Original article

Evaluation of pro- and anti-inflammatory interleukins in the mammary gland of goats experimentally infected with *Staphylococcus chromogenes*

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Abstract

The aim of this work was to evaluate the relative gene expression levels of the cytokines IL-1B, IL-8, IL-12, IFN-γ, IL-4, IL-10 and TGF-β in somatic milk cells of French Alpine breed, anestrous goats that were experimentally infected in the left mammary gland with *Staphylococcus chromogenes* during the lactation peak. Milk samples were obtained from both glands for 21 consecutive days post infection. Total RNA was extracted, and real-time PCR was conducted using primers specific to each cytokine. The relative RNA expression of the evaluated cytokines was determined by the comparative method $2^{-\Delta\Delta CT}$, using milk from the right gland of the goats as a reference (control) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. According to the Wilcoxon test results, IL-1B and IL-12 expression levels showed significant differences compared to those in the control group ($p<0.05$) from 24 hours post infection until the end of lactation; on day three, IL1β, IL8, IL12 and TGF-β had a statistically significant change in expression with respect to those in the control group ($p<0.05$); closer to the end of the lactation period, there is no overexpression of the anti-inflammatory interleukins (IL-4 and TGF-β) which may reflect the effort of the host immune system to eradicate the microorganism from the mammary gland.

Key words: mastitis, *Staphylococcus chromogenes*, goats, qRT-PCR, interleukins

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Introduction

Mastitis is one of the most serious diseases for the dairy industry; in dairy goats, the incidence of clinical mastitis may not exceed 5%, while subclinical mastitis is common and about 6 times more than clinical affection (Wanecka et al. 2019). The main bacterial genera that cause clinical mastitis are *Staphylococcus aureus, Streptococcus agalactiae, S. uberis, S. dysgalactiae* and coliforms (Thompson et al. 2014, Dore et al. 2016, Pirzada et al. 2016, Manzanero et al. 2018, Novac et al. 2019, Kim et al. 2019).

The different pathogens that cause mastitis induce distinct immune responses in the mammary gland, the interactions between pathogens causing mastitis and the host immune system are complex because both have the ability to coevolve, generating ongoing recognition, response and adaptation events. Because of this phenomenon, pathogens have developed several strategies to alter and evade host defences in order to survive. The immune system also adapts and has a broad arsenal to control and eliminate threats. (Thompson et al. 2014).

In goats, coagulase negative staphylococci (CNS) are the main causative agents of mastitis, and it has been reported that their prevalence in herds ranges from 10 to 30% and sometimes up to 71% (Gelasakis et al. 2016, Novac et al. 2019). *Staphylococcus* species that have been frequently reported to cause mastitis in goats include *Staphylococcus chromogenes, S. xylosus* and *S. simulans* (Dore et al. 2016, Novac et al. 2019). In the case of *S. chromogenes*, different *in vitro* studies have shown that this strain is the most virulent CNS (Simojoki et al. 2009). *Staphylococcus* spp exposure often leads to the generation of a humoral and cellular immune response that culminates with the elimination of the pathogen; however, under certain circumstances, the bacterium survives in the host and induces persistent diseases. Despite the large number of studies on the virulence factors that this bacterial genus possesses and the survival strategies used by the bacterium to persist within the host, the immune response during infection with *Staphylococcus* spp., is not yet fully understood (Thompson et al. 2014).

Cytokines are a bridge between the innate immune response and the adaptive immune response. Chemokines are molecules involved in migration and leukocyte recruitment and are considered immunoregulators. (Alluwaimi, 2004). Based on the above findings, the aim of this study was to evaluate the production of interleukins IL1β, IL4, IL10, IL8, IL12, IFN-γ and TGF-β in mammary gland samples from goats experimentally infected with *S. chromogenes* by gene expression analysis, with the purpose of understanding the immune response induced by *S. chromogenes* in the mammary gland of goats.

Materials and Methods

The present study was carried out at the Faculty of Veterinary Medicine and Zootechnics of the National Autonomous University of Mexico and at the Laboratory of Agricultural Microbiology of the Autonomous Metropolitan University. All experimental procedures were approved by the Local Ethics Commitee for Animal Experiments.

Animals and experimental infection

Three 7-month-old French Alpine anestrous goats were used and 5 days before the expected delivery, they were separated from the herd and placed in isolation. After birth, the kids were separated from their mothers. Before and after experimental inoculation, the goats were tested by clinical examination and California mastitis test (CMT) to detect subclinical mastitis (Contreras et al. 1996). To confirm that samples were free of any bacterial agent before the experimental infection, 30 µl of sample was seeded on blood agar and MacConkey agar plates and both plates were incubated at 37°C for 24 hours under aerobic conditions. In the case where no growth was observed, the plates were incubated for 48 further hours until bacterial growth was ruled out (Manzanero et al. 2018). A total of 38 milk samples were taken from each goat from the day of birth with weekly sampling (nine samples in total) until reaching the lactation peak (approximately 56 days postpartum). Upon reaching the maximum milk production, the experimental infection was performed, and from this day, samples were collected daily for 21 consecutive days. Once this period was over, weekly samples were taken until the end of lactation (8 samples). 50 mL of milk was obtained from each gland and placed in new, sterile hermetic bottles. For the experimental infection of goats, a *S. chromogenes* isolate obtained from a case of chronic mastitis in a French Alpine breed goat was used. The left glands of each goat were inoculated with the infectious dose of *S. chromogenes* (2.1×10^6 CFU) according to Simojoki et al. (2009) and resuspended in 6 mL of sterile PBS. The right mammary glands were inoculated with 6 mL of sterile PBS.

qRT-PCR for quantifying bacteria from milk samples

A Gram stain was performed on the colonies recovered from the milk samples to confirm that they corresponded to Gram positive bacteria with cluster group-

Table 1. Sequences and conditions of primers.

Gene	Sequence (5´- 3´)	Conditions	Size (bp)
Extracel Protein	F´- ACGTGAGCAATATATGAACGC R´- TTATAAATACCTGTTAATGCGCC	1 cycle 95ºC/5 m, 35 cycles 95ºC/1 m, 50ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	133
IL-1β	F´-TGCAGCTGGAGGAAGTAGACC R´- GTACAGGACAGACTCAAATTCAACTG	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	112
IL-4	F´-GACGTCTTTGCTGCCCC R´- CCTGTCAAGTCCGCCCA	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	122
IL-8	F´-AGTACAGAACTTCGATGCCAATG R´- GTAAGCTTAACAATGATTTCTGAATTTTC	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	127
IL-10	F´-GCGAAAACAAGAGCAAGGC R´- GCTTCAGTTTTRCATCTTCGTTGTC	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	115
IL-12	F´-CTCAGCAGTTGGTCGTTTCC R´- ACATAAACATTTTTCTCCAGTTCCC	1 cycle 95 ºC/5 m, 40 cycles 95 ºC/1 m, 60 ºC/1 m, 72 ºC/1 m, 1 cycle 72 ºC/5 m	87
IFN- γ	F´-CATCTACTCRCAGGTCCTCTTC R´- GCAATGCGGCTGATGGT	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	120
TGF- β	F´-TTA CAA CAG TAC CCG CGA CC R´- ATT TTGTTGCCGTATTCCACC	1 cycle 95ºC/5 m, 40 cycles 95ºC/1 m, 60ºC/1 m, 72ºC/1 m, 1 cycle 72ºC/5 m	110

ing. To determine the bacterial load in each of the milk samples, from day zero of infection until the last sampling before drying off, DNA extraction with guanidine isothiocyanate and silica was performed according to the protocol described by Cremonesi et al. (2006). Once the DNA was extracted, the bacterial count was measured using primers specific to the extracellular protein gene of *S. chromogenes.* qRT-PCR was performed on the QIAGEN Rotor-Gene Q 5plex HRM Platform thermocycler using 12.5 µl of SYBR Green (Thermo-Fisher Catalogue Number K0251) as a detection system, 1 µl of each primer (forward and reverse) at a concentration of 5 µM, 1 µl of DNA and 25 µl q.s of nuclease-free water for each reaction.

RNA extraction, quantification and reverse transcription

RNA extraction was performed according to Mura et al. (2013), the RNA concentration was quantified by spectrophotometry at an absorbance of 260 nm (OD_{260}). Purity was evaluated by an OD_{260}/OD_{280} ratio between 1.9 and 2. To synthesize complementary DNA (cDNA), the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Catalogue Number 1672) was used according to the manufacturer's instructions. The primers used for interleukins IL1β, IL8, IL12, IFN-γ, IL4, IL10 and TGF-β, and also for the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), utilized as a normalizer in qRT-PCR, were designed using the DNAman programme version 7.02. (Table 1). qRT-PCR was performed on the QIAGEN Rotor-Gene Q 5plex HRM Platform thermocycler using 12.5 µl of SYBR Green, 1 µl of each primer (forward and reverse) at a concentration of 5 µM, 5 µg of cDNA and 25 µl q.s. of nuclease-free water for each reaction. After 40 cycles, the dissociation curves of all samples were analysed to verify the absence of nonspecific products and dimer formation of the primers.

mRNA expression analysis

The $2^{-\Delta\Delta ct}$ comparative method was performed using noninfected mammary gland milk samples as a reference, whereas the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control (Livack and Schmiigen 2001).
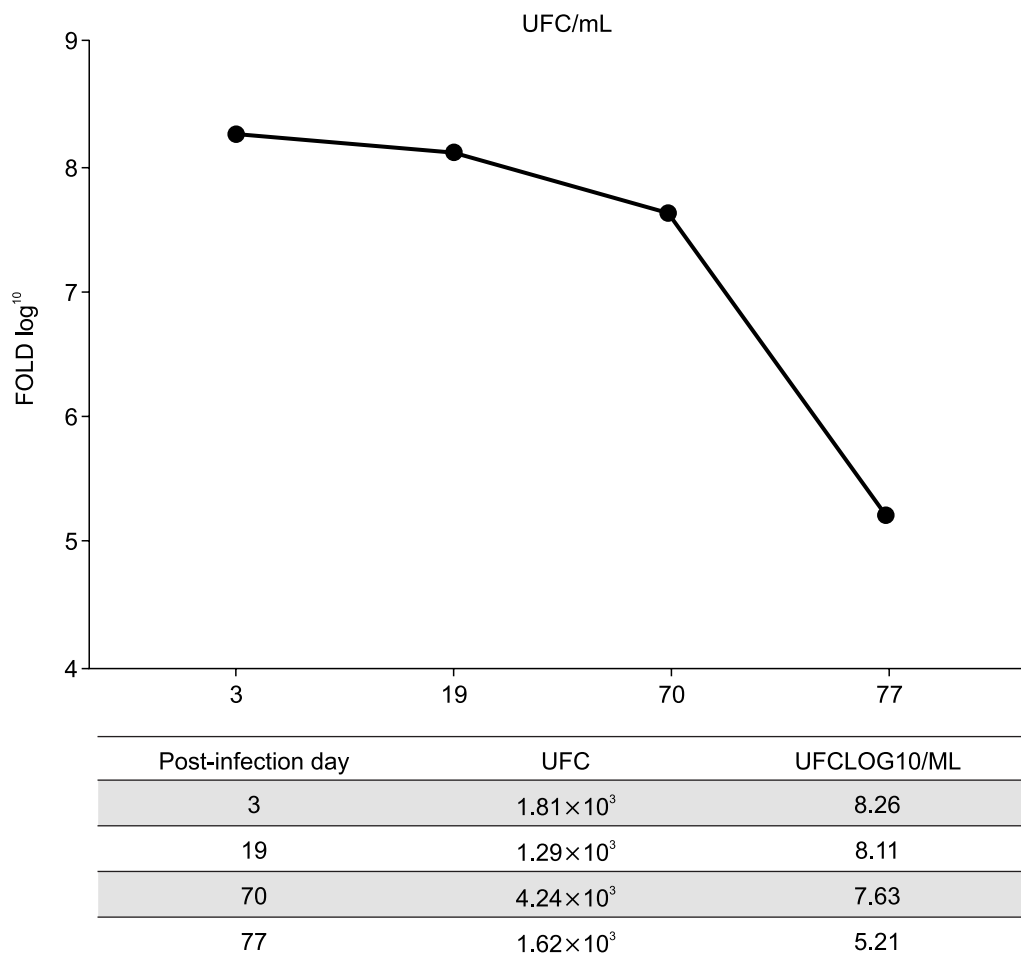
Statistical analysis

To determine the statistical significance of the differences in mRNA transcripts between the infected glands and the control glands, the Wilcoxon test was used for nonparametric data using GraphPad Prism Software version 7.0.

Results

Before experimental inoculation, the mammary glands were clinically healthy and negative to CMT, after inoculation, the left glands of the goats showed signs of clinical mastitis including swelling, heat, redness and pain, CMT was positive at a score of 2 and 3 (2 000 000 - >10 000 000 cells/ml) until the end of lactation and milk samples were positive for growth of bacterial colonies compatible with *Staphylococcus* spp. in the 21 consecutive samples, as in the 8 samples of the final stage of lactation. The right glands remained

Table 2. Relative expression of genes in infected/control quarters with the respective standard deviation of the mean (* p<0.05).

		IL-1β	IL-8	IL-12	IFN-ɣ	IL-4	IL-10	TGF-β
CONTROL	Day 3	-3.3±2.12	-6.15±6.86	0.265±0.52	-5.5±6.36	-4.7±8.91	0.47±2.08	1.055±0.02
	Day 19	-0.575±2.58	-6.52±11.99	0.795±1.12	-4.7±6.65	1.05±0.57	6.65±4.68	0.145±2.33
	Day 70	-0.595±1.85	-7.15±9.69	-0.2±0.14	-6.3±8.06	-6.4±7.92	4.25±9.12	-0.09±1.15
	Day77	-1.75±1.34	-4.25±9.55	0.92±0.57	-5.85±7.28	0.64±2.18	4.985±5.81	1.02±2.29
INFECTED	Day 3	1.79*±2.12	1.935*±1.51	3.235*±1.44	-4.25±3.18	1.06±4.75	1.675±4.35	3.055*±2.74
	Day19	1.64*±2.58	-2.505±6.71	2.96*±3.17	-2.77*±4.43	2.005±2.98	8.56*±2.04	2.55*±3.75
	Day 70	-0.015*± 1.85	-5.05±2.65	2.875*±3.47	3.02*±4.26	2.225±4.42	7.98*±5.26	2.535±3.26
	Day 77	0.535*± 1.34	-3.035±7.05	3.025*±1.36	9.53*±3.35	2.23±4.06	2.63*±3.25	2.535±4.57

Post-infection day	UFC	UFCLOG10/ML
3	1.81×10^3	8.26
19	1.29×10^3	8.11
70	4.24×10^3	7.63
77	1.62×10^3	5.21

Fig. 1. Bacterial count during post-infection samples with S. chromogenes, using the extracellular protein gene.

healthy, since CMT were negative (< 480 000 cells/ml) and no bacterial colonies were observed in the general bacteriological analysis, and the samples were negative for the 16S gene of *Staphylococcus* spp, as demonstrated by PCR. The relative count of *S. chromogenes* was obtained for each post-infection sample collected from the left glands of both goats. The 29 post-infection samples were positive for the presence of bacteria. To quantify pro- and anti-inflammatory interleukins, we selected sampling days that would represent the immune response in the mammary gland during the acute and chronic stages of the disease. The selected days were 3, 19, 70 and 77 post-infection (Fig. 1). Differences in the expression levels of the seven evaluated interleukins from the infected group and the control group with respect to the levels at day zero of infection (that is, before the immune response of the mammary gland was stimulated) were evaluated. On day three, IL1β, IL8, IL12 and TGF-β had a statistically significant change in expression with respect to those in the control group
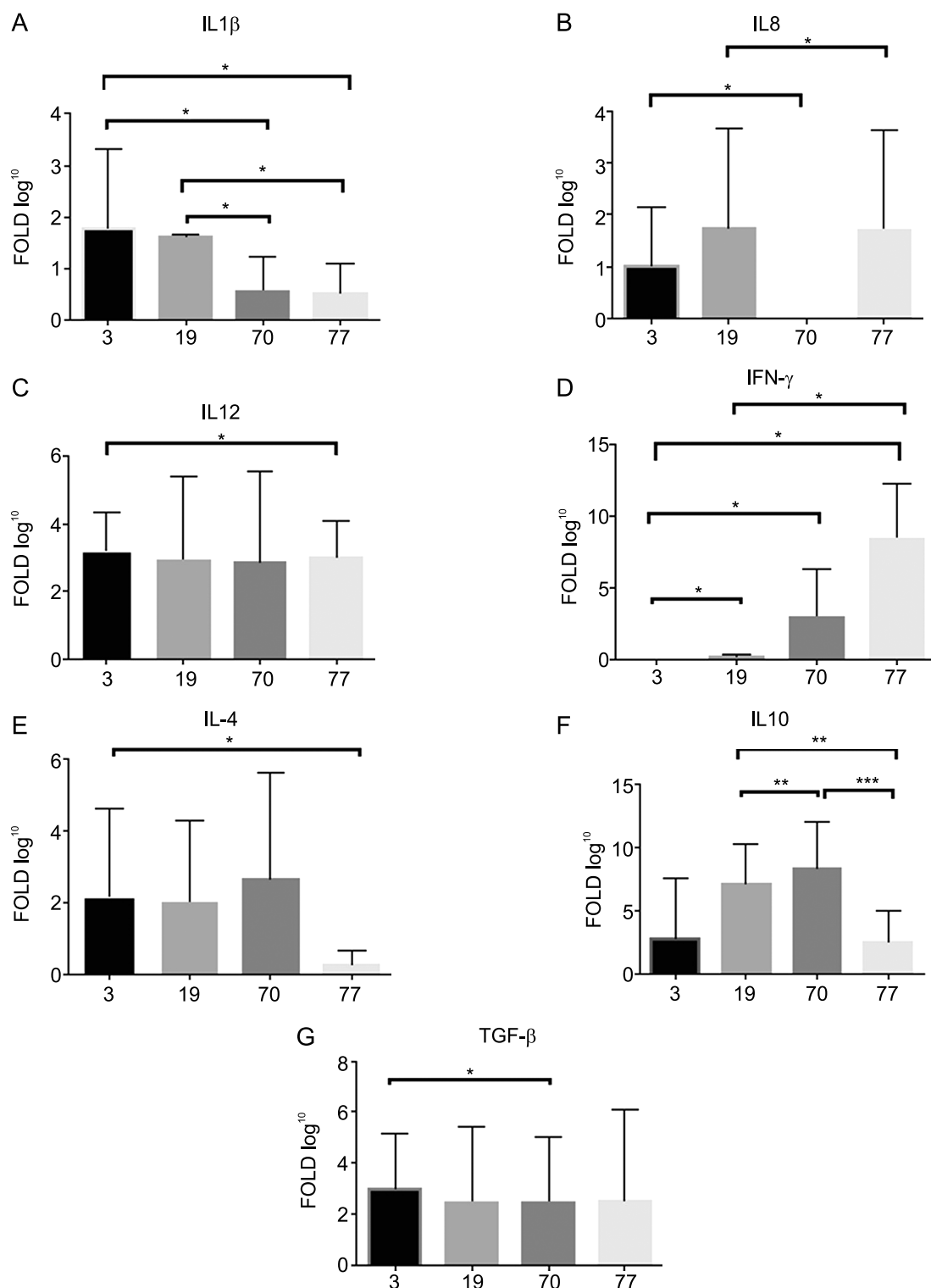
Fig. 2. Difference in the number of mRNA transcripts on the different days post-infection in the infected group. (p>0.05), * p<0.05, ** p< 0.01, *** p< 0.001.

(p<0.05). On day 19 and 70 post infection, IL1β, IL12, IFN-γ, IL10 and TGF-β showed differences with respect to those in the control group (p<0.05), while on day 77, IL1β, IL12, IFN-γ and IL10 expression levels were different with respect to those in the control group (p<0.05) (Table 2)

Changes in the expression of interleukins in the infected group over time

IL1β showed differences in its expression levels on days 3 and 19 compared to days 70 and 77 (p<0.05), whereas IL8 showed significant differences between days 3 and 70 and on days 19 and 77 (p<0.05). IL12

only showed differences in its expression levels between days 3 and 77 ($p<0.05$). IFN-γ showed significant differences on day 3 with respect to days 19, 70 and 77 and on day 19 with respect to day 77 ($p<0.05$). Regarding anti-inflammatory interleukins, IL4 only showed a difference between days 3 and 77 ($p<0.05$), while IL10 showed differences on day 19 with respect to days 70 and 77 ($p<0.01$) and on day 70 with respect to day 77 ($p<0.001$). Finally, for TGF-β, a difference in expression was only observed between days 3 and 70 ($p<0.05$) (Fig. 2).

Discussion

The bacterial presence in the gland was determined from the first 24 hours post infection until the last milk sampling before drying off of the goats (Fig. 1). Previously, experimental infections in six Holstein cows using *S. chromogenes* isolates showed that all animals reached a peak in bacterial growth at 8 hours post infection (Simojoki et al. 2009); however, 46 hours later, no bacterial growth was detected in the culture media, except in one animal that continued to shed bacteria until 14 days post infection. The dose used by Simojoki et al. (2009) was the same as the one we used in this study. In both cases, the mammary gland was infected despite working with different animal species, which developed subclinical mastitis without presenting visible physical changes in the mammary gland or any clinical signs. However, the cows were able to clear the infection, and it should be noted that different strains were used in the two studies, so there may be distinct virulence factors that contribute to the presentation of chronic mastitis and the elimination of microorganisms in animals (Piccart et al. 2016). This inability to eliminate the bacteria by the animals is similar to the deficiency that occurs in mastitis cases caused by *S. aureus* in cattle, where researchers from different countries have indicated that S. *aureus* persists during full lactations and where failure in bacterial recognition by TLR2 has been observed (Yang et al. 2008).

In the research conducted by Yang et al. (2008), it was reported that after infection of bovine mammary gland epithelial cells with *S. aureus* and *E. coli*, both agents induced the expression of IL8 and TNF-α, genes. In the case of *S. aureus,* lipoteichoic acid (LTA), as well as the whole bacterium, failed to activate NF-Kβ in cell cultures. Most likely, there are several mechanisms that block the transduction of the TLR signal. TGF-β blocks the MyD88-dependent signalling pathway through the subsequent ubiquitination and proteasomal degradation of this factor. However, upon inhibition of MYD88, the activation of inducers such as β-defensins and iNOS

(nitric oxide synthetase), or proteins of the acute phase of inflammation (e.g., SAA3 or HP) does not occur, leading to persistent infections (Yang et al. 2008). It has been demonstrated that mastitis caused by *S. aureus* increases TGF-β expression in the mammary gland, which could be indicative of the activation of the above mechanism (Andreotti et al. 2014). There are other mechanisms that directly block the activation of NF-Kβ, such as interference with the IKβ complex or even interference with the translocation to the nucleus of the p65 component of NF-Kβ (Yang et al. 2008).

In this investigation, the presence of bacteria in the mammary gland was demonstrated by traditional bacteriological and molecular methods. When comparing the gene expression of interleukins with that in the control group, it was observed that on day 3 post infection there was increased expression of IL1β, IL8, IL12 and TGF-β with respect to that in the control group (Table 2). Fournier and Philpott (2005) reported that *S. aureus* was able to induce proinflammatory responses with peak interleukin levels at 50-75 hours after infection compared to Gram-negative bacteria, in which the interleukin peak appeared within 5 hours after infection. However, it has also been reported that *Staphylococcus* spp wall antigens such as PG, LTA and other proteins are not enough to increase the presence of proinflammatory interleukins such as TNF-α (Lee and Bannerman 2006, Bulgari et al. 2017).

IL1β transcripts were detected in this study after infection with *S. chromogenes*. IL1β is one of the main proinflammatory cytokines which in turn stimulates the production of IL8, the main neutrophil chemoattractant towards the site of infection (Alluwaimi 2004). In addition, the presence of IL12, which is produced by macrophages, neutrophils, monocytes and B lymphocytes, was also observed. One of the main functions of IL12 is to act as a mediator between the innate immune response and the adaptive immune response by regulating T cell polarization towards a TH1-type response, which is characterized by intense phagocytic activity (Ezzat et al. 2014). Alluwaimi et al. (2004) reported the presence of IL12 at 24 hours post infection with *S. aureus*. IL12 activates macrophages by stimulating the production of IFN-γ by T cells and NK cells; however, on day 3 post infection, the overexpression of IL12 was not observed. IFN-γ also functions as a mediator between the innate and adaptive immune responses and regulates the expression of MHC-I and MHC-II molecules (Alluwaimi 2004). In another study conducted by the same group in 2004, IFN-γ was detected in healthy mammary glands and glands with mastitis, unlike Bougarn et al. (2010), who reported the absence of this interleukin in mastitis caused by *Staphylococcus* spp. Lee and Bannerman (2006) found

that persistent intramammary infections in cows are characterized by an increase in the number of recovered bacteria. Other authors such as Person et al. (1997), who studied sheep experimentally infected with *S. aureus*, found an increase in IFN-γ during the first 24 hours post infection. In this investigation, IFN-γ was expressed on day 19 post infection, which could reflect a trend towards increasing the TH1 immune response to eradicate bacteria that were not eliminated by innate immune response mechanisms (Bannerman, 2009).

TGF-β is a cytokine with anti-inflammatory activity that also regulates the development of the mammary gland. Additionally, it acts on macrophages and other cell types to inhibit proinflammatory responses by decreasing the production of proinflammatory cytokines and chemokines (Persson et al. 1997). Several authors have detected this cytokine even in the absence of experimental infections, in which the TGF-β peak is maintained for up to 72 hours. Other functions of this cytokine include regulating the growth of the galactophore ducts and alveolar development (Lee and Bannerman 2006). An increase in this cytokine has been reported in chronic *S. aureus* infections, which could occur in order to reduce tissue damage and contribute to the repair mechanisms of the mammary gland, since TGF-β is essential to limiting the inflammatory focus (Andreotti et al. 2014). On days 19 and 70 post infection, TGF-β was still expressed, which could be due to the suppression of inflammation and the increase in phagocytosis of bacterial residues and inflammatory cells by this cytokine. TGF-β could also be involved in the repair of injured parenchyma in chronic infections during mammary involution (Andreotti et al. 2014). Most likely, due to this activity, TGF-β was present in the mammary gland, and on day 77 post infection day, there was no difference from the levels in the control group, since the uninfected mammary gland also decreased milk production due to drying off.

For the anti-inflammatory interleukin IL4, no significant differences were found with respect to the control group, consistent with the investigation by Riollet et al. (2000), who also reported the lack of expression of IL4 in cattle infected with *S. aureus*. According to Faoucon et al. (2009), lactating goats negatively regulate IL4 signalling, which is characterized by a decrease in the IL4R and NFATc2 genes. The proteins encoded by these genes are specific to TH2 lymphocytes, which could be related to the inhibition of IL4. On the other hand, IL10 was significantly expressed on day 19 post infection. This interleukin is produced by macrophages and acts during acute and chronic infections, preventing the production of proinflammatory cytokines and chemokines. IL10 has other func-

tions, such as (1) inhibiting antigen-presenting cells (T lymphocytes) by decreasing the expression of MHC-II (Couper et al. 2007); (2) reducing the excessive TH1 response characterized by an overproduction of IFN-γ and TNF-α; (3) reducing inflammation; and (4) decreasing pathological changes by regulating TH2 responses to avoid overproduction of IL4, IL5 and IL13, which can lead to the generation of severe fibrosis. The above characteristics could be the reason why IL4 was not overexpressed in the infected glands. Interleukins IL1β, IL12, IFN-γ, IL10 and TGF-β were overexpressed on both day 19 and 70 post infection (Table 2). The innate immune system was not able to control the infection and eliminate *S. chromogenes*. Based on the expression pattern of interleukins, a TH1 response is likely to have predominated. In the last sampling performed on day 77 post infection, the expression of TGF-β was no longer significant, since it was likely needed for the repair of the mammary gland (Table 2).

The IL1β expression levels were different between days 3 and 70, as well as on days 3 and 77 post infection (p<0.05). The highest expression was observed on day 3 post infection, probably due to the proinflammatory activity at that time. The expression also showed differences between days 19 and 77 post infection, although it was higher. Bougarn et al. (2010) reported that stimulation by LTA and muramyl dipeptide was not sufficient to detect the presence of this cytokine in the cell culture supernatant. These bacterial wall antigens synergistically induce inflammation. Megyeri et al. (2002) mentioned that *S. aureus* and *S. epidermidis* (another species included in the CNS) increase the expression of IL1β by the same magnitude, and this could be caused by the different virulence factors of *Staphylococcus* species (Piccart et al. 2016)

Regarding IL8, there was only a difference in its expression on day 3 post infection. Lee and Bannerman (2006) were not able to detect IL8 after experimental infection with *S. aureus* in cattle, while Lee and Bannerman (2006) reported that IL8 transcripts were considerably lower compared to those in *E. coli* infection. Other research indicates a decrease or complete absence of IL8 expression as a response to intramammary infections caused by *S. aureus* (Piccart et al. 2016). The IL12 and IFN-γ expression levels were always significantly different compared to those in the control group. However, the expression of IFN-γ was not significant on day 3 post infection. IL12 showed a significant difference between days 3 and 77 post infection. IL12 was always expressed; and its greatest expression occurred on day three, when it was most likely acting as a regulator of TNF-α, IL8 and IL10 production. IFN-γ expression was higher as the infection progressed.

IL4 expression showed no significant difference from the control group, although this interleukin was detected between days 3 and 77 post infection, with the greatest expression on day three. The lowest expression was on day 77, which could indicate that IL10 is the main anti-inflammatory interleukin associated with this infection. Fonseca et al. (2009) found no significant differences in the expression of IL4 between healthy and infected glands; the main function of IL4 is to mediate humoral immune responses; mastitis caused by *Staphylococcus* spp. mainly induces cellular immunity. TGF-β only showed significant differences between days 3 and 77 post infection; however, on day three, the expression was slightly higher. According to Lahouassa et al. (2007), TGF-β is normally expressed in the healthy gland due to its functions within the mammary gland.

Experimental infections with *S. chromogenes* have only been performed in cows. Piccart et al. (2016) reported a moderate immune response in these animals. In that study, the authors infected different animals with different *S. chromogenes* isolates, and one of the isolates was able to induce IL1β expression, but the others did not induce any change in gene expression. Leitner et al. (2012) reported that the immune response caused by CNS is similar; however, the animals were not able to eliminate the bacteria.

Conclusions

This study has shown that the mammary gland was unable to eliminate *S. chromogenes*. These results are similar to those previously reported for *S. aureus*; however, closer to the end of the lactation period there is no overexpression of the anti-inflammatory interleukins, which may reflect the effort of the host immune system to eradicate the bacteria. Mastitis caused by CNS requires further studies to clarify the bacterial persistence phenomenon.

Acknowledgements

References

Alluwaimi AM, (**2004**) The cytokines of bovine mammary gland: prospects for diagnosis and therapy. Res Vet Sci 77: 211-222.

Andreotti CS, Pareyra EAL, Baravalle C, Renna MS, Ortega HH, Calvinho LF, Dallard BE (**2014**) *Staphylococcus aureus* chronic intramammary infection modifies protein expression of transforming growth factor beta (TGF-β) subfamily components during active involution. Res Vet Sci 96: 5-14.

Bannerman DD (**2009**) Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J Anim Sci 87: 10-25.

Bougarn S, Cunha P, Harmache A, Fromageau A, Gilber FB, Rainard P (**2010**) Muramyl dipeptide synergizes with *Staphylococcus aureus* lipoteichoic acid to recruit neutrophils in the mammary gland and to stimulate mammary epithelial cells. Clin Vaccine Immunol 17: 1797-1809.

Bulgari O, Dong X, Roca AL, Caroli AM, Loor JJ (**2017**) Innate immune responses induced by lipopolysaccharide and lipoteichoic acid in primary goat mammary epithelial cells. J Animal Sci Biotechnol 8: 29.

Contreras A, Sierra D, Corrales JC, Sánchez A, Marco J (**1996**) Physiological threshold of somatic cell count and California Mastitis Test for diagnosis of caprine subclinical mastitis. Small Ruminant Res 21: 259-264.

Couper KN, Blount DG, Riley EM (**2008**) IL-10: the master regulator of immunity to infection. J Immunol 180: 5771-5777.

Cremonesi P, Castiglioni B, Malferrari G, Biunno I, Vimercati C, Moroni P, Morandi S, Luzzana M (**2006**) Technical Note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J Dairy Sci 89: 163-169.

Ezzat Alnakip M, Quintela-Baluja, Böhme K, Fernández-No I, Caamaño-Antelo S, Calo-Mata P, Barros-Velázquez J (**2014**) The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. J Vet Med Article ID 659801.

Faoucon F, Rebours E, Bevilacqua C, Helbling JC, Aubert J, Makhzami S, Dhorne-Pollet S, Robin S, Martin P (**2009**) Terminal differentiation of goat mammary tissue during pregnancy requires the expression of genes involved in immune functions. Physiol Genomics 40: 61-82.

Fonseca I, Vendramini SP, Lange CC, Guimaraes MF, Weller MM, Sousa KR, Lopes PS, Guimaraes JD, Guimaraes SE (**2009**) Expression profile of genes associated with mastitis in dairy cattle. Genet Mol Biol 32: 776-778.

Fournier B, Philpott DJ (**2005**) Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 18: 521-540.

Gelasakis AI, Angelidis AS, Giannakou R, Filioussis G, Kalamaki MS, Arsenos G (**2016**) Bacterial subclinical mastitis and its effect on milk yield in low-input dairy goat herds. J Dairy Sci 99: 3698-3708.

Kim SJ, Moon DC, Park SC, Kang HY, Na SH, Lim SK (**2019**) Antimicrobial resistance and genetic characterization of coagulase negative staphylococci from bovine mastitis milk samples in Korea. J Dairy Sci 102: 11439-11448.

Lahouassa H, Moussay E, Rainard P, Riollet C (**2007**) Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli.* Cytokine 38: 12-21.

Leitner G, Merin U, Krifucks O, Blum S, Rivas AL, Silanikove N **(2012)** Effects of intra-mammary bacterial infection with coagulase negative staphylococci and stage of lactation on shedding of epithelial cells and infiltration of leukocytes into milk: comparison among cows, goats and sheep. Vet Immunol Immunopathol 147: 202-210.

Lee JW, Bannerman DD, Paape MJ, Huang MK, Zhao X **(2006)** Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR. Vet Res 37: 219-229.

Livack KJ, Schmittgen TD **(2001)** Analysis of relative gene expression data using real-time quantitive PCR and the $2^{-\Delta\Delta ct}$ method. Methods 25: 402-408.

Manzanero-Martínez SP, Ruiz-Romero RA, Cervantes OR, Espinosa OV, Duocing WA **(2018)** Identification of and antimicrobial resistance in bacteria causing caprine mastitis in three states and a city in Central Mexico under manual and mechanical milking conditions. J Dairy Vet Anim Res 7: 115-118.

Megyeri K, Mandi Y, Degre M, Rosztóczy I **(2002)** Induction of cytokine production by different staphylococcal strains. Cytokine 19: 206-212.

Mura MC, Daga C, Bodano S, Paludo M, Luridiana S, Pazzola M, Dettori ML, Vacca GM, Carcangiu V **(2013)** Development of a RNA extraction method from milk for gene expression study in the mammary gland of sheep. Mol Biol Rep 40: 2169-2173.

Novac CS, Andrei S, Fit NL **(2019)** An overview of specific pathogens in goat mastitis. Bull UASVM Vet Med 76: 118-125.

Persson-Waller K, Colditz IG, Seow HF **(1997)** Accumulation of leucocytes and cytokines in the lactating ovine udder during mastitis due to *Staphylococcus aureus* and *Escherichia coli*. Res Vet Sci 62: 63-66.

Piccart K, Verbeke J, De Visscher A, Piepers S, Haesebrouck F, De Vliegher S **(2016)** Local host response following an intramammary challenge with *Staphylococcus fleuretti* and different strains of *Staphylococcus chromogenes* in dairy heifers. Vet Res 47: 56.

Pirzada M, Malhi KK, Kamboh AA, Rind R, Abro SH, Lakho SA, Bhutto KR, Huda N **(2016)** Prevalence of subclinical mastitis in dairy goats caused by bacterial species. J Anim Health Prod 4: 55-59.

Rainard P, Gitton C, Chaumeil T, Fassier T, Huau C, Riou M, Tosser-Klop G, Krupova Z, Chaize A, Gilbert FB, Rupp R, Martin P **(2018)** Host factors determine the evolution of infection with *Staphylococcus aureus* to gangrenous mastitis in goats. Vet Res 49: 72.

Riollet C, Rainard P, Poutrel B **(2000)** Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. Clin Diagn Lab Immunol 7: 161-167.

Simojoki H, Orro T, Taponen S, Pyörälä S **(2009)** Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. Vet Microbiol 134: 95-99.

Thompson-Crispi K, Atalla H, Miglior F, Mallard BA **(2014)** Bovine mastitis: frontiers in immunogenetics. Front Immunol 5: 493.

Wanecka A, Król J, Twardón J, Mrowiec J, Korzeniowska-Kowal A, Wzorek A **(2019)** Efficacy of MALDI-TOF mass spectometry as well as phenotypic methods in identification of staphylococci other than *Staphylococcus aureus* isolated from intramammary infections in dairy cows in Poland. J Vet Diagn Invest 31: 523-530

Yang W, Zerbe H, Petzl W, Brunner RM, Günther J, Draing C, Von Aulock S, Schuberth HJ, Seyfert HM **(2008)** Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *Escherichia coli*, *but Staphylococcus aureus* fails to both activate NF-Kappaβ in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder. Mol Immun 45: 1385-1397.