Research Article

**N°6-Methyladenosine Modification Profile in Bovine Mammary Epithelial Cells Treated with Heat-Inactivated Staphylococcus aureus**

Ting Li,1 Yifan Zhu,2,3 Changjie Lin,1 Jie Chen,4 Yiya Yin,1 Xin Tang,1 Yingyu Chen,2,3 Aizhen Guo,2,3 and Changmin Hu1

1Department of Clinical Veterinary Medicine, Faculty of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070 Hubei Province, China
2Department of Preventive Veterinary Medicine, Faculty of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070 Hubei Province, China
3State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, 430070 Hubei Province, China
4Institute of Animal Husbandry and Veterinary Medicine, Wuhan Academy of Agricultural Sciences, Wuhan, 430023 Hubei Province, China

Correspondence should be addressed to Changmin Hu; hcm@mail.hzau.edu.cn

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The symptoms of mastitis caused by *Staphylococcus aureus* (*S. aureus*) in dairy cows are not obvious and difficult to identify, resulting in major economic losses. N°6-Methyladenosine (m°6A) modification has been reported to be closely associated with the occurrence of many diseases. However, only a few reports have described the role of m°6A modification in *S. aureus*-induced mastitis. In this study, after 24 h of treatment with inactivated *S. aureus*, MAC-T cells (an immortalized bovine mammary epithelial cell line) showed increased expression levels of the inflammatory factors IL-1β, IL-6, TNF-α, and reactive oxygen species. We found that the mRNA levels of METLL3, METLL14, WTAP, and ALKBH5 were also upregulated.

Methylated RNA immunoprecipitation sequencing analysis revealed that 133 genes were m°6A hypermethylated, and 711 genes were m°6A hypomethylated. Biological functional analysis revealed that the differential m°6A methylated genes were mainly related to oxidative stress, lipid metabolism, inflammatory response, and so on. In the present study, we also identified 62 genes with significant changes in m°6A modification and mRNA expression levels. These findings elucidated the m°6A modification spectrum induced by *S. aureus* in MAC-T cells and provide the basis for subsequent m°6A research on mastitis.

1. Introduction

Mastitis is one of the most common diseases in dairy cows, with a high incidence rate, and its treatment remains a challenge. Most cases of mastitis are caused by pathogenic microorganisms that invade the mammary gland tissues [1]. To date, more than a hundred pathogenic microorganisms have been isolated from bovine mammary gland tissues [2]. When the gland is stimulated by pathogenic microorganisms, bovine mammary epithelial cells activate the innate immune response to resist invasion. Innate immunity plays a major role in the early stages of infection. Pathogen-associated molecular patterns are recognized by receptors on the surface of bovine mammary epithelial cells, causing an immune response that wipes out pathogenic microorganisms [3]. Among the many pathogenic microorganisms that cause mastitis, *Staphylococcus aureus* is one of the most common gram-positive bacteria [1, 4]. Previous studies have shown that *S. aureus* is involved in many pathological processes, including breast tissue destruction and chronic inflammation [5, 6]. The main toxins produced by *S. aureus*, such as endotoxins, and modifications to their peptidoglycan layer and lipoteichoic acid moieties, can cause mastitis [7]. Exotoxins secreted by *S. aureus* can destroy the basic
structure of the mammary gland, leading to the degeneration and necrosis of mammary epithelial cells. After *S. aureus* infection, bovine mammary epithelial cells release inflammatory factors through the toll-like receptors (TLRs) and nuclear factor-kappa B (NF-κB) signaling pathway and activate innate immunity [8–10]. In addition, *S. aureus* has been reported to induce fibrosis [11]. Mastitis caused by *S. aureus* infection is usually recursive or subacute; thus, it is not easily detected in clinical settings. Owing to the high infection rate and drug resistance of *S. aureus*, *S. aureus*-induced mastitis is difficult to cure [7, 12]. Therefore, further studies on the mechanism of *S. aureus*-induced mastitis are of great significance for clinical diagnosis, treatment, and prevention.

N6-Methyladenosine (m6A) modification is a type of RNA modification during which the N6 position of adenine in RNA is methylated. As a reversible dynamic modification [13, 14], its biological function is mainly determined by the methylase system [15]. The system includes “Writer,” methyltransferases, including methyltransferase-like 3 (METTL3) and 14 (METTL14), and Wilms tumor 1-associated protein (WTAP) [16, 17]; “Eraser,” demethylase such as fat mass and obesity-associated gene (FTO) and alkB homolog 5 (ALKBH5) [17]; and “Reader,” m6A methylated reading proteins [18, 19], such as the YTH family of proteins, IGF2BP protein, and eukaryotic initiation factor (eIF3) protein [20]. Studies have shown that most diseases are accompanied by changes in the levels of methylating enzymes; methylation enzymes are often involved in the regulation of diseases [21–23]. Changes in m6A modifications are closely related to immune regulation [24]. Feng et al. found that m6A levels are responsible for lipopolysaccharide-induced inflammatory reactions in human dental pulp cells [25]. In addition, m6A can also regulate T-cell homeostasis, resistance to viruses and bacteria, and antitumor immunity [26–28]. Furthermore, Jiaxing et al. summarized the role of m6A modification in stem cell death and survival and further explained the role of m6A modification in immune response, cell apoptosis, autophagy, and senescence [29]. Moreover, m6A modification has been confirmed to play an important role in the development of many diseases. However, whether m6A affects *S. aureus*-induced mastitis in bovines has not been reported.

In this study, we detected changes in the expression levels of methyltransferase and demethylase in bovine mammary epithelial cells induced by *S. aureus* and analyzed the detected differential m6A modified transcripts by using methylated RNA immunoprecipitation sequencing (MeRIP-seq). These findings shed light on the role of m6A modification in *S. aureus*-induced mastitis.

2. Materials and Methods

2.1. Bacterial Strains and Cell Line. MAC-T cells (an immortalized bovine mammary epithelial cell line) were kindly donated by Professor Mark Hanigan of Virginia Tech University. We used DME/F12 medium (Hyclone, Tauranga, New Zealand) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY, United States) to culture the cells, with incubation at 37°C in a 5% CO2 incubator. We used 0.25% trypsin and 0.02% EDTA to digest and passage the cells.

*Staphylococcus aureus* (ATCC 29213) was donated by Professor Zhou Rui of Huazhong Agricultural University. A 100 µL of *S. aureus* suspension was inoculated into 10 mL Luria-Bertani (LB) liquid medium at a ratio of 1:100 and grown in a shaker incubator at 220 rpm/min and 37°C. Then, 100 µL of the bacterial culture was serially diluted from 10⁻¹ to 10⁻⁸, spread plated onto LB solid medium, and incubated overnight in a bacterial incubator at 37°C. The number of bacteria was counted, and the remaining bacteria in the broth culture were inactivated in a water bath at 63°C for 30 min.

2.2. Sample Collection and RNA Extraction. The MAC-T cells were seeded at a density of 10⁵ cells per well in a cell culture dish (Corning, New York, NY, USA). The cells were set up in triplicate per group. After 12 h, the DMEM was replaced with 2% FBS in the cell culture dish. Then, the inactive bacterial cells were added to the dish at a 10:1 ratio (bacteria: cells) [30]. In the control group, an equal volume of LB medium was added, and the cells were incubated for 24 h. After incubation, we discarded the medium and then used cold phosphate-buffered saline (PBS) to wash the cells three times.

Total RNA was extracted from the MAC-T cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. The RNA concentrations were measured using a NanoDrop 2000 instrument (Thermo, Waltham, MA, USA). Ribonucleic acid integrity and gDNA contamination were assessed using agarose gel electrophoresis.

2.3. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The RNA samples were reverse transcribed using HiScript III Reverse Transcriptase (Vazyme, Nanjing, China). Then, we used AceQ qPCR SYBR Green Master Mix (Vazyme) to configure the sample to be tested by RT-qPCR. The samples were placed in a Viia™ 7 Real-Time PCR System instrument (Applied Biosystems Inc., Foster, CA, USA), and the qPCR program was run. Data were analyzed using the 2⁻ΔΔCT method. The relevant primer information is summarized in Table 1.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). The supernatants of the MAC-T cells exposed to the bacteria for 24 h and of the control group were collected. The concentrations of the inflammatory factors (IL-1β, IL-6, and TNF-α) in the supernatant were then measured using an ELISA kit (Cusabio, Wuhan, Hubei, China) following the manufacturer’s instructions.

2.5. Cellular Reactive Oxygen Species Detection. Cellular reactive oxygen species (ROS) were detected using a commercially available kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. Specifically, the supernatant of the MAC-T cells was discarded, and the cells were washed three times with cold PBS. Next, trypsin without EDTA was used to collect the cells. The cells were then
resuspended in PBS. Finally, flow cytometry (Beckman Coulter, Indianapolis, IN, USA) was used to detect the fluorescence intensity at 488 nm and 525 nm, respectively.

2.6. Methylated RNA Immunoprecipitation Quantitative Polymerase Chain Reaction (MeRIP-qPCR). The following experiments were performed in accordance with the manufacturer’s instructions (Millipore, Bedford, MA, USA): (1) total RNA was fragmented by Zn²⁺ at 94°C; (2) magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) and m⁶A antibody (Abcam, Cambridge, UK) were incubated for 1 h at room temperature; (3) the system was incubated with the fragmented RNA at 4°C for 2 h; (4) elution buffer was used to elute the mixture twice at 4°C for 1 h; and (5) the collected eluate was subjected to RNA extraction and reverse transcription (Vazyme). In accordance with manufacturer’s the instructions, cDNA was detected by RT-qPCR using the AceQ SYBR qPCR Master Mix (Vazyme). The data were analyzed by % input; that is, 

\[
\%\text{input} = \frac{2^{-\Delta\text{CT}}}{} - \log_2(\text{input dilution factor})
\]

\(\Delta\text{CTRIP}\) means the CT value of the RNA immunoprecipitation (IP RNA) samples, and \(\Delta\text{CTinput}\) means the CT value of the input RNA samples. The primers for the relevant methylated RNA were as follows (Table 2).

2.7. MeRIP-seq and mRNA-seq. The collected RNA was sent for MeRIP-seq and ribonucleic acid sequencing (RNA-seq) at Cloud-Seq Biotech (Shanghai, China) (GSE161050). In this study, the m⁶A-MeRIP kit (Millipore, Burlington, MA, USA) was used to perform the m⁶A RNA immunoprecipitation reaction. RNA sequencing libraries were constructed from the input RNA samples and IP RNA samples after immunoprecipitation with the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). After library quality control, high throughput sequencing was performed with Illumina HiSeq (Illumina, San Diego, CA, USA).

2.8. Bioinformatic Analysis. Clean reads of high quality were obtained after Q30 quality control and removal of the connector using the Cutadapt (v1.9.3 software). Then, HISAT2 (v2.0.4 software) was used to match the clean reads of the samples to the reference genome (bosTau9), and the MACS (v1.4.2 software) was used to identify RNA m⁶A methylation. Enrichment analyses were performed using Gene Ontology (GO, http://www.geneontology.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg) for the differentially methylated genes.

2.9. Statistical Analysis. In this study, Prism v7.0 (GraphPad software) was mainly performed using for data analyses. The results are presented as the mean values (±SD) of three independent experiments, and \(p\) values <0.05 were considered statistically significant.

3. Results

3.1. Heat-Inactivated S. aureus Induced Inflammation and Oxidative Stress in the MAC-T Cells. After the MAC-T cells were stimulated with heat inactivated S. aureus at an MOI ratio of 10:1 for 24 h, the expression of inflammatory factors was detected using RT-qPCR and ELISA. Compared with the control group, the S. aureus group showed significantly increased mRNA and protein levels of IL-1β, IL-6, and TNF-α (Figures 1(a) and 1(b)). In previous studies, mastitis was also accompanied by oxidative stress [31]. As shown in Figure 1(c), compared with the control group, the expression level of ROS in the S. aureus group was significantly increased. These data thus indicated that heat-inactivated

### Table 1: Primer sequence of mRNA in RT-qPCR.

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| β-Actin   | AGATCAAGATCATCCTGGGCC   | TAACCGCAGCTAACAGTCCGG |
| IL-1β     | TTTCCATTCTCTTGGGATAGA  | AAATGAACCGAGAAGTGGTTT |
| IL-6      | AGCCAGTCCATTGGTTGTTGG  | CTGTTGTCAATCAGCAGAT   |
| TNF-α     | TCTTCTCAAGCTAAGTAAACGCC | CCATGAAGGCTATTGCCATAC |
| METTL3    | GGAACACTGCTTGGGTTGTTG  | GGTTGACATTTGTGGTGTGTC |
| METTL14   | TTGGGAGCAAGGTTTCTACCG  | CATTTTACGCTCCAACTGCA |
| FTO       | CTCGGTCTGGAGAGGATTCA   | TGCTCCTTTGTGGCTAGTGC |
| WTAP      | CTCGGTCTGGAGAGGATTCA   | CTGCTGCTGAGATTCTGCTG |
| ALKBH5    | CCCATCACATCTTCGAGCG    | AGCAGGCTATCCACTGAGC |

### Table 2: Primer sequence of m⁶A modified part in mRNA in RT-qPCR.

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| TNF-α     | AATATGTCGGTCAGGCTGG | TCCCTTATGTTGTTGTTGG |
| PDGFRA    | GACCAGCAGGTCTAGTCTAAAT | GCAGGAGGCAAAAGAGAAC |
| TRAF1     | ATGAGGAGGAGGAGGCGAAGA | CAGAGTCCACCTCCAGCTTC |
| TLR4      | CCGGTGTTTGGGAGAAT  | ATGGTCAGGTTGCACTGAC |

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S. aureus induces inflammation and oxidative stress in MAC-T cells.

3.2. Abnormal Expression Levels of m^6^A Transferase/Demethylase Were Induced by S. aureus in the MAC-T Cells. The m^6^A enzyme system plays an important role in RNA m^6^A modification [18, 19]. Some studies have indicated that the occurrence of disease is related to the abnormal expression of the m^6^A enzymes [32]. In this study, the expression levels of methyltransferases METTL3, METTL14, and WTAP and demethylases ALKBH5 and FTO were detected using RT-qPCR. The results showed that compared with the control group, the S. aureus group had significantly increased mRNA expression levels of METTL3, METTL14, WTAP, and ALKBH5 (Figures 2(a)–2(d)), but no significant difference in the expression level of FTO was observed (Figure 2(e)). This suggests that m^6^A modification may be related to the inflammatory response and oxidative stress induced by S. aureus in the MAC-T cells.

3.3. Overview of the m^6^A Methylation Map in the Control and S. aureus Groups. Based on the expression levels of the m^6^A enzymes, we speculated that m^6^A methylation differed between the control and the S. aureus groups, which was further detected using MeRIP-seq. Compared with the control group, the S. aureus group obtained 1,006 significantly distinct m^6^A peaks in 844 mRNAs (p < 0.0001, fold change > 2, Table S1), among which 133 mRNAs had 135 hypermethylated sites such as PDGFRA and 711 mRNAs had 871 hypomethylated sites such as TNF and TRAF1 (Figures 3(a) and 3(b)). In addition, by MeRIP-qPCR, this study confirmed the hypermethylation of PDGFRA and hypomethylation of TNF and TRAF1 in S. aureus-induced MAC-T cells by MeRIP-qPCR (Figure 3(c)). Table 3 lists the top 20 differential m^6^A peaks.
**Figure 2:** Variation in the mRNA expression levels of the m^6^A enzymes in *S. aureus*-induced mastitis. The mRNA expressions of the methyltransferases METTL3 (a), METTL14 (b), and WTAP (c) and the demethylases ALKBH5 (d) and FTO (e) were detected by RT-qPCR. ns: not significant, *p* < 0.01 and **p** < 0.001.

**Figure 3:** Analysis of differential m^6^A-modified genes between the control and *S. aureus* groups. (a) Volcano plot analysis of differential m^6^A methylation genes (*p* < 0.00001, fold change > 2). (b) Visualization of PDGFRA, TNF, and TRAF1 using the IGV software. (c) Verification of PDGFRA, TNF, and TRAF1 by MeRIP-seq.
Further analysis revealed the differential methylation sites on all chromosomes, especially chromosomes 3, 4, and 5 (Figure 4(a)). To obtain the preferred location distribution of the m\(^6\)A methylating peaks in the genes, this study performed a statistical analysis of the transcriptome. We found that the m\(^6\)A peak was mainly enriched in the coding sequence, stop codon, and 3′-untranslated regions in the two groups (Figures 4(b) and 4(c)). The most enriched motif sequence of the m\(^6\)A peaks was GGACU in the control group (Figures 4(b) and 4(c)). The most enriched motif sequence was GGACU in the control and 6(b)). Compared with the control group, 848 differentially methylated sites were found in 135 hypermethylated sites (fold change > 2 and \(p < 0.05\), Table S2); among those genes, there are 249 upregulated genes and 599 downregulated genes. Table 4 shows the top 20 DEGs in the 871 differentially methylated and expressed genes (DEGs) were found in S. aureus groups (fold change > 2 and \(p < 0.05\), Table S2); among those genes, there are 249 upregulated genes and 599 downregulated genes. Table 4 shows the top 20 DEGs in the control and S. aureus groups. Meanwhile, we verified that the mRNA expression of the genes PHOAPHO2 and MYH11 was downregulated by RT-qPCR (Figures 6(c)–6(f)). We presented 15 differentially methylated and expressed genes (Table 5). The KEGG analysis of the 62 genes revealed that these genes were mainly enriched in pyruvate metabolism, the TGF-β, NF-κB, and Hippo signaling pathways (Figure 5(d)), which are associated with the progression of mastitis.

### Table 3: The top 20 differential m\(^6\)A-modified peaks between S. aureus and con based on \(p\) value.

| Gene name | Peak region | Peak start | Peak end  | Chromosome | Log10 (\(p\) value) | Log2 (fold change) | Hyper/hypo |
|-----------|-------------|------------|-----------|------------|---------------------|-------------------|------------|
| KDM3A     | CDS         | 48394594   | 48394800  | NC_037338.1| -7.35               | 1.27              | Hyper      |
| C18H19orf81| StopC      | 56747061   | 56748820  | NC_037345.1| -6.59               | 1.09              | Hyper      |
| LTZS1     | CDS         | 67371075   | 67371555  | NC_037335.1| -6.49               | 1.21              | Hyper      |
| USP26     | CDS         | 16901441   | 16901740  | NC_037357.1| -6.12               | 3.89              | Hyper      |
| CDH4      | CDS         | 55180735   | 55180969  | NC_037340.1| -6.07               | 4.30              | Hyper      |
| CRCT1     | StopC      | 18184161   | 18184773  | NC_037330.1| -5.98               | 5.74              | Hyper      |
| KLHL6     | CDS         | 83597549   | 83597780  | NC_037328.1| -5.86               | 5.90              | Hyper      |
| SPECC1    | 5′UTR       | 33638477   | 33638721  | NC_037346.1| -5.86               | 1.03              | Hyper      |
| LOC781261 | 5′UTR       | 74730061   | 74730340  | NC_037335.1| -5.69               | 5.94              | Hyper      |
| GAB2      | 3′UTR       | 17794541   | 17795150  | NC_037356.1| -5.67               | 1.52              | Hyper      |
| LOC101903326| StopC  | 1089721    | 1090900   | NC_037341.1| -8.60               | 8.19              | Hyper      |
| LOC616254 | StopC      | 48235186   | 48235992  | NC_037346.1| -8.43               | 8.73              | Hyper      |
| LOC100848799| CDS      | 52319261   | 52319860  | NC_037334.1| -8.06               | 4.30              | Hyper      |
| FAM1998B  | StartC     | 40796022   | 40797166  | NC_037344.1| -8.01               | 8.43              | Hyper      |
| TACR2     | StopC      | 25769941   | 25770440  | NC_037355.1| -7.94               | 4.06              | Hyper      |
| GPR132    | StopC      | 69438921   | 69439920  | NC_037348.1| -7.90               | 1.95              | Hyper      |
| FRMPD1    | CDS         | 61863921   | 61865028  | NC_037335.1| -7.88               | 3.09              | Hyper      |
| ANGPT4    | StopC      | 60234003   | 60234780  | NC_037340.1| -7.88               | 8.61              | Hyper      |
| OR9Q2     | CDS         | 81371461   | 81372540  | NC_037342.1| -7.87               | 5.35              | Hyper      |
| SAA3      | CDS         | 26414395   | 26414676  | NC_037356.1| -7.87               | 4.68              | Hyper      |

3.4. Differentially m\(^6\)A Methylated RNAs Were Involved in Mastitis-Related Processes. To further explore the biological function of m\(^6\)A methylation in bovine mammary epithelial cells stimulated by S. aureus, gene with different m\(^6\)A peaks was analyzed by GO and KEGG analyzed. The GO analysis showed that the m\(^6\)A hypermethylated genes in the S. aureus group were more closely associated with the regulation of vascular-associated smooth muscle cell migration and phosphatidylethanolamine metabolic process (biological process, [BP]), COP9 signalosome (cellular component, [CC]), and phospholipase activity (molecular function, [MF]) (Figure 5(a)). The hypomethylated genes were significantly involved in transcription regulation, DNA-templated synthesis (BP), nucleoplasm (CC), and transcription regulator activity (MF, Figure 5(b)).

Remarkably, according to the KEGG analysis, we identified that the m\(^6\)A hypermethylated genes were significantly associated with fatty acid degradation and adipocytokine signaling pathway, amongst others (Figure 5(c)), whereas the hypomethylated genes were mainly enriched in the TGF-β, NF-κB, and Hippo signaling pathways (Figure 5(d)), which are associated with the progression of mastitis.

3.5. Conjoint Analysis of Differential m\(^6\)A Modification and mRNA. RNA-seq was used to detect the mRNA expressions in the control and S. aureus groups (Figures 6(a) and 6(b)). Compared with the control group, 848 differentially expressed genes (DEGs) were found in S. aureus groups (fold change > 2 and \(p < 0.05\), Table S2); among those genes, there are 249 upregulated genes and 599 downregulated genes. Table 4 shows the top 20 DEGs in the control and S. aureus groups. Meanwhile, we verified that the mRNA expression of the genes PHOAPHO2 and MAPKBP1 was upregulated, and that of CHRNB1 and MYH11 was downregulated by RT-qPCR (Figures 6(c)–6(f)). By crossanalysis of the MeRIP-seq and RNA-seq data, we discovered that in 135 hypermethylated sites (fold change > 2 and \(p < 0.05\), four genes were upregulated (called “hyper-up”), and one gene was downregulated (called “hyper-down,” fold change > 2 and \(p < 0.05\). In the 871 hypomethylated sites (fold change > 2 and \(p < 0.05\), six genes were upregulated (called “hypo-up”), and 50 genes were downregulated (called “hypo-down,” fold change > 2 and \(p < 0.05\), Figure 6(g)). We presented 15 differentially methylated and expressed genes (Table 5). The KEGG analysis of the 62 genes revealed that these genes were mainly enriched in pyruvate metabolism, the TGF-β, and Hippo signaling pathway (Figure 6(h)).
4. Discussion

In mastitis in dairy cows, pathogenic microorganisms usually induce breast inflammation. *Staphylococcus aureus* is one of the most common pathogens that often causes subclinical mastitis [34]. Previous studies have shown that bovine mammary epithelial cells are the first line of defense against the invasion of mammary glands by microorganisms such as *S. aureus* [1, 35], which leads to the release of various chemokines and cytokines [36]. Although there is currently much research into mastitis, the molecular mechanism of mastitis caused by *S. aureus* is still unclear. There are many challenges in improving mastitis diagnosis, treatment, and prevention. m<sup>6</sup>A methylation modification can affect RNA splicing, transcription, and translation [37–39] and thereby participating in the initiation and progression of many diseases such as cancer and cardiovascular diseases [26, 40, 41]. However, only a few studies have reported m<sup>6</sup>A methylation modification in mastitis. To our knowledge, this is the first study to report the m<sup>6</sup>A map of *S. aureus*-induced mastitis, which provides a clue for further study of m<sup>6</sup>A modification in mastitis.

![Figure 4: m<sup>6</sup>A modification map of *S. aureus*-induced mastitis. (a) The distribution of the differential m<sup>6</sup>A-modified genes on bovine chromosomes. (b) Gene position distribution of the differential m<sup>6</sup>A modification sites in the control and *S. aureus* groups. (c) Peak density distribution of the differential m<sup>6</sup>A modification sites in the control and *S. aureus* groups. (d) Motif sequence analysis of m<sup>6</sup>A-modified genes in the control and *S. aureus* groups.](image)
In bovine, studies have shown that *S. aureus* induces an inflammatory response and finally leads to the secretion of cell factors, such as TNF-α, IL-6, and IL-1 [42, 43]. Oxidative stress is a state of imbalance between oxidation and antioxidation, that increases the production of ROS [44]. In mastitis, the release of ROS is key to the inflammatory response [45]. On the one hand, ROS plays an important role in inflammation, apoptosis, and cell growth [46, 47]. On the other hand, ROS can cause oxidation of proteins and DNA, inducing damage to nearby tissues. In this study, we used inactivated *S. aureus* to stimulate MAC-T cells, and we found that the expression levels of the inflammatory factors and ROS increased significantly (Figure 1), consistent with the results of other related studies [47, 48].

*m^4*A modification is considered a reversible dynamic modification. In addition, the methylase system determined its biological function [13]. Studies have shown that most diseases are accompanied by changes in methylases [21, 23]. Wu et al. reported the mRNA expression levels of *m^4*A related enzymes in bovine mammary epithelial cells treated with aflatoxins B1 and M1 [49]. In this study, the mRNA levels of methyltransferase (METLL3, METLL14, and WTAP) and a demethylase (ALKBH5) were upregulated (Figure 2), which suggests that *m^4*A modification may be related to the inflammatory response and oxidative stress induced by *S. aureus* in the MAC-T cells.

With analysis the MeRIP-seq results, we speculated that *S. aureus* may have induced the *m^4*A modification of some
Figure 6: Continued.
RNA molecules in MAC-T cells. Therefore, the m^6^A modification map of *S. aureus*-induced MAC-T cells was described using the MeRIP-seq technique. We found 1,006 differential methylation sites in 844 genes, some of which are closely related to the occurrence and development of mastitis. PDGFRA (Figure 3(b)), a hypermethylated molecule, reportedly induced constitutive phosphorylation of Akt, ERK1/2, and STAT3 [50]. Among the hypomethylated molecules, TNF (Figure 3(b)), a cytokine, and NFxB1 and NFxB2 (Table S1), which are important transcription factors, are essential for inflammation and innate immunity [51, 52]. TGF-β2 (Table S1) is a member of the TGF factor superfamily that plays an important role in regulating the initiation, maintenance, and resolution of immune responses and epithelial-mesenchymal transition [53, 54]. TRAF1 (Figure 3(b)) plays an important role in mediating cell survival, differentiation, proliferation, and death [54], and inhibition of TRAF1 can effectively inhibit inflammation, oxidative stress, and apoptosis [55]. We confirmed the m^6^A hypermethylation of PDGFRA and hypomethylation of TNF and TRAF1 through MeRIP-qPCR (Figure 3(c)). However, whether these molecules function through m^6^A modification requires further verification.

Abnormal lipid metabolism and oxidative stress are important factors that lead to the development of inflammatory diseases. Similar metabolic abnormalities have been reported in the early stages of mastitis [56, 57]. Researchers believed that lipids and their metabolites could be used as predictive diagnostic markers, preventive tools, and early treatment interventions for mastitis [57, 58]. In this study, GO analysis of differential m^6^A methylated genes revealed that the BP functions were mainly enriched in the phosphatidylethanolamine metabolic process, glycerophospholipid catabolic process (Figure 5(a)), and so on. The KEGG analysis revealed that the differential m^6^A methylated genes were mainly involved in the fatty acid degradation signaling pathway (Figure 5(c)). Thus, we speculated that m^6^A modification may affect the occurrence of *S. aureus*-induced mastitis through lipid metabolism and oxidative stress. In addition, previous studies have confirmed that bovine mammary epithelial cells transmit inflammatory signals mainly through the TGF, NF-κB, and TNF signaling pathway and other signaling pathways [59, 60]. The KEGG pathway analysis revealed that the differential m^6^A methylated genes were also enriched in the TGF and NF-κB signaling pathways (Figure 5(d)) in the current study. This suggests that m^6^A

Table 4: The top 20 differential mRNA expression in *S. aureus* vs. con.

| gene_id    | LogFC | Log10 (p value) | Regulation |
|------------|-------|-----------------|------------|
| ADGRE3     | 6.42  | -4.08           | Up         |
| STAR0D7    | 6.37  | -3.93           | Up         |
| TRIP01     | 6.21  | -3.97           | Up         |
| NTRK1      | 6.05  | -3.40           | Up         |
| KRT08      | 6.05  | -3.19           | Up         |
| LOC527796  | 6.00  | -3.15           | Up         |
| RAB0A4     | 5.87  | -2.80           | Up         |
| MAPKBP1    | 5.85  | -2.77           | Up         |
| PHOSPH02   | 5.81  | -2.84           | Up         |
| LOC100849008 | 5.81 | -2.72           | Up         |
| FBN1       | -9.80 | 7.29            | Down       |
| MYH11      | -9.19 | 6.27            | Down       |
| ACP2       | -7.80 | 5.34            | Down       |
| PRR29      | -7.71 | -5.01           | Down       |
| LOC11244598 | -7.59 | 4.99            | Down       |
| CHRNB1     | -7.55 | 4.56            | Down       |
| PSIP1      | -7.41 | 3.92            | Down       |
| DXO        | -7.37 | 4.36            | Down       |
| DSG1       | -7.29 | 3.61            | Down       |
| GLC        | -7.25 | 3.58            | Down       |

Figure 6: Joint analysis of differential mRNA expression and m^6^A modification. (a) Volcano map showing the differential mRNA expressions between the control and *S. aureus* groups (p<0.05, fold change >2). (b) Heat map demonstrating the differential mRNA expressions between the three control input samples and three *S. aureus* input samples. (c)–(f) mRNA expressions of PHOSPH02, MAPKBP1, CHRN1B1, and MYH11 detected by RT-qPCR. (g) Quadrant diagram showing the genes with differential mRNA expression and m^6^A modification (p<0.00001, fold change >2) and differential mRNA expression (p<0.05, fold change >2). (h) KEGG analysis of the biological functions of 62 genes with differential m^6^A modification and mRNA expression.
Table 5: 15 transcripts of differential m\(^6\)A modification and mRNA expression in *S. aureus* vs. con.

| Gene name | Change | Chromosome | Peak start | Peak end | m\(^6\)A modification change | Fold change | Log10 (p value) | mRNA expression change | LogFC | Log10 (p value) |
|-----------|--------|------------|------------|----------|-----------------------------|-------------|-----------------|-----------------------|-------|-----------------|
| ALK       | Hyperu | NC_037338.1| 70660375   | 70661760 | 1385                        | CDS         | 7.70            | 5.10                  | 5.19  | -6.82           |
| ERO1A     | Hyper-u| NC_037337.1| 11498514   | 11498840 | 326                         | 5\(^{\prime}\)UTR | 5.13            | 5.13                  | 5.02  | -2.55           |
| RYBP      | Hyperu | NC_037349.1| 29277201   | 29277420 | 219                         | 3\(^{\prime}\)UTR | 5.21            | 5.21                  | 4.06  | -1.41           |
| SERPINE1  | Hyperu | NC_037352.1| 35605621   | 35605860 | 239                         | 5\(^{\prime}\)UTR | 5.22            | 5.22                  | 1.90  | -1.81           |
| HEATR4    | Hyperdown| NC_037337.1| 84967915   | 84968140 | 225                         | 5\(^{\prime}\)UTR | 5.15            | 5.15                  | -5.94 | -1.89           |
| FBN1      | Hypodown| NC_037337.1| 61917881   | 61918380 | 499                         | CDS         | -22.53         | -7.75                  | -8.47 | -7.29           |
| CD70      | Hypodown| NC_037334.1| 17908292   | 17908687 | 395                         | CDS         | -17.43         | -5.40                  | -7.24 | -3.51           |
| LHFPL4    | Hypodown| NC_037349.1| 17088303   | 17088792 | 489                         | CDS         | -2.46          | -5.03                  | -7.03 | -3.19           |
| KMT2E     | Hypodown| NC_037331.1| 46268266   | 46268531 | 265                         | CDS         | -2.37          | -6.93                  | -6.96 | -3.26           |
| PRPF38A   | Hypodown| NC_037330.1| 93935321   | 93935880 | 559                         | 3\(^{\prime}\)UTR | -2.63         | -6.14                  | -6.95 | -3.02           |
| EXT1      | Hypoup  | NC_037341.1| 46477327   | 46477877 | 550                         | CDS         | -2.05          | -6.07                  | 5.56  | -2.60           |
| RPGR      | Hypoup  | NC_037357.1| 10498266   | 104983554| 893                         | CDS         | -2.47          | 7.23                   | 5.04  | -1.69           |
| ARSI      | Hypoup  | NC_037334.1| 61646441   | 61648420 | 1979                        | 3\(^{\prime}\)UTR | -5.82         | -5.60                  | 4.99  | -1.67           |
| ACSS2     | Hypoup  | NC_037340.1| 64232901   | 64233260 | 359                         | 3\(^{\prime}\)UTR | -4.76         | -5.39                  | 4.64  | -1.50           |
| APC       | Hypoup  | NC_037334.1| 43874981   | 43877340 | 2359                        | CDS         | -4.16          | -5.01                  | 3.99  | -9.68           |
modification may be associated with mastitis. m\(^6\)A modification is well known that affects mRNA splicing, translation, and stability [37–39]. In the GO analysis, we also found that the differential m\(^6\)A modification was mainly related to biological progress items such as RNA biosynthetic process and transcription. Meanwhile, we conducted a joint analysis of differential m\(^6\)A molecules and mRNA. We found 62 genes whose m\(^6\)A modification and mRNA expression levels had changed significantly ($p < 0.05$, Figure 6(c)), and the changes in mRNA expression levels might have been caused by the change in the m\(^6\)A modification. Biological analysis of these 62 genes revealed that they were related to pyruvate metabolism, fatty acid biosynthesis, TGF-beta signaling pathway, and so on (Figure 6(d)).

The heat-inactivated S. aureus mastitis model retained the main infectious components while avoiding bacterial overgrowth and excessive cell death. There are many ways to inactivate S. aureus, such as ultraviolet irradiation (UV) and chemical treatment. However, it has been suggested that gram-positive bacteria are more resistant to ultraviolet light than gram-negative bacteria [61, 62]. The inactivation of S. aureus using chemical treatment, such as formaldehyde, is not completely effective [63]. In addition, residual chemical agents may have some effect on mammalian cells during subsequent infection experiments. The heat inactivation method can not only effectively inactivate S. aureus but is also simple and easy to perform. Therefore, the establishment of a mastitis model with heat-inactivated S. aureus has been recognized and applied by many researchers [30, 64, 65].

Through the above analysis, we speculate that, in MAC-T cells treated with S. aureus, m\(^6\)A modification will affect the transcription and translation of mRNA, thus affecting the physiological and pathological processes of inflammation, oxidative stress, and lipid metabolism (Figure 7). However, the mechanism by which m\(^6\)A regulates mastitis in bovine is still unclear. This study thus provides a clue to the mechanism of m\(^6\)A modification in S. aureus-induced mastitis and should be explored further in future studies.

5. Conclusions

The results of this study clearly show the changes in the m\(^6\)A modification spectrum in S. aureus-induced mastitis. We found that the different m\(^6\)A-modified molecules were involved in lipid metabolism, oxidative stress, inflammatory reactions, and other mastitis-related biological processes. This study broadens the research direction for dairy cow mastitis and lays the foundation for further research that the function of m\(^6\)A modification in mastitis.
Data Availability
The data obtained in this study are available from the corresponding author upon request.

Conflicts of Interest
There is no conflict of interest.

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Supplementary Materials
Table S1: differentially methylated RNA sites for S. aureus and con. Table S2: differential mRNA expression for S. aureus and Con. (Supplementary Materials)

References
[1] A. Ashraf and M. Imran, “Causes, types, etiological agents, prevalence, diagnosis, treatment, prevention, effects on human health and future aspects of bovine mastitis,” Animal Health Research Reviews, vol. 21, no. 1, pp. 36–49, 2020.
[2] H. Chu and S. K. Mazmanian, “Innate immune recognition of the microbiota promotes host-microbial symbiosis,” Nature Immunology, vol. 14, no. 7, pp. 668–675, 2013.
[3] Y. Wu, Y. Sun, X. Dong et al., “The synergism of PGN, LTA and LPS in inducing transcriptome changes, inflammatory responses and a decrease in lactation as well as the associated epigenetic mechanisms in bovine mammary epithelial cells,” Toxins, vol. 12, no. 6, p. 387, 2020.
[4] C. Lin, Y. Zhu, Z. Hao et al., “Genome-wide analysis of LncRNA in bovine mammary epithelial cell injuries induced by Escherichia coli and Staphylococcus aureus,” International Journal of Molecular Sciences, vol. 22, no. 18, p. 9719, 2021.
[5] T. Matsunaga, S. I. Kamata, N. Kakiichi, and K. Uchida, “Characteristics of Staphylococcus aureus isolated from peracute, acute and chronic bovine mastitis,” The Journal of Veterinary Medical Science, vol. 55, no. 2, pp. 297–300, 1993.
[6] T. Tollersrud, K. Penny, D. A. Caugant, and A. Lund, “Characterisation of isolates of Staphylococcus aureus from acute, chronic and subclinical mastitis in cows in Norway,” APMIS, vol. 108, no. 9, pp. 565–572, 2000.
[7] L. Sutra and B. Poutrel, “Vulurex factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus,” Journal of Medical Microbiology, vol. 40, no. 2, pp. 79–89, 1994.
[8] J. I. Jun and L. F. Lau, “CCN1 is an opsonin for bacterial clearance and a direct activator of toll-like receptor signaling,” Nature Communications, vol. 11, no. 1, p. 1242, 2020.
[9] B. Liu, Y. Che, M. Zhang et al., “IFN-γ activates the TRLR4-CCL5 signaling through reducing arginine level, leading to enhanced susceptibility of bovine mammary epithelial cells to Staphylococcus aureus,” Inflammation, vol. 43, no. 6, pp. 2209–2221, 2020.
[10] J. L. Lai, Y. H. Liu, C. Liu et al., “Indirubin inhibits LPS-induced inflammation via TLR4 abrogation mediated by the NF-kB and MAPKs signaling pathways,” Inflammation, vol. 40, no. 1, pp. 1–12, 2017.
[11] Y. Bi, Y. Ding, J. Wu, Z. Miao, J. Wang, and F. Wang, “Staphylococcus aureus induces mammary gland fibrosis through activating the TLR/NF-κB and TLR/AP-1 signaling pathways in mice,” Microbial Pathogenesis, vol. 148, article 104427, 2020.
[12] N. Pumipuntu, S. Kulpeanprasit, S. Santajit et al., “Screening method for Staphylococcus aureus identification in subclinical bovine mastitis from dairy farms,” Veterinary World, vol. 10, no. 7, pp. 721–726, 2017.
[13] Z. Bi, Y. Liu, Y. Zhao et al., “A dynamic reversible RNA N6-methyldenosine modification: current status and perspectives,” Journal of Cellular Physiology, vol. 234, no. 6, pp. 7948–7956, 2019.
[14] H. C. Duan, Y. Wang, and G. Jia, “Dynamic and reversible RNA N6-methyladenosine methylation,” Wiley Interdiscip Rev RNA, vol. 10, no. 1, article e1507, 2019.
[15] C. Gu, X. Shi, C. Dai et al., “RNA m6a modification in cancers: molecular mechanisms and potential clinical applications,” The Innovation, vol. 1, no. 3, article 100066, 2020.
[16] T. Tollersrud, K. A. Doxtader, and Y. Nam, “Structural basis for cooperative function of Mettl3 and Mettl14 methyltransferases,” Molecular Cell, vol. 63, no. 2, pp. 306–317, 2016.
[17] X. Wang, J. Huang, T. Zou, and P. Yin, “Human m6A writers: two subunits, 2 roles,” RNA Biology, vol. 14, no. 3, pp. 300–304, 2017.
[18] H. Shi, J. Wei, and C. He, “Where, when, and how: context-dependent functions of RNA methylation writers, readers, and erasers,” Molecular Cell, vol. 74, no. 4, pp. 640–650, 2019.
[19] H. Huang, H. Weng, and J. Chen, “m6A modification in coding and non-coding RNAs: roles and therapeutic implications in cancer,” Cancer Cell, vol. 37, no. 3, pp. 270–288, 2020.
[20] D. Dominissini, S. Moshitch-Moshkovitz, S. Schwartz et al., “Topology of the human and mouse m 6 A RNA methylomes revealed by m6A-seq,” Nature, vol. 485, no. 7397, pp. 201–206, 2012.
[21] H. Zhang, X. Shi, T. Huang et al., “Dynamic landscape and evolution of m 6 A methylation in human,” Nucleic Acids Research, vol. 48, no. 11, pp. 6251–6264, 2020.
[22] Y. Qin, L. Li, E. Luo et al., “Role of m 6 A RNA methylation in cardiovascular disease (review),” International Journal of Molecular Medicine, vol. 46, no. 6, pp. 1958–1972, 2020.
[23] J. Wang, J. Wang, Q. Gu et al., “The biological function of m 6 A demethylase ALKBH5 and its role in human disease,” Cancer Cell International, vol. 20, p. 347, 2020.
[24] C. Zhang, J. Fu, and Y. Zhou, “A review in research progress concerning m 6 A methylation and immunoregulation,” Frontiers in Immunology, vol. 10, p. 922, 2019.
[25] Z. Feng, Q. Li, R. Meng, B. Yi, and Q. Xu, “METTL3 regulates alternative splicing of MyD88 upon the lipopolysaccharide-induced inflammatory response in human dental pulp cells,” Journal of Cellular and Molecular Medicine, vol. 22, no. 5, pp. 2558–2568, 2018.
[26] H. B. Li, J. Tong, S. Zhu et al., “m 6 A mRNA methylation controls T cell homeostasis by targeting the IL-7/STAT5/SOCS pathways,” Nature, vol. 548, no. 7667, pp. 338–342, 2017.
[27] G. Lichinchi, S. Gao, Y. Saleroti et al., “Dynamics of the human and viral m(6)A RNA methylomes during HIV-1
infection of T cells,” *Nature Microbiology*, vol. 1, article 16011, pp. 1–9, 2016.

[28] D. Han, J. Liu, C. Chen et al., “Anti-tumour immunity controlled through mRNA m6A methylation and YTHDF1 in dendritic cells,” *Nature*, vol. 566, no. 7743, pp. 270–274, 2019.

[29] J. Chen, C. Wang, W. Fei, X. Fang, and X. Hu, “Epitranscriptomic m6A modification in the stem cell field and its effects on cell death and survival,” *American Journal of Cancer Research*, vol. 9, no. 4, pp. 752–764, 2019.

[30] W. Jin, E. M. Beagha-Awenu, G. Liang, F. Beaudoin, X. Zhao, and L. L. Guan, “Transcriptome microRNA profiling of bovine mammary epithelial cells challenged with *Escherichia coli* or *Staphylococcus aureus* bacteria reveals pathogen directed microRNA expression profiles,” *BMGC Genomics*, vol. 15, p. 181, 2014.

[31] X. Tang, C. Liu, T. Li et al., “Gambogic acid alleviates inflammation and apoptosis and protects the blood-milk barrier in mastitis induced by LPS,” *International Immunopharmacology*, vol. 86, article 106697, 2020.

[32] Y. Yang, P. J. Hsu, Y. S. Chen, and Y. G. Yang, “Dynamic trans- scriptomic m6A decoration: writers, erasers, readers and functions in RNA metabolism,” *Cell Research*, vol. 28, no. 6, pp. 616–624, 2018.

[33] J. E. Harper, S. M. Miceli, R. J. Roberts, and J. L. Manley, “Sequence specificity of the human mRNA N6-adenosine methlyase *Vitrin*,” *Nucleic Acids Research*, vol. 18, no. 19, pp. 5735–5741, 1990.

[34] L. S. Rocha, D. M. Silva, M. P. Silva et al., “Comparative genomics of *Staphylococcus aureus* associated with subclinical and clinical bovine mastitis,” *PLoS One*, vol. 14, no. 8, article e0220804, 2019.

[35] M. Cai, W. Fan, X. Li et al., “The regulation of *Staphylococcus aureus*-induced inflammatory responses in bovine mammary epithelial cells,” *Frontiers in Veterinary Science*, vol. 8, article 683886, 2021.

[36] Y. Zheng, G. Liu, W. Wang et al., “Lactobacillus casei Zhang counteracts blood-milk barrier disruption and moderates the inflammatory response in *Escherichia coli*-induced mastitis,” *Frontiers in Microbiology*, vol. 12, article 675492, 2021.

[37] I. A. Roundtree, M. E. Evans, T. Pan, and C. He, “Dynamic RNA modifications in gene expression regulation,” *Cell*, vol. 169, no. 7, pp. 1187–1200, 2017.

[38] Z. M. Zhu, F. C. Huo, and D. S. Pei, “Function and evolution of RNA N6-methyladenosine modification,” *International Journal of Biological Sciences*, vol. 16, no. 11, pp. 1929–1940, 2020.

[39] S. D. Kasowitz, J. Ma, S. J. Anderson et al., “Nuclear m6A reader YTHDC1 regulates alternative polyadenylation and splicing during mouse oocyte development,” *PLoS Genetics*, vol. 14, no. 5, article e1007412, 2018.

[40] Z. X. Rong, Z. Li, J. J. He et al., “Downregulation of Fat Mass and Obesity Associated (FTO) promotes the progression of intrahepatic cholangiocarcinoma,” *Frontiers in Oncology*, vol. 9, p. 369, 2019.

[41] Y. G. Chen, R. Chen, S. Ahmad et al., “N6-Methyladenosine modification controls circular RNA immunity,” *Molecular Cell*, vol. 76, no. 1, pp. 96–109, 2019.

[42] S. Von Aulock, S. Morath, L. Hareng et al., “Lipoteichoic acid from *Staphylococcus aureus* is a potent stimulus for neutrophil recruitment,” *Immunobiology*, vol. 208, no. 4, pp. 413–422, 2003.

[43] S. Knapp, S. Von Aulock, M. Leendertse et al., “Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor,” *Journal of Immunology*, vol. 180, no. 5, pp. 3478–3484, 2008.

[44] H. Sies, “Oxidative stress: a concept in redox biology and medicine,” *Redox Biology*, vol. 4, pp. 180–183, 2015.

[45] C. Castillo, J. Hernandez, A. Bravo, M. Lopez-Alonso, V. Pereira, and J. L. Benideto, “Oxidative status during late pregnancy and early lactation in dairy cows,” *Veterinary Journal*, vol. 169, no. 2, pp. 286–292, 2005.

[46] M. Liu, C. Zhang, X. Xu et al., “Ferulic acid inhibits LPS-induced apoptosis in bovine mammary epithelial cells by regulating the NF-κB and Nrf2 signalling pathways to restore mitochondrial dynamics and ROS generation,” *Veterinary Research*, vol. 52, no. 1, p. 104, 2021.

[47] A. A. I. Arbab, X. Lu, I. M. Abodalla et al., “Metformin inhibits lipoteichoic acid-induced oxidative stress and inflammation through AMPK/NRF2/NF-κB signaling pathway in bovine mammary epithelial cells,” *Frontiers in Veterinary Science*, vol. 8, article 661380, 2021.

[48] R. Li, H. Fang, J. Shen et al., “Curcumin alleviates LPS-induced oxidative stress, inflammation and apoptosis in bovine mammary epithelial cells via the NFκB signaling pathway,” *Toxins*, vol. 13, no. 3, p. 208, 2021.

[49] K. Wu, S. Jia, J. Zhang et al., “Transcriptomics and flow cytometry reveals the cytotoxicity of aflatoxin B1 and aflatoxin M1 in bovine mammary epithelial cells,” *Ecotoxicology and Environmental Safety*, vol. 209, article 111823, 2021.

[50] C. K. M. Ip, P. K. S. Ng, K. J. Jeong et al., “Neomorphic PDGFRα extracellular domain driver mutations are resistant to PDGFRα targeted therapies,” *Nature Communications*, vol. 9, no. 71, article 4583, 2018.

[51] D. Wallach, “The cybernetics of TNF: old views and newer ones,” *Seminars in Cell & Developmental Biology*, vol. 50, pp. 105–114, 2016.

[52] B. Hoesel and J. A. Schmid, “The complexity of NF-κB signaling in inflammation and cancer,” *Molecular Cancer*, vol. 12, p. 86, 2013.

[53] W. Chen and P. Ten Dijke, “Immunoregulation by members of the TGFβ superfamily,” *Nature Reviews. Immunology*, vol. 16, no. 12, pp. 723–740, 2016.

[54] M. Maleszewskia, J. R. Moonen, N. Huijkman, B. van de Sluis, G. Krenning, and M. C. Harmens, “IL-1β and TGFβ2 synergistically induce endothelial to mesenchymal transition in an NFκB-dependent manner,” *Immunobiology*, vol. 218, no. 4, pp. 443–454, 2013.

[55] W. Bin, X. Ming, and C. Wen-Xia, “TRAFl mediates lipopolysaccharide-induced acute lung injury by up regulating JNK activation,” *Biochemical and Biophysical Research Communications*, vol. 511, no. 1, pp. 49–56, 2019.

[56] E. Derwish, G. Zhang, D. Hailenmariam, S. M. Dunn, and B. N. Ametaj, “Innate immunity and carbohydrate metabolism alterations precede occurrence of subclinical mastitis in transition dairy cows,” *Journal of Animal Science and Technology*, vol. 57, p. 46, 2015.

[57] F. Zandkari, J. Vanegas, X. Fern, C. S. Maier, and G. Bobe, “Metabotypes with elevated protein and lipid catabolism and inflammation precede clinical mastitis in prepartal transition dairy cows,” *Journal of Dairy Science*, vol. 101, no. 6, pp. 5531–5548, 2018.
[58] L. M. Sordillo, G. A. Contreras, and S. L. Aitken, “Metabolic factors affecting the inflammatory response of periparturient dairy cows,” Animal Health Research Reviews, vol. 10, no. 1, pp. 53–63, 2009.

[59] J. Wu, Y. Ding, J. Wang, and F. Wang, “Staphylococcus aureus induces TGF-β1 and bFGF expression through the activation of AP-1 and NF-κB transcription factors in bovine mammary epithelial cells,” Microbial Pathogenesis, vol. 117, pp. 276–284, 2018.

[60] I. Ali, M. Yang, Y. Wang et al., “Sodium propionate protects the blood-milk barrier integrity, relieve lipopolysaccharide-induced inflammatory injury and cells apoptosis,” Life Sciences, vol. 270, article 119138, 2021.

[61] H. P. Farrell, M. Garvey, M. Cormican, J. G. LaFey, and N. J. Rowan, “Investigation of critical inter-related factors affecting the efficacy of pulsed light for inactivating clinically relevant bacterial pathogens,” Journal of Applied Microbiology, vol. 108, no. 5, pp. 1494–1508, 2010.

[62] E. Gayán, D. García-Gonzalo, I. Alvarez, and S. Condón, “Resistance of Staphylococcus aureus to UV-C light and combined UV-heat treatments at mild temperatures,” International Journal of Food Microbiology, vol. 172, pp. 30–39, 2014.

[63] P. Nikolic, P. Mudgil, and J. Whitehall, “The in vitro antibacterial effect of permethrin and formaldehyde on Staphylococcus aureus,” Microbiology, vol. 9, no. 8, article e1054, 2020.

[64] F. B. Gilbert, P. Cunha, K. Jensen et al., “Differential response of bovine mammary epithelial cells to Staphylococcus aureus or Escherichia coli agonists of the innate immune system,” Veterinary Research, vol. 44, no. 1, pp. 1–23, 2013.

[65] E. M. Ibeagha-Awemu, A. E. Ibeagha, S. Messier, and X. Zhao, “Proteomics, genomics, and pathway analyses of Escherichia coli and Staphylococcus aureus infected milk whey reveal molecular pathways and networks involved in mastitis,” Journal of Proteome Research, vol. 9, no. 9, pp. 4604–4619, 2010.