the reference gene. Primer pairs for *VWF* (F: 5′-TCGGGATTGGGGACCAGTAT-3′, R: 5′-CAC TGACGAACCCAGAGCAT-3′), *ITGB3* (F: 5′-GCT CCCAGATTACCCAAG-3′, R: 5′-TCTTCC ACCTGC CGAACT-3′), *F2RL3*(F: 5′-CTGCTGCTG TTGCAC TTCTC-3′, R: 5′-CTGAACTCGGCAGACAC-3′), *PRKG1* (F: 5′-CTGGAGAAGCGGCTGTCA-3′, R: 5′-TCG TGGAAGGACCTGTAGG-3′), *GDA* (F: 5′-CCTGGG GCTGGATAGAGAGA-3′, R: 5′-GCCCAC ATACAC CTCCTCAA-3′), *HNMT* (F: 5′-ACACTG CTGGTC TTATCTCTCC-3′, R: 5′-AGCCCCCTGAAA CCTAGT TG-3′), *GPT2* (F: 5′-AGTCCATGAACCCGC AGG-3′, R: 5′-GCACGGATGACCTCAGTGA-3′) and *β-actin* (F: 5′-ATCACCATCGGCAATGAGCGGTTC-3′, R: 5′-CGG ATGTCGACGTCACACTTCATGA-3′), were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). After amplification, the relative fold change of the differentially expressed genes was calculated using the $2^{-\Delta\Delta CT}$ algorithm.

Results

Observations in experimental and control animals and pathogen prevalence in their milk samples. The characteristics of the seven dairy cows are given in Table 1. The results showed that the cows in the M group had higher SCC values (ranging from 456 to 623 × 10^4/mL) than those in the NM group. Changes in milk appearance (flakes and clots) were observed in the M group. Notably, the cows in the M group had a lower daily milk yield than the cows in the NM group. Samples were positive in 55% (11/20), 20% (4/20) and 30% (6/20) proportions for *S. aureus*, *E. coli*, and *Streptococcus agalactiae*, respectively, and 15% (3/20) were positive for all three pathogens. The three CM cases selected for RNA-seq were positive for *S. aureus* only (Fig. 1), and negative for *E. coli* and *Streptococcus agalactiae*.

Characterisation of transcriptome sequencing data. Seven RNA sequences were generated from the four healthy and three mastitic dairy cows. The results are listed in Table 2. After the quality control of the raw data, we obtained an average of approximately 47.60 Mbp clean reads for each sample. The Q20 and Q30 of each sample ranged from 97.78% to 97.96% and 91.07% to 91.82%, respectively. By aligning clean reads to the bovine reference genome, we obtained an average mapping ratio of 81.10% (from values ranging from 76.37% to 84.97%). These results suggested that the data quality was suitable for subsequent analysis.

Identification of differentially expressed genes. A total of 16,544 genes were detected. Their expression profiles are shown in Fig. 2A. Of these genes, 4,286 were indicated to be DEGs based on the threshold level (log2 (fold change) ≥ 1 and FDR < 0.05) (Fig. 2B). Among them, 3,085 DEGs were classified as upregulated, and the remaining 1,201 as downregulated. Figure 2C displays the hierarchical cluster expression patterns of the DEGs among all the studied groups.

Gene ontology/pathway enrichment analysis of DEGs. Investigating the relationship between DEGs and the inflammatory response by GO analysis, we found that most upregulated DEGs were annotated to 549 GO terms including 402 biological processes (BP), 70 cellular components (CC), and 77 molecular functions (MF), while most downregulated DEGs were enriched into 297 GO terms consisting of 225 BP, 39 CC, and 33 MF (Fig. 3).

For the upregulated DEGs, the top three BP were the cellular process, response to stimulus, and biological regulation. The cellular anatomical entity, intracellular component, and organelle were the three most predominant CC, while binding, catalytic activity, and protein binding were the major three MF. These results suggested that the invasion of the pathogen might induce changes in various membrane structures and cellular components.

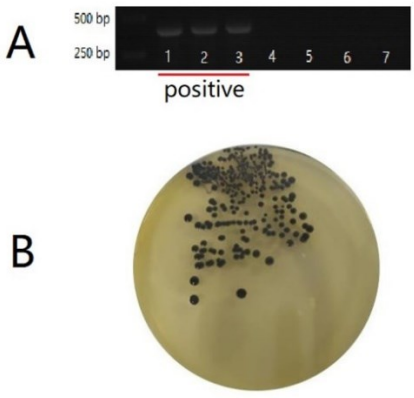

Fig. 1. Identification of *Staphylococcus aureus* isolated from milk samples from Holstein cows. A − specific PCR and electrophoresis map of the *S. aureus* protein A (*spa*) gene (323 bp); B − morphology of *S. aureus* colony on Baird-Parker agar

For the downregulated DEGs, the same three foremost BP as for the upregulated DEGs were observed. The cellular anatomical entity, membrane, and intrinsic component of the membrane were the three most important CC, while binding, ion binding, and catalytic activity were the leading three MF. These results suggested that this pathogenic infection might be related to dynamic changes in gene expression in specific cellular biological processes.

Table 1. Characteristics of the seven experimental and control dairy cows

Animals	Group	Lactation (d)	SCC ($\times 10^4$/mL)	Milk yield (kg/day)	Milk morphology	Bacterium
NM002	NM	288	4.5	23	normal	–
NM003	NM	256	5.1	19	normal	–
NM013	NM	241	3.9	18	normal	–
NM182	NM	198	4.4	20	normal	–
M008	M	267	623	5.0	thin, clots	*Staphylococcus aureus*
M010	M	213	456	4.6	flakes	*Staphylococcus aureus*
M021	M	186	503	3.0	clots	*Staphylococcus aureus*

SCC – somatic cell count; – – samples without bacterial infection

Table 2. RNA-seq data for the seven experimental and control dairy cows

Samples	Total clean reads (bp)	Clean reads Q20 (%)	Clean reads Q30 (%)	Total mapping ratio (%)
NM002	47,493,260	97.78	91.07	82.86
NM003	47,472,822	97.83	91.31	82.25
NM013	47,646,180	97.87	91.34	83.81
NM182	47,588,374	97.84	91.22	84.97
M008	47,642,372	97.92	91.64	79.93
M010	47,710,376	97.96	91.82	76.37
M021	47,664,652	97.92	91.75	77.52

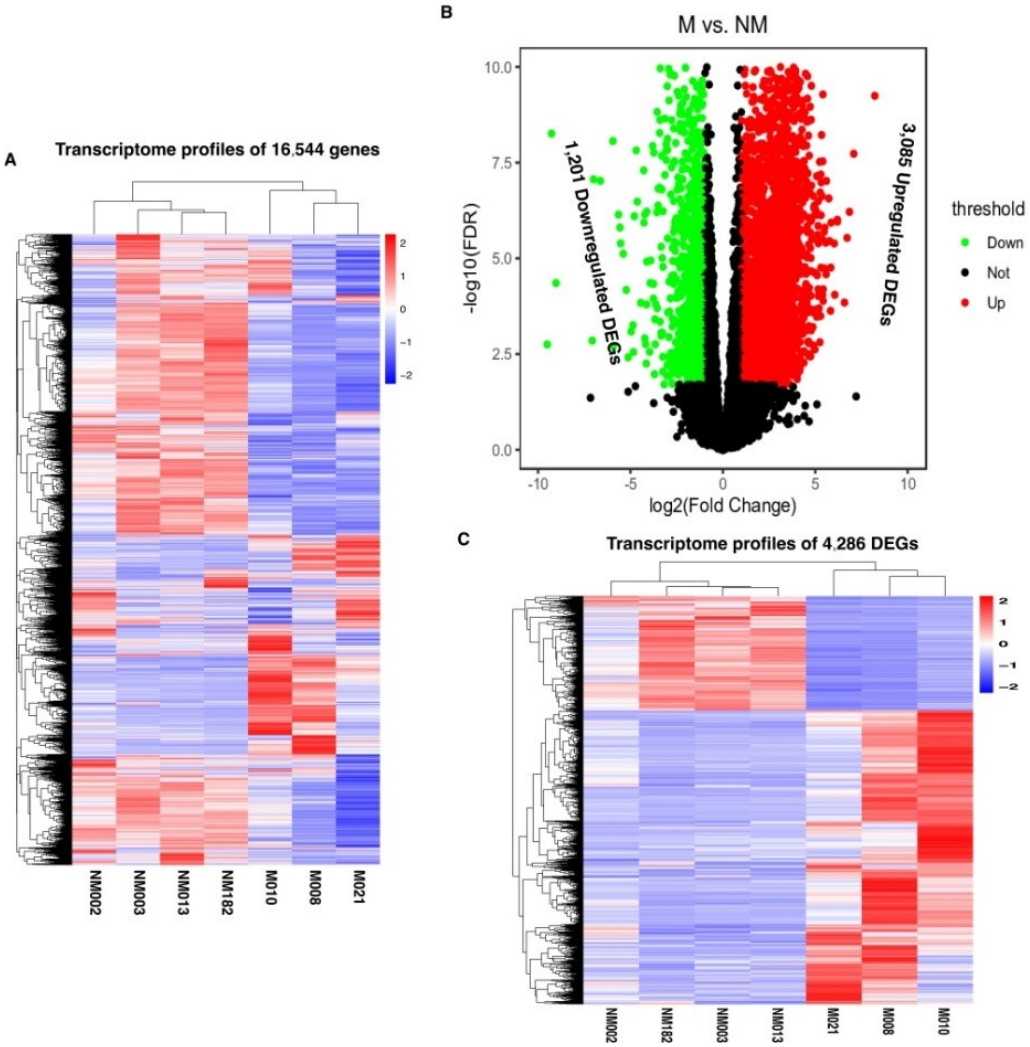

Fig. 2. Transcriptome profiles of 16,544 genes identified by mRNA in peripheral blood leukocytes (PBL) from all the samples (A); volcano plot of global differentially expressed genes (DEGs) in PBL between the mastitic (M) and non-mastitic (NM) groups (B); and transcriptome profiles of 4,286 DEGs between the M and NM groups (C)

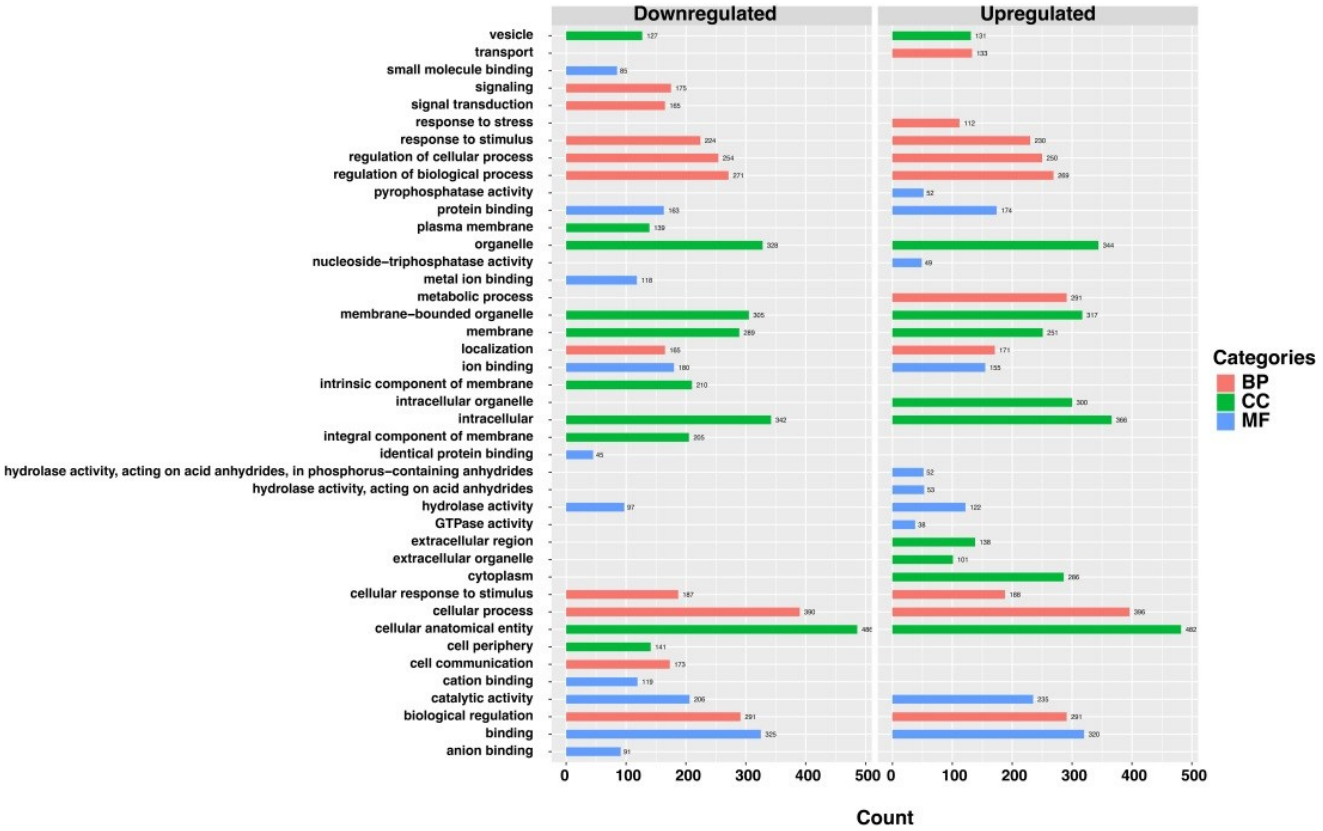

Fig. 3. The top 30 up- and downregulated differentially expressed genes analysed by Gene Ontology
BP – biological processes; CC – cellular components; MF – molecular functions

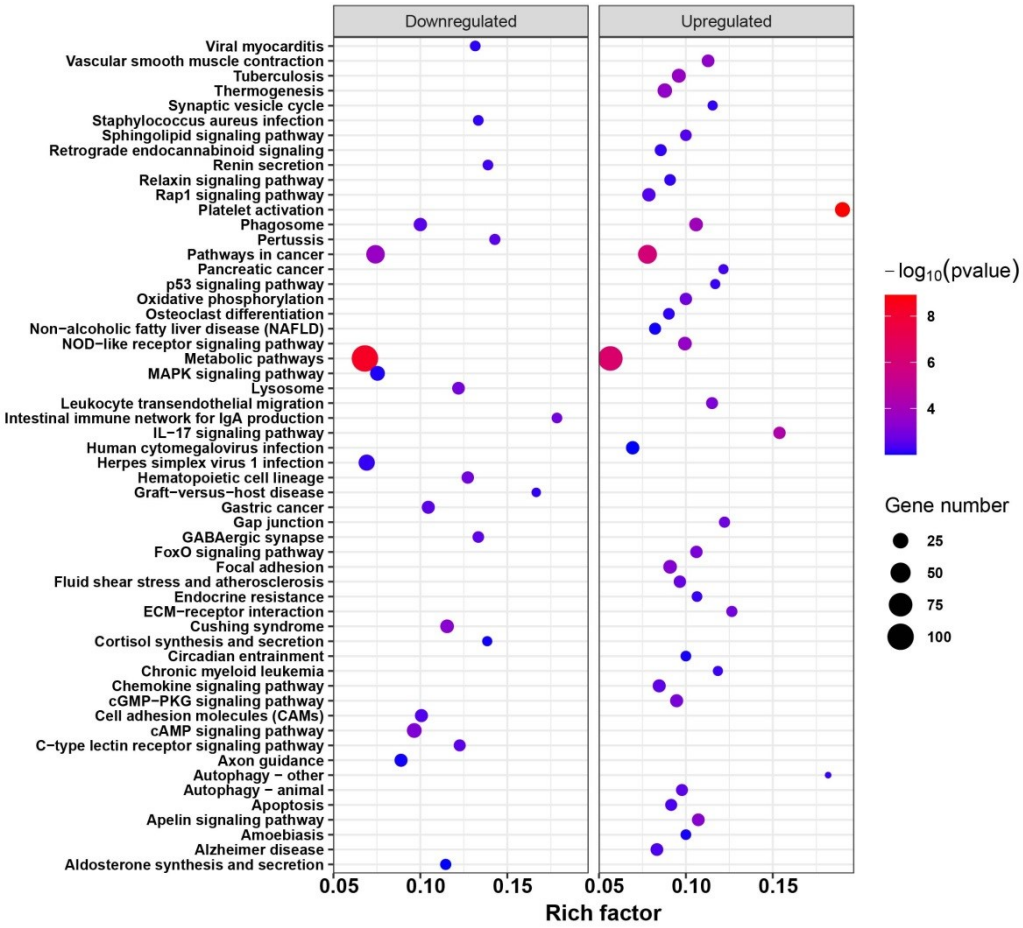

Fig. 4. Pathways significantly enriched with the upregulated and downregulated DEGs, as observed by KEGG enrichment

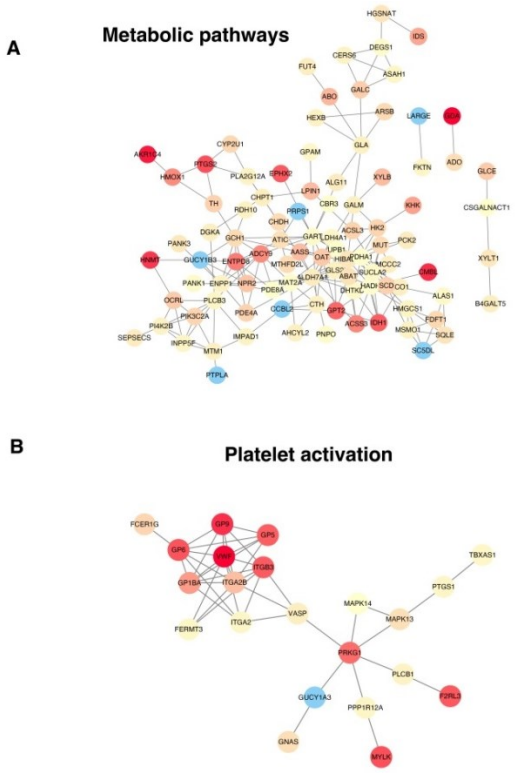

Fig. 5. DEGs downregulated in the metabolic pathway (A) and upregulated in platelet activation (B) observed by protein–protein interaction (PPI) analysis. The different colours represent degrees of connectivity

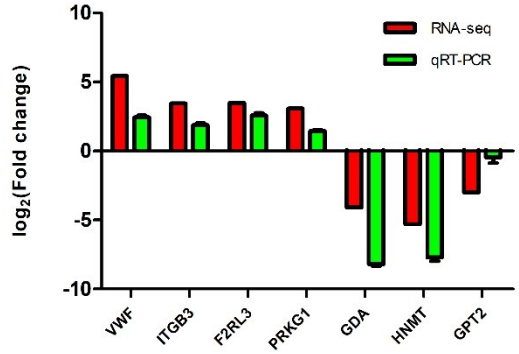

Fig. 6. Concordance of gene expression profiles obtained by RNA-seq and qRT-PCR methods for four upregulated genes (*VWF*, *ITGB3*, *F2RL3*, and *PRKG1*) and three downregulated genes (*GDA*, *HNMT*, and *GPT2*). The *β-actin* gene was used as an internal reference control gene

To further explore the DEGs involved in the pathway of the inflammatory response, the KEGG database was employed to identify key candidate genes. We observed that 37 and 22 pathways were significantly enriched with upregulated and downregulated DEGs, respectively (Fig. 4). For the upregulated DEGs, the top three pathways were platelet activation, metabolic pathways, and pathways in cancer. As for the downregulated DEGs, the three principal pathways were metabolic pathways, pathways in cancer, and Cushing syndrome. These results suggest that both upregulated DEGs in platelet activation and downregulated DEGs in the metabolic pathway might play a vital role in the inflammatory response.

Differentially expressed genes associated with metabolic pathways and platelet activation. The results of the PPI analysis showed that the downregulated DEGs in the metabolic pathway clustered into four PPIs (Fig. 5A), while the upregulated DEGs in platelet activation clustered into one PPI (Fig. 5B). For the downregulated DEGs, the largest PPI network contained 95 genes, 6 of which had high degrees of connectivity and a threshold for $\log_2$ fold change < −3.0. These genes were *AKR1C4*, *PTGS2*, *HNMT*, *EPHX2*, *CMBL*, and *IDH1*. For the upregulated DEGs, the PPI network consisted of 22 genes, 8 of which had high degrees of connectivity and a threshold for $\log_2$ fold change > 3.0. These genes were *VWF*, *GP9*, *MYLK*, *GP6*, *F2RL3*, *ITGB3*, *GP5*, and *PRKG1*.

Confirmation of gene expression with qPCR. The results of RNA-seq validation in NM to M comparisons showed that the gene expression patterns obtained using sequencing were consistent with the results generated from qRT-PCR (Fig. 6).

Discussion

Clinical mastitis is one of the most frequent and costly diseases in dairy cows, causing serious inflammatory diseases and economic losses. Currently, there is no optimal treatment or effective drug for CM. Our understanding of the host immune response to *S. aureus* infection in dairy cows with CM is limited, making it difficult to design novel therapeutics. A bacterial infection usually causes massive alterations in the host transcriptome, leading to an aberrant host metabolism and a modulated immune response which is ideal for bacterial invasion. In this study, we describe a transcription dataset of PBL samples in dairy cows with CM and in age-matched control samples. A total of 4,286 DEGs were observed in the CM group relative to the control group. Of these, 3,085 genes were expressed relatively more intensely, and the remaining 1,201 were expressed relatively less intensely. The number of DEGs identified here through RNA-seq analysis exceeds the number of DEGs previously reported by Wang *et al.* (31) for the same RNA sample types (17 DEGs consisting of 7 upregulated and 10 downregulated genes). The reason for this difference is that the mastitic individuals in our study had a more serious disease status. It means that a more comprehensive clinical study and a larger scale of transcriptome profiling with more cases are needed to evaluate the pathogenesis of CM with *S. aureus* infection.

The functional annotation analysis of DEGs indicated that both upregulated and downregulated genes were assorted into categories including cellular anatomical entity, cellular process, binding and membrane-bounded organelle, which all play important roles in bacterial invasion of the host. The upregulated DEGs were found to mainly be involved in platelet

activation and the downregulated DEGs to mainly be involved in the metabolic pathways. Numerous studies have shown that regardless of an inflammatory challenge being transient acute, sustained subacute, or repeated transient, such a challenge can directly affect the metabolic function in lactating dairy cattle (7).

The upregulated DEGs in the platelet activation pathway clustered into one PPI network. We observed several genes displaying high degrees of connectivity. Of these, three upregulated DEGs (*VWF, ITGB3,* and *MYLK*) were reported by other authors to be associated with inflammation (16, 18, 33). In particular, evidence revealed that the *VWF* gene is a plasma glycoprotein with a crucial haemostatic role in serving as a molecular bridge linking platelets to subendothelial components following vascular injury (18). Interestingly, several glycoprotein Ib-IX-V complex genes, including *GP9*, *GP6*, *GP5,* and *GP1BA*, were observed to be receptors for *VWF* and ascribed high importance in mediating *VWF*-dependent platelet adhesion to blood vessels (14). In addition, *S. aureus* was found to bind directly to *VWF* using its von Willebrand binding protein (11) and form small bacteria-platelet-fibrin microaggregates, which strongly adhere to *VWF* exposed on the vessel wall (30). The levels and multimer size of *VWF*s tend to increase during inflammatory and septic states caused by *S. aureus* (24). An additional gene with inflammatory activity is *F2RL3*, because it encodes coagulation factor II (thrombin) receptor-like 3, and its methylation contributes to inflammation through platelet activation (9). Jaeger *et al.* (16) found that *ITGB3* gene expression was increased in porcine mammary epithelial cells after a challenge with *S. aureus*. In addition, *PRKG1*, a key regulator of adipokine secretion, has an important regulatory function for milk fatty acid metabolism and may be related to feeding intake and feed conversion efficiency in dairy cattle (28). Milk secreted from CM cows was very thin compared with milk from the control group in our study, suggesting that increased expression of *PRKG1* in CM cows might be the cause of milk changes. These results suggested that upregulated DEGs in the platelet activation pathway may play a vital role in the inflammatory response of dairy cows infected with *S. aureus*.

The downregulated DEGs in the metabolic pathway clustered into four PPI networks. The biggest contained 95 genes, 6 of which had high degrees of connectivity and a threshold for $\log_2$ fold change < -3.0. Two downregulated DEGs (*PTGS2* and *IDH1*) were reported to be associated with inflammation (4, 23). It is well known that *AKR1C4* belongs to a family of enzymes that regulate the metabolism of progesterone. Evidence showed that the transcript abundance of *AKR1C4* was greater in the endometrium of cows with higher postovulatory progesterone, suggesting that increased expression of *AKR1C4* may stimulate vascularisation of the endometrium to support blastocyst growth and embryo implantation (2). Some studies indicated that CM cows were at a higher risk of abortion than cows without mastitis during early lactation (12). The expression of *AKR1C4* was significantly decreased in CM cows in this study. Future studies are needed to analyse the molecular mechanisms underlying the regulation of *AKR1C4* and their effect upon reproductive performance in CM cows. A new study found that *HNMT* disruption resulted in a robust increase in brain histamine concentration (34). Histamine is a potent inflammatory mediator commonly associated with allergic reactions, promoting inflammatory and regulatory responses that contribute to pathological processes (8). We can speculate that the decreased expression of *HNMT* in CM cows may be related to the inflammatory response. The *PTGS2* gene, also known as cyclooxygenase-2 (COX-2), induced by inflammatory stimuli, hormones, and growth factors, is a more important source of prostanoid formation in inflammation and proliferative diseases (13). The activity of *EPHX2* is considered a major determinant of the bioavailability of anti-inflammatory epoxyeicosatrienoic acid metabolites in the body. Research indicated that the inflammation response was attenuated in *EPHX2* knockout mice (21). We can speculate that the decreased expression of *EPHX2* in the CM cows may be related to the degree of inflammation in the disease course. These results suggest that the downregulated DEGs in the metabolic pathway might play an important role in the inflammatory response in dairy cows infected with *S. aureus*.

In conclusion, a total of 3,085 upregulated DEGs and 1,201 downregulated DEGs were found in the CM groups compared to the control. Functional analysis of GO showed that both the upregulated and downregulated DEGs were mainly enriched in the cellular process, biological regulation, and regulation of the biological process. Analysis of the KEGG pathway showed that the upregulated and downregulated DEGs mainly participated in the platelet activation pathway and metabolic pathway, respectively. Several key candidate genes are known to be related to the inflammatory response. These findings improve our understanding of the biological processes of the CM response to *S. aureus* infection, enabling us to identify specific genes for diagnostic markers and possibly for targeted therapy.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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