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Infective mastitis due to bovine-associated *Streptococcus dysgalactiae* contributes to clinical persistent presentation in a murine mastitis model

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Abstract

Background: Mastitis caused by *Streptococcus dysgalactiae* (GCS) is a major pathology of dairy cows. The mechanisms by which GCS intramammary infection is established and maintained involve not only bacterial adherence and invasion but also modulation of the cytokines and TLR immune response.

Objectives: The study aimed to evaluate characteristics of persistent infection of GCS collected from bovine mastitis milk in a murine mastitis model whose mammary structure is similar to that of dairy cows; dairy cow mastitis can be well simulated by using mice as models. HLJ2019 was tested for its ability to persistently infected mice by intramammary inoculation.

Methods: As antibiotics tested, establish an intramammary infection model in murine, histopathology analyses, relative expression of inflammatory cytokines mRNA and adherence and invasion in mMECs.

Results: It induced a robust inflammatory reaction in the mammary gland, characterized by histopathological changes, increased myeloperoxidase activity and induced expression of inflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-10, IL-1α and IL-1β) and TLR2/4, the exhibited strong LDH release, adhesion and invasive abilities in contact with mMECs.

Conclusion: These results contribute to increase the available information on host-pathogen interaction and point out the need for further research to expand the knowledge about these interactions for developing new strategies to intervene in the intramammary persistent infection progress.

KEYWORDS

adherence, cytokines, intramammary persistent infection, murine mastitis model, *Streptococcus dysgalactiae*

1 | INTRODUCTION

Bovine mastitis is the most common and important disease that produces huge economic losses to the dairy industry around the world (Hogeveen et al., 2011). GCS, submitted α-hemolysis (Jordal et al., 2015), is a frequent cause of bovine mastitis and was reported to be associated with toxic shock-like syndrome in bovine (Chenier et al., 2008), GCS was isolated in 7.5% of the mastitic milk samples (Zhang et al., 2018), also highly associated with outbreaks in fish farms and has sporadically been found associated with mastitis or cause infective arthritis in sheep, even there has also been a single report of GCS causing neonatal mortality in puppies (Vela et al., 2006). In recent years, many outbreaks of GCS of aquatic origin have occurred all over the world (Koh et al., 2020). GCS is always considered exclusively animal pathogens (Rato et al., 2013), while the first suspected zoonotic fish-associated GCS infection was described in Singapore (Koh et al., 2009). GCS strains are reported to comprise a wide variety of genes for prominent virulence factors that impair the host immune system by mediating adhesion to host epithelial cells and resisting opsonophagocytosis through the binding of fibrinogen and massive cytokine release (Cole et al., 2011). Adhesion to host epithelial cells and the presence of wall-associated plasminogen binding proteins are prerequisites to Streptococcus infection (Abdelsalam et al., 2015).

However, few studies report the use of a murine model for GCS as a mastitis pathogen. The development of experimental intramammary infection (IMI) models facilitates the study of mastitis pathogenesis. Of interest is to determine whether GCS is capable of inducing innate immune and inflammatory responses during IMI and characterize the type of mastitis produced. Therefore, the objective was to further explore the pathogenicity of HLJ2019 isolates from mastitis cases, based on the capacity to produce systemic and mammary infections using murine models and mouse mammary epithelial cells (mMECs).

2 | MATERIALS AND METHODS

2.1 | Bacterial strain

HLJ2019 was isolated from a clinical mastitis sample collected from Heilongjiang province in China. The presumptive identification of isolates recovered from 2019 was performed by standard phenotypic tests such as colony morphology on 5% defibrinated sheep blood plate and Gram staining, hemolysis in blood agar plates, followed by the confirmation of the bovine mastitis *S. dysgalactiae* isolates with a sequence of 16S rRNA gene and comparison between sequencing data and the GenBank database. Sequence analysis showed between 98.5% and 100.0% nucleotide identity to *S. dysgalactiae* strain ATCC 43,078 (accession number: NR_115147.1) deposited in the NCBI database. Before each experiment, fresh bacterial suspensions were prepared from frozen stocks by culture on THA (Hopebio, Qingdao, China) incubated at 37°C for 24 hr. Thereafter, strains were subcultured on THB (Hopebio) for 12 hr to mid-log phase. The Colony forming units (CFUs)/ml were determined from the 1/100 diluted bacterial preculture by spectrophotometric measurements at OD600 and were confirmed by plating on THA (Hopebio) plates. The pathogens were suspended in PBS to achieve the required concentration.

2.2 | Methods

2.2.1 | Antibiotic susceptibility testing

Antibiotic susceptibility was determined by the standardized agar diffusion test on MH agar (MH, Hopebio, Qingdao, China) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2020). The isolates were categorized as susceptible and resistant. *S. aureus* ATCC 25,923 was used as quality control.

2.2.2 | Murine mammary infection model for HLJ2019

A murine model of an intramammary challenge with bovine mastitis pathogens has been successfully used to assess bacterial infection and tissue damage (Pereyra et al., 2017). To determine the pathogenic role of HLJ2019 during IMI, female 8–10 week old specific-pathogen-free pregnant C57BL/6 mice were purchased from Yisi Laboratory Animal Technology (Changchun, China). Mice were kept in germ-free isolators and fed ad libitum in a controlled environment with 12 hr light and 12 hr dark cycles. The pups were removed 4 hr before the intramammary inoculation. At 5–7 days after parturition, mice were anesthetized. The mammary gland ducts of the fourth pair of the mammary gland were exposed by cutting the teat tip, and the sample was slowly intraductal injected at a volume of 100 μl with a 33 gauge blunted needle. Two groups ($n = 30$ per group) of mice were allocated: one group with HLJ2019 (10^8 CFUs), compared to a negative control group (sterile PBS). Mice were sedated and euthanized by cervical dislocation and mammary glands aseptically collected at 1–10 days post-inoculation (PI) (three mice in each group).

2.2.3 | Bacterial burden and histopathological evaluation

At necropsy, inoculated mammary glands were excised, weighed, placed in 1 ml of PBS, minced and homogenized. For bacterial burden, 100 μl of various dilutions of each tissue were plated onto THA and incubated overnight at 37°C. Numbers of viable colonies were counted and expressed as CFUs/gland (Liu et al., 2019). Mammary gland tissue was fixed in 10% formalin buffer, embedded, sectioned and stained with H&E. Histological scoring was performed according to tissue necrosis, polymorphonuclear neutrophilic granulocyte

inflammation and lymphocytic inflammation (Liu et al., 2019). Each feature was graded semi-quantitatively in 10 different high power fields at 200× magnification, and an average score for each category was calculated.

2.2.4 | Measurement of myeloperoxidase activity

Measurement of myeloperoxidase (MPO) activity was analysed with the test kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Briefly, the mammary gland tissues were homogenized and then fluidized in extraction buffer to obtain 5% of homogenate, heated to 37°C in water for 15 min with 100 μl reaction buffer and 900 μl homogenate. The activity was evaluated by measuring the change in absorbance at 460 nm using a spectrophotometer.

2.2.5 | Mammary epithelial cell culture

Primary mMECs were isolated from the fourth mammary glands of female lactating ICR mice, as described previously with minor modifications (Li et al., 2014; Wan et al., 2020). Following aseptic lymph node removal, mammary tissue was finely minced and placed in DMEM/ F12 (CORNING, USA) containing 1.5 mg/ml collagenase I, 1.5 mg/ml collagenase II, 1.5 mg/ml trypsin and 100 U/ml penicillin/streptomycin at 37°C for 3 hr. After enzymatic dissociation, the tissue suspensions were centrifuged at 250 × *g* for 5 min. The fatty top layer and pellets were collected in DMEM/F12 with 10% FBS and then centrifuged, resuspended and filtered. The trapped cells were collected in growth media (DMEM/F12, 10% FBS, 10 ng/ml EFG, 5 μg/ml insulin, 5 μg/ml hydrocortisone, 100 U/ml penicillin/streptomycin) and plated in culture plates at 37°C and 5% CO_2 in a humidified atmosphere. The primary cultured mMECs were divided into different indicated groups and allowed to acclimate for 24 hr before any treatments in all experiments.

2.2.6 | Cytotoxic LDH release assay

A lactate dehydrogenase (LDH) assay was used to evaluate cytotoxic effects of HLJ2019 on mMECs (LDH Cytotoxicity Assay Kit; Beyotime). Cells cultured at 37°C with 5% CO_2 in 96-well plates (JET BIOFIL) were challenged with HLJ2019, at a multiplicity of infection (MOI, the ratio of HLJ2019 to cells) of 10:1 for 0.5, 1, 2, 3, 6, 9 and 12 hr. Non-infected cells were similarly incubated as control. After incubation, supernatants were collected and centrifuged at 400*g* for 5 min. Supernatants were collected and LDH release quantified by absorbance at 490 nm (ELISA reader ELx808, BioTek). Cytotoxicity (%) was calculated using LDH Kit reagent treatment cells as a positive control (100% cytotoxicity), while cells only treated with cell culture medium were used as a negative control (0% cytotoxicity), according to the following equation:

$$\%\,\text{cytotoxicity} = \left(\text{Abs}_{\text{experiment}} - \text{Abs}_{\text{negative control}}\right) / \left(\text{Abs}_{\text{positive control}} - \text{Abs}_{\text{negative control}}\right) \times 100.$$

2.2.7 | HLJ2019 adhesion and internalization assays

Adherence and internalization are vital virulence mechanisms in bacterial IMI (Dogan et al., 2006; Pereyra et al., 2016). In previous trials, 100 μg/ml of gentamycin for 2 hr was used for the internalization assay of HLJ2019, as described (Liu et al., 2019), with minor modifications. The mMECs cultured in six-well plates (NEST) and infected with either HLJ2019 at an MOI of 10:1 for 0.5, 1, 2, 3, 6, 9 and 12 hr. Following incubation, cells were washed with sterile PBS in triplicate and treated with 100 μg/ml gentamycin for 2 hr to kill extracellular HLJ2019. Supernatants were collected and plated on THA to verify killing. Infected cells without antibiotic treatment were used as a control group. Cells were washed with sterile PBS in triplicate and lysed with 0.5% (v/v) Triton X-100. Cell lysates cultured onto THA and incubated at 37°C for 24 hr to count CFUs. To assess the adhesive capacity of HLJ2019 to mMECs, an adhesion assay was performed as described above except for the gentamicin procedure with slight modifications (Liu et al., 2019). Invasion and adhesion assays were repeated three times, and each experiment was performed in triplicate. The invasion and adhesion rate was determined as follows:

Invasion rate of bacteria =Lysate of infected cells with antibiotics treatment (CFUs/ml)/Cell lysates and treated bacterial supernatants in the control group (CFUs/ml) × 100,

Adhesion rate of bacteria = Cell lysate of infected group (CFUs/ml)/Cell lysates and treated bacterial supernatants in the control group (CFUs/ml) × 100,

Invasion rate of adhered HLJ2019 = Invasion rate of HLJ2019/ Adherence rate of HLJ2019.

2.2.8 | Gene expression of inflammatory cytokines and Toll-like receptors in mMECs and mammary gland tissue challenged by HLJ2019

mMECs were challenged with either HLJ2019 (MOI of 10:1) for 0.5, 1, 2, 3, 6, 9 and 12 hr. Mammary gland tissue were challenged with either HLJ2019 (1×10^8 CFU/gland) for 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hr. Total RNA was isolated from samples using Trizol Reagent (Invitrogen) and reverse-transcribed using Revert Aid First Strand cDNA Synthesis Kit (Monad). A relative quantitative real-time polymerase chain reaction was performed at 95°C for 30 s, 40 cycles of 95°C for 15 s and 60°C for the 30 s. Primer sequences are shown in Table 1 (Liu et al., 2019; Pereyra et al., 2017). Gene expression levels were standardized to the corresponding β-actin threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ comparative method.

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)	Amplicon size (bp)
TLR2	F-CAAGTACGAACTGGACTTCTCC	148
	R-CAGGTAGGTCTTGGTGTTCATT	
TLR4	F-TGCCTTCACTACAGGGACTTT	101
	R-TGGGACACCACGACAATAAC	
IL–1α	F-CTCTGAGAACCTCTGAAACGTC	116
	R-GAAACTCAGCCGTCTCTTCTT	
IL–1β	F-ACCTGTGTCTTTCCCGTGG	162
	R-TCATCTCGGAGCCTGTAGTG	
TNF-α	F-GTCGTAGCAAACCACCAAGT	149
	R-TTGAAGAGAACCTGGGAGTAGA	
IFN-γ	F-CAAGTGGCATAGATGTGGAAGA	164
	R-GACGCTTATGTTGTTGCTGATG	
IL–6	F-AGTTGTGCAATGGCAATTCTGA	223
	R-CCCCAGCATCGAAGGTAGA	
IL–10	F-ATACTGCTAACCGACTCCTTAAT	140
	R-TCAAATGCTCCTTGATTTCTGG	
β-actin	F-AACTCCATCATGAAGTGTGA	248
	R-ACTCCTGCTTGCTGATCCAC	

2.2.9 | Statistical analyses

Data were presented as means ± standard deviation (*SD*), and each experiment was repeated three times. The differences between groups were assessed using one-way ANOVA, followed by the least significant difference (LSD) multiple comparisons of treatment means. Statistical analyses were performed using SPSS 23.0 (SPSS, Inc.).

3 | RESULTS

Figure 1 shows the number of CFU recovered from mice mammary glands inoculated with 1×10^8 CFU/gland HLJ2019 at different time points. HLJ2019 was present in all inoculated mammary glands.

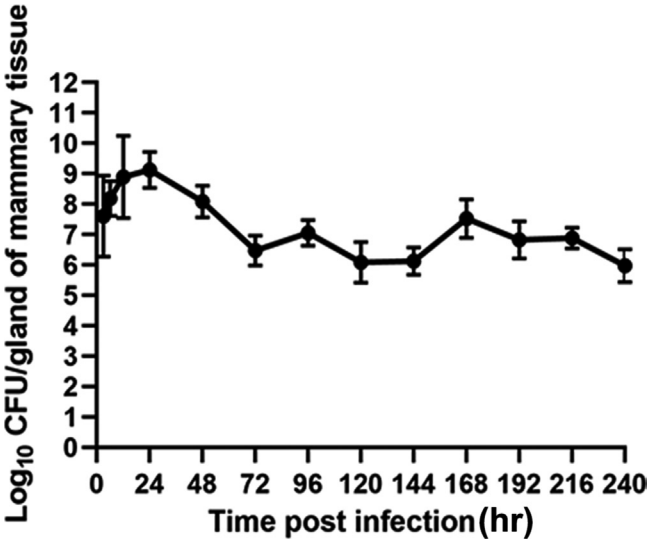

FIGURE 1 Bacterial burden after intramammary infection with 1×10^8 CFU/ml of HLJ2019 in female lactating C57BL/6 murine mammary gland at different time points. Each value represents mean ± *SD* of three mice

The mean viable counts decreased from 7.61 logs CFU/ml at time 3 to 5.97 log CFU/ml at time 10 days throughout the study. Figure 1 shows the logarithms of the total number of CFU recovered from mammary glands, inoculated with 1×10^8 CFU, over, although bacterial counts were rapidly increased until 1 day ($p < .001$ to initial inoculum), reaching the highest number of CFUs (1.3×10^9 CFUs/gland). Then, it showed a downward trend until the end of the test. In the process, a slight increase in the bacterial load was observed at 4 and 7 days compared to the previous time point. A slow decrease was followed up to 10 days, and no clearance of bacteria was observed during the assay.

Antibiotic susceptibility testing showed that HLJ2019 was resistant to the majority of commonly used in bovine mastitis antibiotics was observed. The results obtained are presented in Table 2.

Figure 2 shows gross examination of HLJ2019 infected mammary gland showed slight redness and congestion at 1 day PI; then, obvious swelling at 2 days, tissue degeneration, atrophy and pustules

TABLE 2 Susceptibility of HLJ2019 antimicrobials isolated from bovine mastitis

Antimicrobial agent	Zone diameter (mm)	Breakpoints zone diameter (mm)			Susceptibility
		S	I	R	
P	—	≥20			R
AM	—	≥24	—	—	R
AMX	—	—	—	—	R
OT	15	≥28	25–27	≤24	R
TC	10	≥28	25–27	≤24	R
E	—	≥21	16–20	≤15	R
GTF	8	≥21	18–20	≤17	R

Abbreviations: AM, ampicillin, 10 μg; AMX, amoxicillin, 10 μg; E, erythromycin, 15 μg; GTF, gatifloxacin, 5 μg; OT, oxytetracycline, 30 μg; P, penicillin, 10 μg; TC, tetracycline, 30 μg.

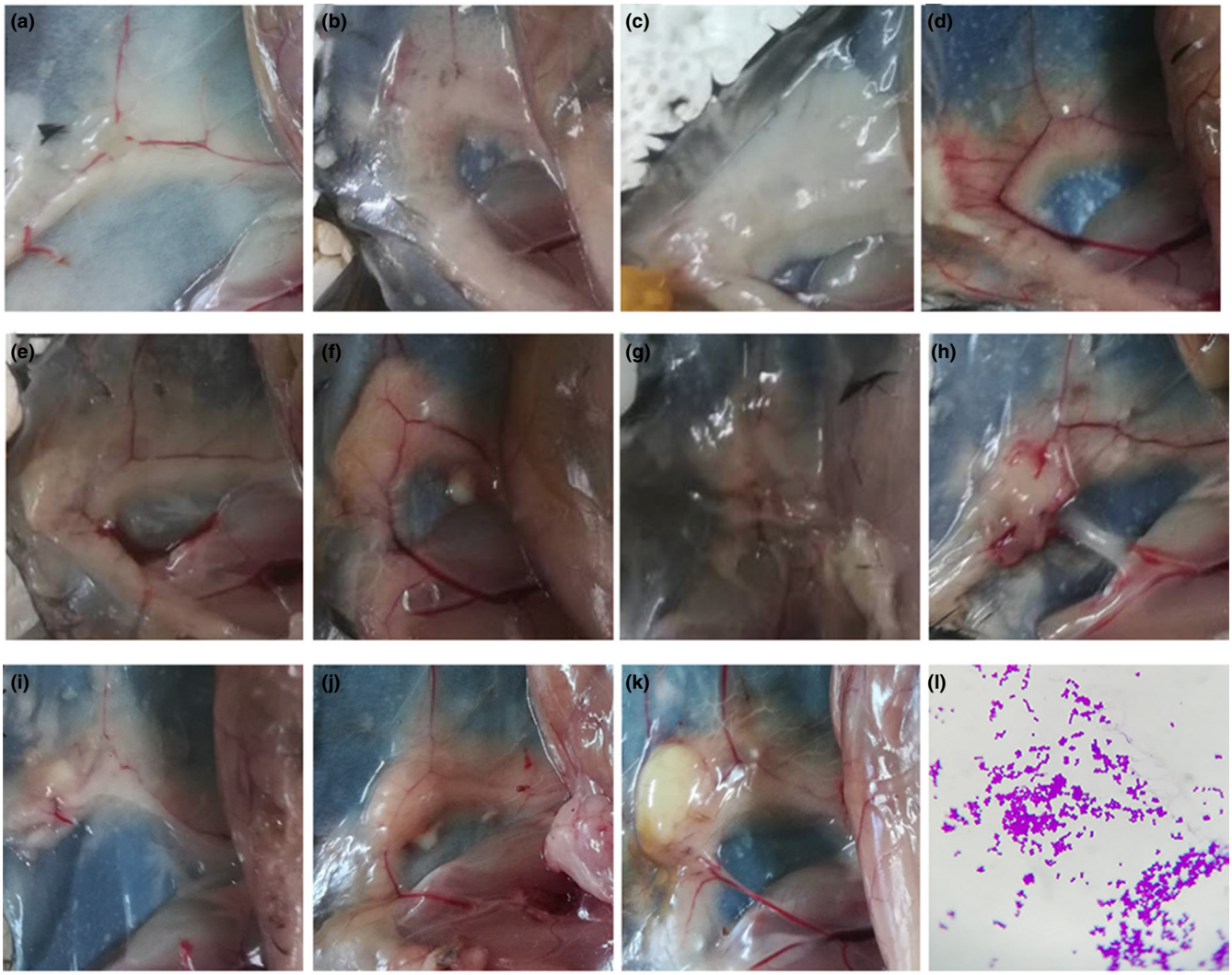

FIGURE 2 The gross pathology changes of mammary glands. Conditions are (a) control group; (b–k) inoculated with GCS (1×10^{11} CFU/gland) and sacrificed at 1–10 days post-inoculation (PI), respectively; (l) GCS in mammary gland at 10 days PI of Gram staining

were observed at 3 days. Pyogenic granuloma was found in the mammary glands of the mice on the 10 days PI. GCS could still be found in the mammary gland at 10 days PI. Histopathological analysis of HE-stained tissues revealed the establishment of HLJ2019 induced mastitis persistent (Figure 3). The breast tissue of the control group displayed a normal breast alveolar structure, and no histopathologic alterations were observed (Figure 3a). In contrast, the breast tissue of the infected group exhibited characteristic histopathologic changes, showing focalized areas of monocyte and neutrophil in infiltration and cell shedding even then fewer interstitial lymphocytes exudate in the alveolar lumen and ducts with epithelial structure preservation, Especially, there was red blood cell infiltration in the interstitial space, Moreover, multifocal necrotic areas were observed with structure loss of connective tissue. Epithelial and luminal cell discontinuities and lining epithelium exhibit various stages of degeneration, necrosis and sloughing PI (Figure 3b–f).

To substantiate these results, we used the MPO assay to compare the levels of neutrophil infiltration into the mammary glands following administration of HLJ2019 (Figure 4). Relative MPO activity was significantly higher after strain challenge in mice than in non-infected mice. Whereas strain challenge resulted in a marked increase of MPO activity in the mammary glands of mice from 3 to 8 days, MPO activity in mammary glands of mice remained low at 2, 9 and 10 days, similar to that found in control glands.

Inflammatory cytokines and toll-like receptors in mammary glands mRNA expression are shown in Figure 5. Gene mRNA expression of IL-1β, IL-1α, IL-6, IL-10, TNF-α and INF-γ in mammary tissue from the three groups at every sampling period determined by qRT-PCR were increasingly exposed to PBS groups (Figure 5a–c, e–g). From 1 to 7 days PI, an increase of IL-1β gene expression in mammary glands showing higher relative gene expression levels compared with mammary glands inoculated with controls ($p < .001$). At 4 and 10 days PI, IL-1α gene expression in mammary glands inoculated with strain was higher than that observed in controls ($p < .001$), showing a peak at 10 days PI for strain. From 4 to 8 days PI, a gradual decrease of mRNA expression for IL-1α in mammary glands was observed,

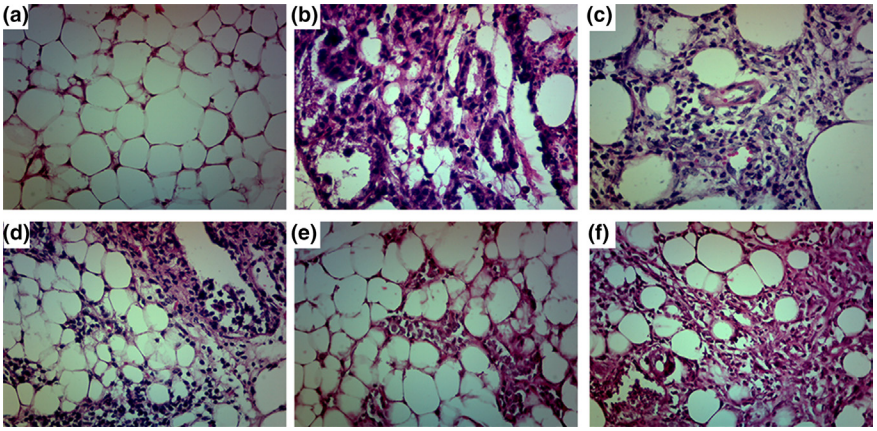

FIGURE 3 Dynamic analysis of histopathology of mice after challenged with GCS. Representative HE stained tissue sections from each group are shown. Representative photographs from dissected mice are shown. Conditions are (a) negative control group; (b–f) inoculated with GCS (1×10^{11} CFU/gland) and sacrificed at 1, 3, 5, 8 and 10 days (PI), respectively. Original magnification ×400

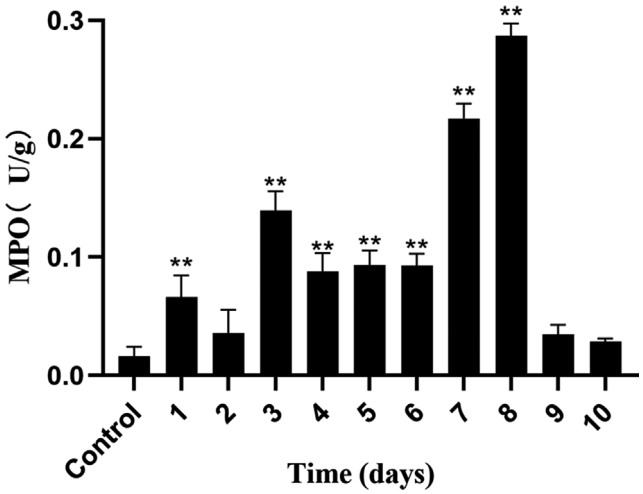

FIGURE 4 MPO activity in HLJ2019-induced murine mastitis. MPO activity was measured in the mammary glands of different sampling times. The mammary gland tissues of the healthy mice were used as controls. All data are shown as the mean ± *SD* ($n = 3$ each group). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared to control group

but at 9 and 10 days PI, a marked increase of IL-1α gene expression in mammary glands inoculated was observed, showing higher relative gene expression levels compared with mammary glands inoculated with HLJ2019 and controls ($p < .01$ and $p < .001$). Only at 5 and 9 days PI, IL-6 gene expression showed higher relative gene expression levels between treatments and control groups ($p < .05$). At 10 days PI, IL-10 gene expression in mammary glands showed a peak in gene expression. At 4 days PI, IL-10 gene expression decreased sharply in mammary glands, showing a more pronounced decrease in mammary glands was maintained at 4 and 5 days PI. This elevated gene expression for TNF-α in mammary glands was maintained from 2 days until 9 days PI. At 4 days PI, a marked increase of IFN-γ gene expression shows a peak in gene expression ($p < .001$). The mRNA expression for TLR2 was influenced by times of sampling (Figure 5d), showing significant differences between the treatments and PBS groups. At 6 days PI, TLR2 gene expression in mammary glands shows a peak in gene expression for inoculated strain. At 2, 3, 5, 8 and 10 days PI, no differences were observed in TLR2 gene expression between treatments, whereas expression of TLR4 increased at 5 and 9 days PI in mammary glands infected with HLJ2019 (Figure 5h).

Our results indicate that cytokines and TLRs transcript levels increased following stimulation of mMEC with HLJ2019 (Figure 6). RT-qPCR of the expression of *TLR2* shows that it increased significantly at 2 hr post-exposure to HLJ2019, which expression tended to be highest at 12 hr (Figure 6d). Stimulated mMECs secreted IL-6 and TNF-α, inducing a higher response than IL-1β (Figure 6a–c). Transcripts of three other chemokines by RT-qPCR, including IL-1α, IL-10 and IFN-γ, were among the most up-regulated (Figure 6e–g).

LDH, an enzyme, is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis and other forms of cellular damage (Kumar et al., 2018). As shown in Figure 7a, there was a significant release ($p < .05$) of LDH at 3 hr PI; however, at 6, 9 and 12 hr PI, there was a significant increase ($p < .001$) of LDH as compared with control groups. This indicated that HLJ2019 seriously damages the cell membranes of mMECs with time. Pathogenicity of HLJ2019 included cytotoxic effects on mMECs as this strain disrupted cell membranes and increased LDH release from cells, reaching 34.86% of LDH released at 12 hr pi (Figure 7b).

To determine pathogenic mechanisms used by HLJ2019 to induce mastitis, the interaction between HLJ2019 and mMECs (Figure 8), our results showed different time points of adherence and invasion of HLJ2019. The HLJ2019 (15.6%) maximumly (2 hr) adhered to mMECs, greater than the control group ($p < .01$), however, slightly decreases at 6 hr (Figure 8c). Besides, it showed the ability to internalize in mMECs. Indeed, it had its peak invasion at 6 hr (Figure 8d). The preliminary quantitative study is shown in Table 3. The adhesion rate increased from 2.6% to 16.2% within 12 hr, and this increase was time dependent, whereas the invasion rate also showed a time-dependent increase from 1.6% to 3.5% within 3 hr, slightly. Since then, it has remained unchanged or even declined from 6 to 12 hr. On the other hand, the invasion rate of adhered bacteria was persistent at a range of 69.1%–19.0% within 12 hr, indicating that as time goes on, the proportion of invading bacteria decreases gradually. In short, these data showed the adhesive and invasive capabilities of HLJ2019 with mMECs.

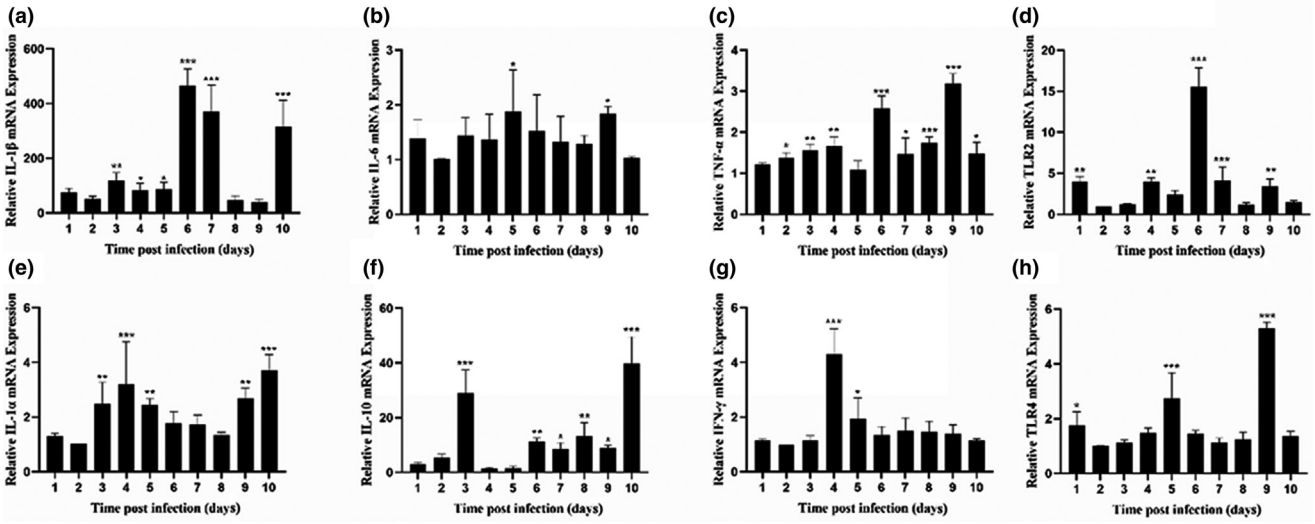

FIGURE 5 mRNA levels of cytokines and toll like receptors in mammary glands of female lactating C57BL/6J mice challenged by HLJ2019 at different time points. Levels of (A) IL-1β, (B) IL-6, (C)TNF-α, (D) TLR2, (E) IL-1α, (F) IL-10, (G) IFN-γ and (H) TLR4 were measured by real-time PCR assay. Values are presented as mean ± *SD* ($n = 3$). $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ significantly different from the PBS control groups (data not shown)

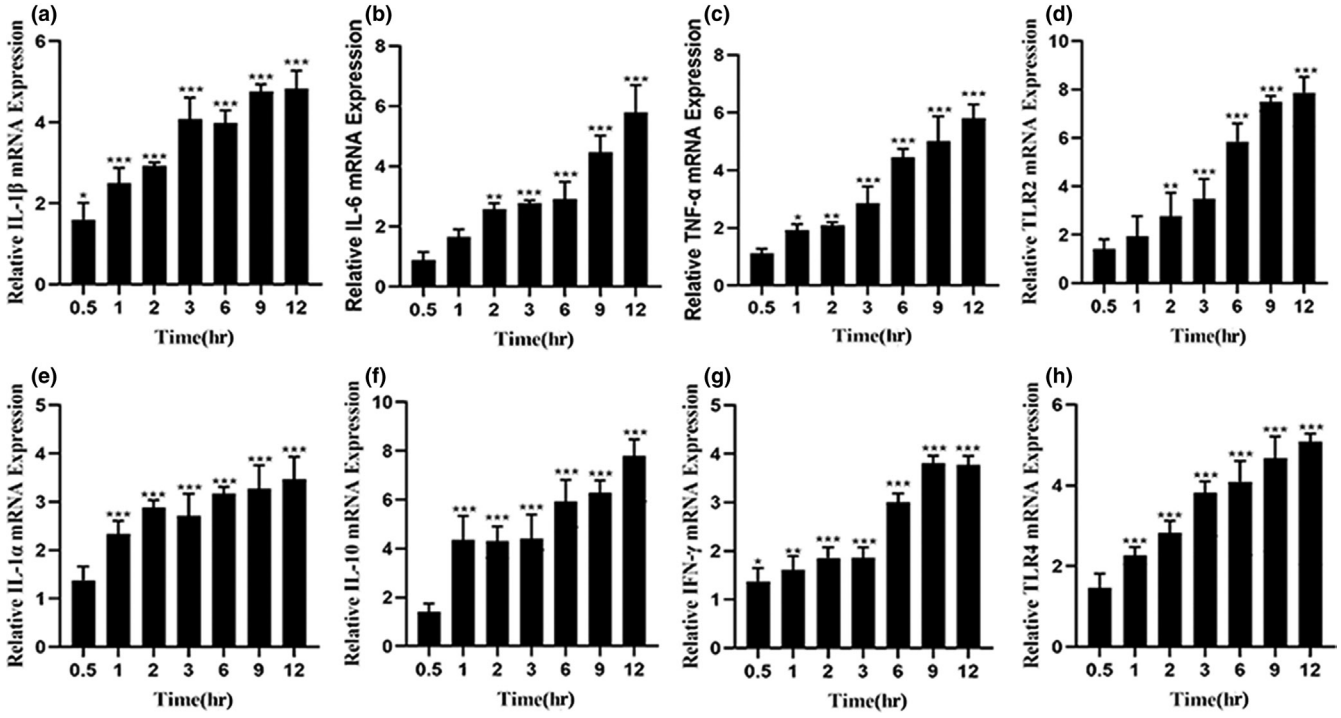

FIGURE 6 mRNA levels of cytokines and toll like receptors in mMECs of female ICR mice challenged by HLJ2019 at different time points. Levels of (A) IL-1β, (B) IL-6, (C)TNF-α, (D) TLR2, (E) IL-1α, (F) IL-10, (G) IFN-γ and (H) TLR4 were measured by real-time PCR assay. Values are presented as mean ± *SD* ($n = 3$). $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ significantly different from the PBS control groups (data not shown)

4 | DISCUSSION

The isolated HLJ2019 from bovine mastitis was resistant to all common antibiotics included tetracycline. GCS can escape host immune mechanisms and antibiotic effects, resulting in poor antibiotic treatment, mastitis and deep breast tissue colonization (Calvinho & Oliver, 1998). Differently, pathogenic streptococcus isolated from mastitic cows was frequently resistant to tetracycline and penicillin (Cheng et al., 2019). Different studies have reported different susceptibility of streptococcus to antibiotics. The resistance of tetracycline was the most common (Minst et al., 2012), followed by resistance to erythromycin; also, tetracycline resistance

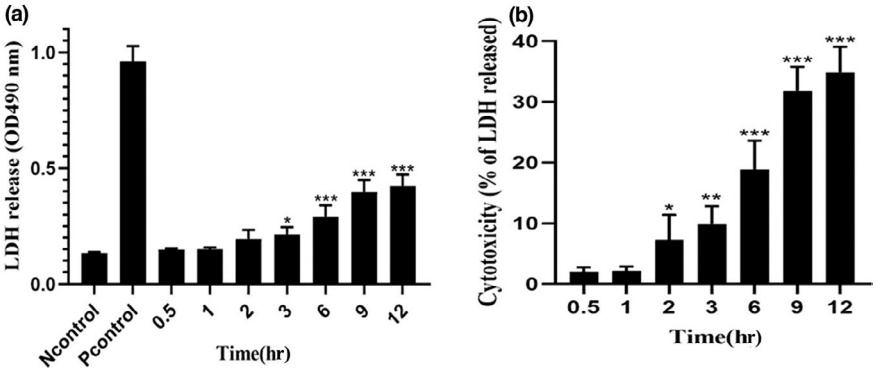

FIGURE 7 LDH release assay in the medium caused by HLJ2019 infected with mMECs. The LDH release assay was repeated three times, and each experiment was performed in triplicate. (a) The LDH released in the supernatant of cultured cells was measured at indicated time, (b) the cytotoxicity of mMECs at indicated time. Results were presented as mean ± *SD*. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ as compared to the control or non-infected groups

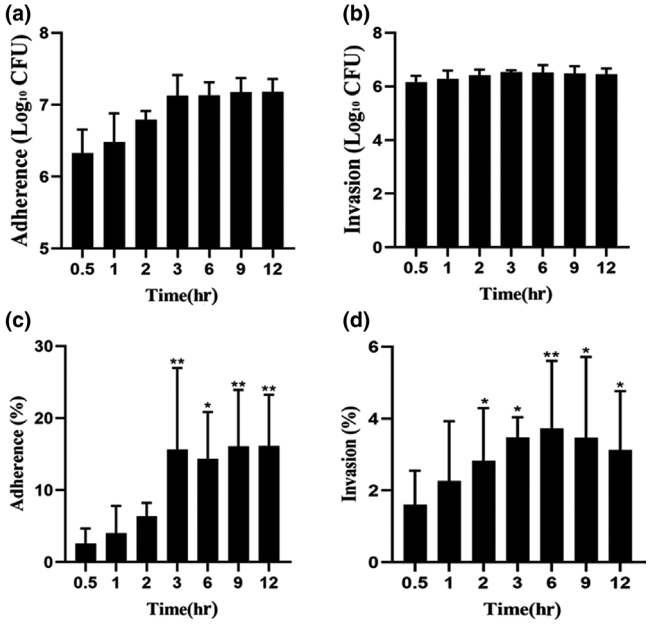

FIGURE 8 Adhesion and invasion analysis of GCS HLJ2019 to cultured mMECs. (a, b) Adhesion and invasion CFU of GCS HLJ2019 to mMECs, respectively. (c, d) Adhesion and invasion rate of GCS HLJ2019 to mMECs, respectively. Results were presented as mean ± *SD* of three independent experiments. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ as compared to the control groups

was very high but for erythromycin resistance was low (de Jong et al., 2018).

We selected the isolated HLJ2019 in milk from bovine mastitis and evaluated its virulence in a murine mastitis model. Mice were infected by intramammary route with 1×10^8 CFU of HLJ2019 to establish an infection in mammary glands. The bacteria could not be removed from mammary glands over 10 days. The number of *Enterococcus faecium* strains recovered from the mammary gland had a strong decrease in the bacterial load was reported at 1 day, followed by a slight increase at 2 days PI (Montironi et al., 2020). A murine mastitis model of *Aerococcus viridans* using BALB/c mice and reported that the pathogen was able to colonize the mammary gland after the intramammary inoculation with 2×10^3 CFUs at 9 days PI (Liu et al., 2019). These results agree partly with those observed in this study suggesting that HLJ2019, as other mastitis agents, may persist on the mammary gland after intramammary inoculation.

Also, the HLJ2019 strain could not be isolated from liver, spleen and kidney homogenates of inoculated mice at different times assayed PI. This result revealed that the HLJ2019 strain could not diffuse from the mammary gland and had not the capacity to invade other organs like the liver, spleen and kidney, while *Enterococcus faecium* was able to disseminate from mammary glands to the liver and kidney (Montironi et al., 2020).

TABLE 3 Adhesion and invasion rate of HLJ2019 to mMECs

	Rate (%)						
Item	0.5 hr	1 hr	2 hr	3 hr	6 hr	9 hr	12 hr
Adherence rate	2.6 ± 2.1	4.0 ± 3.8	6.4 ± 1.9	15.6 ± 11.3	14.4 ± 6.5	16.1 ± 7.1	16.2 ± 7.1
Invasion rate	1.6 ± 1.0	2.3 ± 1.7	2.8 ± 1.5	3.5 ± 0.6	3.7 ± 1.9	3.5 ± 2.3	3.1 ± 1.6
Invasion rate of adhered	69.1 ± 15.0	63.7 ± 12.4	49.8 ± 33.1	30.3 ± 17.9	32.5 ± 21.8	26.7 ± 25.7	19.0 ± 2.4

The data obtained in this study were slightly different from the results of different studies, which reported that the inoculation of a pathogenic strain in a mouse mastitis model caused mastitis symptoms. The results of the histopathology analysis almost the same with the bacterial growth in the mammary gland inoculated with HLJ2019 1×10^8 CFU. The intensity of the inflammation observed in the mammary glands of inoculated groups may be due to the presence of a constant number of bacteria from 1 to 10 days. Also, the presence of the bacteria in the mammary gland during all times tested showed that HLJ2019 could not be removed by effector cells of the innate immune system, as PMNs and macrophages. Our results are consistent with massive infiltration of PMNs after 2×10^3 CFUs on Day 9 of *Aerococcus viridans* inoculation (Liu et al., 2019). The differences found in different studies may be related to the pathogens, virulence factors and the number of inoculated bacteria. It should be noted that the experimental infection degree of environmental pathogens such as streptococcus is considered to be highly variable, which is lower than that of infectious pathogens such as staphylococcus. Also, mice may be slightly sensitive to streptococcus infection (Montironi et al., 2020).

MPO, one of the most important protein components of neutrophils, is a functional and activation marker of neutrophils, MPO induced neutrophil apoptosis and necrosis, which, in turn, worsened tissue damage and can cause oxidative stress and tissue damage during inflammation (Xiang et al., 2017; Zhao et al., 2019). Previous work demonstrates that neutrophil MPO plays a paradoxical role in bacterial clearance and tissue damage in pneumococcal (Xiang et al., 2017). Our results have shown that MPO activity in the infected group was considerably increased compared with the control group but the MPO activity was reduced in 9 and 10 days PI, which indicated the status of persistent infection was not clear enough. On the other hand, the results were not consistent with those observed on pathological sections.

Studies have shown that MECs are associated with pathogens in the mRNA expression of inflammatory factors and chemokines. MECs are the effector cells to clear pathogens and release chemokines and cytokines after contact with pathogens (Wellnitz et al., 2012). GCS isolated from different severity mastitis cases induced different immune responses in vitro, the mRNA expression of some cytokines in MECs induced by the isolates increased more significantly. In the present study, we observed that specific RNA for IL-1α, IL-1β, IL-6, IL-10, TNF-α, IFN-γ, TLR2 and TLR4 was expressed in mammary gland tissue during 1–10 days relative to GCS IMI. In the present study, we evaluated the expression of TLR2 during the GCS IMI. TLR2, one of the activators of the inflammatory response and a link between innate and adaptive immune responses, expressed in immune cells and is involved in innate immune recognition of bacteria through recognition of peptidoglycan and lipoteichoic acid in gram-positive pathogens (Thompson et al., 2014). Research showed that the absence of TLR2 in mMECs has significant damage to cells in the process of *S. uberis* infection (Wan et al., 2020). Moreover, TLR2-mediated mROS has a significant effect on *S. uberis*-induced host defense responses in mammary glands as well as in MECs (Wan et al., 2020). TLRs are also associated with the activation of the NF-κB proinflammatory pathway that ultimately activates the transcription of inflammatory cytokines (Moyes et al., 2014). Although the law of growth and decline cytokines and TLRs were not assessed, both TLR2 and TLR4 are detecting *S. uberis* infection in MECs, and TLR2 is the principal receptor, the role of the PI3K/Akt/mTOR pathway in inflammatory regulation is independent of the activation of TLRs/NF-κB, cross-talk between PI3K/Akt/mTOR and TLRs/NF-κB signalling pathways promote inflammation (Li et al., 2018). TLR4 has been identified as an important receptor for the detection of Gram-negative bacteria, while a recent study demonstrated that this receptor could also be induced either by detecting LTA directly or through recognition of endogenous mediators induced by the interaction between LTA and TLR2 (Liu et al., 2019). This was following our finding that the increased expression of TLR4 (9 days PI) followed the expression of TLR2 (6 days PI). In this study, future studies are needed to examine whether the changes in the determinants of inflammation are a result of an increased function of single-factor compared with multi-factor, as well as, variation changes in the expression of cytokines and TLRs related to innate and adaptive immune cells (epithelium, fibroblast, dendritic cell and macrophage) intramammary which stimulated by GCS. Such knowledge may provide new strategies for improving the regulation function and dairy cows' health during mastitis susceptibility.

After exposure to mMECs, GCS infection led to perforation in cell membranes, the release of LDH, fragmentation of nuclear membrane, chromatin condensation and mitochondrial degeneration, which can cause premature cell collapse (Chen et al., 2017). The invasion process did not seem to affect the viability of MECs, but cellular damage was induced, as indicated by a time-dependent release of increasing amounts of LDH (Almeida & Oliver, 1995). However, *S. uberis* strains in MAC-T cells for an extended time without causing apparent cell damage or death (Tamilselvam et al., 2006).

We also assayed HLJ2019 was able to adhere and internalize mMECs. Similar results were reported in GCS in human cells (Alves-Barroco et al., 2019). Adhesion and invasion of bacteria played important roles in the formation of infection, and that to the MECs are important facts for the development of the infection (Chen et al., 2017; Montironi et al., 2020). GCS could invade bMECs, if this phenomenon occurs in vivo, the invasive ability of this bacterium may be associated with the persistence of IMIs (Almeida & Oliver, 1995). Bovine SDSD strains could adhere to and internalize human primary keratinocyte cells (Roma-Rodrigues et al., 2016). *S. aureus* is capable of invading and replicating inside bMECs (Almeida et al., 1996). The invasion of MECs and intracellular survival play an important role in the pathogenesis of persistent *E. coli* mastitis (Dogan et al., 2006). The quantitative analysis of adhesion and invasion of GCS showed that HLJ2019 possesses adhesion and invasion abilities. We observed that HLJ2019 could promptly adhere to and invade into the mMECs within 3 hr, as well as maintain steady state until 12 hr. However, according to our data, adhesion level and percentage of invasion did not seem to be related to each other, and also, the levels of adhesion and

invasion were lower than those of other bovine-associated mastitis pathogens (Dogan et al., 2006; Liu et al., 2019; Montironi et al., 2020). Variation in expression of bacterial surface properties can be related to changes in the environment and are considered as an adaptive response to either favour bacterial adherence to host cells or to avoid attachment to phagocytes, to survive in the early stages of infection (Calvinho et al., 1996).

The establishment of a mastitis challenge infection model is necessary to test new intramammary anti-microbial compounds, other novel treatments, and study the pathogenesis of bovine mastitis (Khazandi et al., 2015). The mammary structure of mice is similar to that of dairy cows; thus, the characteristic of dairy cow mastitis can be well simulated by using mice as models. The results suggested that IMI may be an effective method to establish a mastitis model. The results of this study indicate that persistent HLJ2019 IMI correlates with chronic inflammatory changes in histological sections of mammary tissue. It is possible that an intramammary reservoir of HLJ2019 could trigger such a longstanding inflammation. It is unknown why the bacteria were not completely removed from the mammary gland during persistent IMI. One explanation for the intramammary persistence of bacteria would be the intracellular survival of HLJ2019 in mMECs. Our in vitro data support this concept. We detect a small number of intracellular HLJ2019, but this could have resulted from the small size of the intracellular reservoir.

In conclusion, our aim of developing a reliable GCS mastitis challenge model in murine during active lactation was achieved. This is the first report that studied GCS strain in a lactating mouse model of mastitis. This finding suggests that intracellular persistence of GCS may be associated with the spread of the infection to deeper tissues and the development of persistent IMI. This investigation shows that mice represent a valuable model for the study of the mastitis pathogenesis caused by GCS considering the high costs of using cows for mastitis research and will help in the understanding of the pathogenesis of GCS IMIs of dairy cows.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICAL NOTE

The present study involving animals was carried out in strict accordance with the recommendations of the Regional Animal Ethics Committee and compliance with Animal Welfare Act regulations as well as the Guide for the Care and Use of Laboratory Animals. All animal experiments were planned to minimize suffering.

PEER REVIEW

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