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Abstract

Mastitis represents one of the major economic and health threats to the livestock sector associated with reduction in milk quality, loss of production and represents a main reason for culling. Somatic cell score (SCS) is used as a criterion in breeding programmes to select for cows genetically less susceptible to mastitis. The relevance of SCS as a predictor of udder health and susceptibility to mastitis is still untested for goats. In this study, two lines of French Alpine goats selected for extreme breeding values for the somatic cell score, one line with high SCS (HSCS) and the other with low SCS (LSCS), were used to test the hypothesis that the mammary response and function differed between the lines. The aim of the present study was to investigate differences in the early immune response in caprine mammary gland tissues challenged with Staphylococcus aureus, one of the main pathogens responsible for the intra-mammary infection in small ruminants, using transcriptomic and histopathology analyses. The comparison between HSCS and LSCS goat lines, showed differences in the inflammatory response at the histological level for inflammation, presence of neutrophils and micro-abscess formation, and molecular level in the expression of CXCL8, IL-6, NFKBIZ and IL-1□. CXCL8 and CXCL2 genes showed a higher level of expression in the experimentally infected HSCS line. The molecular data and histopathology both suggested that following S. aureus infection, mobilization, recruitment, infiltration and chemotaxis of neutrophil, leads to a more severe inflammation in the HSCS compared to LSCS animals. Our results represent an initial basis for further studies to unravel the genetic basis of early mastitis inflammatory responses and to the selection of dairy animals more resistant to bacterial mastitis.

Keywords	goat; somatic cell count; mammary tissue; transcriptome; inflammatory response; Staphylococcus aureus

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Dear Editor,

I wish you consider the paper entitled “Comparison of the response of mammary gland tissue from two divergent lines of goat with high and low milk somatic cell scores to an experimental Staphylococcus aureus infection” for publication in Veterinary Immunology and Immunopathology Journal.

The aim of the present study was to investigate differences in the early immune response in caprine mammary gland tissues challenged with *Staphylococcus aureus,* one of the main pathogens responsible for the intra-mammary infection in small ruminants, using transcriptomic and histopathology analyses. Our results represent an initial basis for further studies to unravel the genetic basis of early mastitis inflammatory responses and to the selection of dairy animals more resistant to bacterial mastitis.

The manuscript has been seen and approved by all authors, who accept full responsibility for the content. The authors had full access to the data and their analysis and agree with the decision that the work has not been published previously and is not under consideration for publication elsewhere. All authors have contributed to it in a meaningful way.

Sincerely,
Dr. Paola Cremonesi

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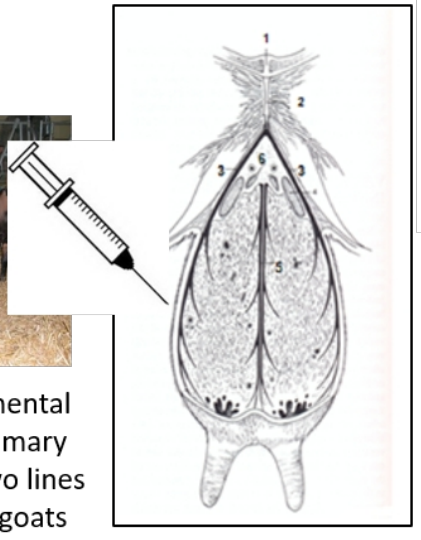
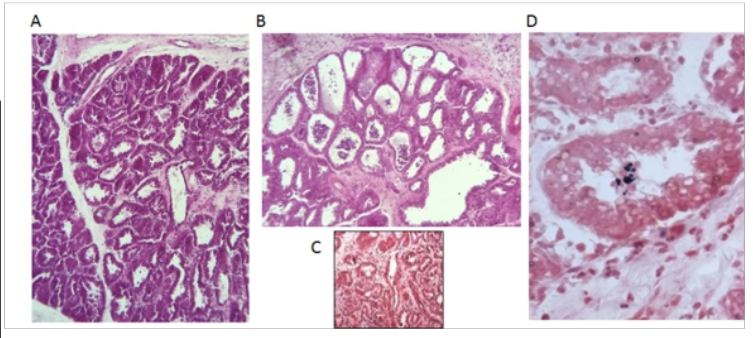
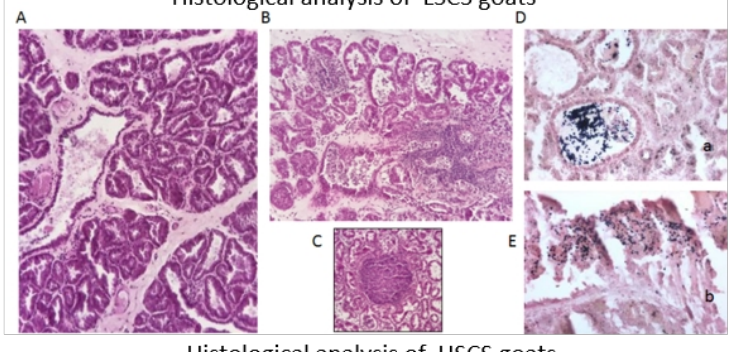


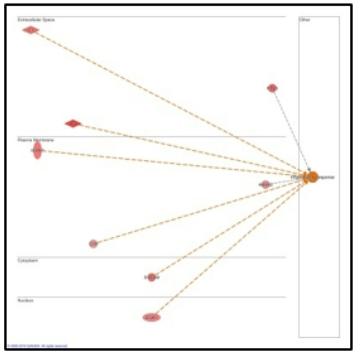
S. aureus experimental infection of mammary gland tissues of two lines of French Alpine goats



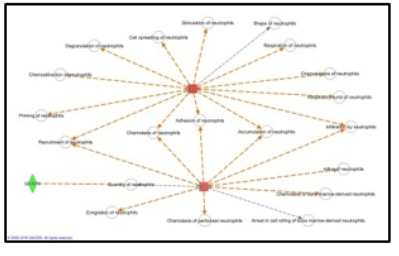
Histological analysis of LSCS goats



Histological analysis of HSCS goats



DEGs had an important role in chemotaxis of cells and in the inflammatory response



CXCL8 and CXCL2, were up regulated in the HSCS compared to the LSCS line

Comparison of the response of mammary gland tissue from two divergent lines of goat with high and low milk somatic cell scores to an experimental *Staphylococcus aureus* infection.

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Authorship

RC and PC extracted the RNA, prepared RNA for hybridization, performed the microarray experiments and drafted the manuscript. BC collaborated in microarray experiments and drafted the paper. GP performed the challenge, collected samples. PR carried out the histopathology analyses, drafted the paper. FR and JF carried out the immunohistochemistry and Real time PCR analyses. FR performed the statistical analyses and drafted the paper. MDC performed the statistical analyses for microarray results. RR selected and provided the animals. AS and RR designed the experiment. AS supervised the experimental study. JLW was participate in the study design and in revising the

manuscript. GP, PM performed the challenge and collected samples. GP, BC, PR, FR, PM revised the manuscript critically. All the authors approved the final version.

Abstract

Mastitis represents one of the major economic and health threats to the livestock sector associated with reduction in milk quality, loss of production and represents a main reason for culling. Somatic cell score (SCS) is used as a criterion in breeding programmes to select for cows genetically less susceptible to mastitis. The relevance of SCS as a predictor of udder health and susceptibility to mastitis is still untested for goats. In this study, two lines of French Alpine goats selected for extreme breeding values for the somatic cell score, one line with high SCS (HSCS) and the other with low SCS (LSCS), were used to test the hypothesis that the mammary response and function differed between the lines. The aim of the present study was to investigate differences in the early immune response in caprine mammary gland tissues challenged with *Staphylococcus aureus,* one of the main pathogens responsible for the intra-mammary infection in small ruminants, using transcriptomic and histopathology analyses.

The comparison between HSCS and LSCS goat lines, showed differences in the inflammatory response at the histological level for inflammation, presence of neutrophils and micro-abscess formation, and molecular level in the expression of CXCL8, IL-6, NFKBIZ and IL-1β. CXCL8 and CXCL2 genes showed a higher level of expression in the experimentally infected HSCS line. The molecular data and histopathology both suggested that following *S. aureus* infection, mobilization, recruitment, infiltration and chemotaxis of neutrophil, leads to a more severe inflammation in the HSCS compared to LSCS animals. Our results represent an initial basis for further studies to unravel the genetic basis of early mastitis inflammatory responses and to the selection of dairy animals more resistant to bacterial mastitis.

Highlights

- ✓ High Somatic Cell Score goats present an increased PMN interlobular infiltration
- ✓ Goat lines have a different pattern of innate immune related molecules
- ✓ Gene expression profiling involved inflammatory response and lipid metabolism

Keywords: goat, somatic cell count, mammary tissue, transcriptome, inflammatory response, *Staphylococcus aureus*

Abbreviations: SCC, Somatic Cell Count; IMI, intramammary infection; SCS, somatic cell scores; HSCS, high somatic cell scores; LSCS, low somatic cell scores; PMN, polymorphonuclear neutrophil.

1. Introduction

Mastitis, an inflammation of the mammary gland, which could be caused by different microorganisms, represents one of the most economically important health traits for milk production, which makes it among the major concerns for the livestock sector. *Staphylococcus aureus* mastitis is a frequent and costly disease for dairy ruminants, associated with subclinical, clinical and gangrenous mastitis. Infection leads to discarding milk, reduced milk production, treatments cost and culling animals, which makes mastitis a concern for farmers. Infections are generally associated with changes in the defence responses of the mammary gland (Sordillo, 2009). Upon infection, leukocytes are recruited into the mammary gland tissue. Monocytes and neutrophils, (Paape et al., 2003), migrate to the site of infection in response to a variety of inflammatory mediators, to phagocytise and kill bacteria by releasing potent oxidative reagents (Bonnefont et al, 2011). Mammary epithelial cells are conjunctively involved in the early innate immune response to reinforce neutrophil recruitment that results in an increase in milk SCC (Lutzow et al., 2008). Somatic cells composed of macrophages, PMNs, lymphocytes, and epithelial cells, represent a correlated measurement for early diagnosis for intramammary infection (IMI).

Response to infections involve a series of complex traits under multi-genic control, which make it difficult to develop adequate selection strategies to improve immune response (Cremonesi et al., 2012; Pighetti et al., 2011). Currently, the use of somatic cell scores (SCS) in cattle, is used as a parameter in genetic selection for resistance to mastitis (Pighetti et al., 2011; Rupp et al., 2003; Heringstad et al., 2000; Rupp et al., 2000). Selection for lower lactation average SCC in cattle is expected to decrease the incidence of pathogen-specific mastitis, especially caused by *Streptococcus uberis*, *Strep. dysgalactiae*, and, to a lesser extent, by *S. aureus* and *Escherichia coli* (Sorensen et al., 2009; Rupp et al., 2003).

Little information is available for small ruminants regarding the genetic basis of resistance and functional complexity of the host-pathogen interaction during infection. Therefore, developing better knowledge of the genetic basis of mastitis resistance is among the top priorities to improve breeding management of small ruminants. Studies of two divergent selected lines of Lacaune sheep (Bonnefont et al., 2011; Bonnefont et al., 2012; Rupp et al., 2009) have investigated genetic mechanisms that operate in response to *Staphylococcus* infection in animals with a different degree of susceptibility. Rupp and co-workers (2009) demonstrated that a selection for decreased SCS was

associated with a reduction in IMI. More recently, Banos and co-authors (2017) investigated the genomic architecture of mastitis resistance in Chios dairy sheep confirming genetic variability among animals in mastitis resistance, and identifying genomic regions associated with specific mastitis resistance traits. Rupp and co-workers (2011) estimated the heritability of milk SCC, milk production and udder type, was between 0.2 and 0.25, while the correlation was low (-0.13) suggesting that a reduction in SCC could be achieved by selection while still improving milk production and udder type. Variation in mastitis resistance is likely to be related to allelic difference and expression levels of genes in milk somatic cells, in other immune cells, and/or in the mammary gland. However, a transcriptomic analysis of blood and milk somatic cells in goat lines divergent for SCC challenged with *S. aureus* showed no significant differences in gene expression between the two lines (Cremonesi et al., 2012). The present study used the same animals as Cremonesi and co-workers to investigate the response of caprine mammary gland tissues to experimental challenge with *S. aureus*, using transcriptomic and histopathology analyses. Mammary gland tissues were analysed by (i) histopathology in order to evaluate the severity of mastitis (tissue morphology, cells infiltration and distribution), (ii) immunohistochemistry to better understand the different immune response between the two goat lines, and by (iii) transcriptomic analysis to reveal differences in gene expression. The results were used to compare the responses between the two divergent lines of goat.

2. Materials and Methods

2.1 Animals

Six primiparous French Alpine goats from divergent selection for extreme breeding values for the somatic cell score were selected to have similar milk production (3.2 ± 0.5 kg/d) as described in Rupp et al. (2011). Out of the 6 animals, 3 were from a LSCS caprine line and 3 from the HSCS caprine line. The animals were transferred two months before kidding to "Centro Zootecnico Didattico Sperimentale, Università degli Studi di Milano-Italy" for experimental infection as previously described by Cremonesi et al., (2012). The goats were monitored for intramammary infections from parturition to the day of challenge, with weekly bacteriological analysis of milk samples. Before the challenge, all animals were free of any mastitis pathogens and had a SCC below 250,000 cells/mL. Mammary tissue samples were collected post slaughter. All experimental animal procedures were performed according to the Italian legislation, following approval by the ethics committee of the University of Milan.

2.2 Experimental design

The left half udder was inoculated with a solution containing *S. aureus* strain DV137 at a final concentration of 10^3 CFU/mL. This strain was originally isolated from a chronic case of caprine mastitis (Vimercati et al., 2006). The right half udder was sham inoculated with sterile Phosphate Buffered Saline (PBS, Invitrogen, Milan, Italy). Thirty hours post inoculation, the animals were sacrificed and tissue samples from superficial (M1), mid (M2) and deep (M3) areas of the right and left mammary glands of each goat were obtained (3 samples for each half udder for a total of 6 mammary samples per goat) and simultaneously one suprammamary lymphnode was collected from each goat.

2.3 Histopathology

Tissue samples were fixed in 10% buffered formalin and processed for histopathological examination by embedding in paraffin wax, cutting into 5 μm thick sections and staining with haematoxylin, eosin and gram stains. The presence and severity of mastitis lesions were morphologically evaluated, and semi-quantitatively scored as follows: = (<5%), +/- (5-10% of tissue affected;), + (> 10-25% of tissue affected), ++ (> 25-50% of tissue affected), and +++ (>50-75% of tissue affected), ++++ (>75% of tissue affected) (Johnson et al., 2004). Severity of bacterial colonization was also scored after Gram staining as follows: - (absent), + (mild), ++ (moderate), and +++ (severe) infiltration.

2.4 Immunohistochemistry

Tissue slices of 3 μm were processed as previously described (Filipe et al., 2018; Filipe et al., 2019) for immunostaining with polyclonal anti-αhPTX3 antibody recognizing PTX3 at 1:400 dilution (kindly provided by Prof. Cecilia Garlanda, Humanitas University, Rozzano, Italy) and anti-αhIL-1R8 antibody recognising IL1R-8 at 1:1000 dilution (AF990, R&D Systems, Minneapolis, MN, USA) primary antibodies. Negative controls were isotype-matched, irrelevant monoclonal antibody recognizing epitopes from another species (anti-human CD8, clone C8/144B, Dako, Glostrup, Denmark). Antibodies were incubated overnight in a humidified chamber at 4°C. Secondary detection was performed with the Avidin-Biotin enzyme Complex (ABC kit, Vectastain®, Burlingame, CA, U.S.A.) for 30 minutes. The reaction was developed with the peroxidase Amino-9-ethyl-carbazole (AEC) substrate kit (Dako®, Glostrup, Denmark). Smears were counterstained with Mayer's hematoxylin for 1 minute and cover-slipped with an aqueous mounting media (Glicerine, Sigma-Aldrich®, St. Louis, MO, U.S.A.). Immunohistochemical reactions were evaluated in the micro-anatomical regions of the normal and pathological mammary glands, including ductal epithelium, glandular epithelium, luminal secretum, macrophages, interstitial

lymphoplasmacytic to follicular inflammation and, neutrophil inflamed mammary glands. The level of the reaction was scored as follows: - (Negative), +/- (< 25%), + (25-50%), ++ (50-75%), and +++ (> 75%). Intensity of staining was scored as follows: + (weak), ++ (moderate), and +++ (intense).

2.5 Total RNA extraction from tissue

Total RNA was extracted from tissue samples using Trizol following the instructions of the supplier (Invitrogen, Milan, Italy). RNA was further purified using a RNeasy MinElute spin column (Qiagen, Milan, Italy) and eluted in RNase free water. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and quality-checked using a Bioanalyser 2100 (Agilent, Santa Clara, CA). RNA samples with RNA Integrity Number (RIN) values between 7.0 and 10.0 were used for the microarray analysis.

2.6 Reverse-transcription and Real Time PCR

One μg of total RNA from each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions and as previously described (Filipe et al., 2018). The cDNA samples were stored at -20°C.

The cDNA obtained from each sample was used as a template for Real Time PCR as previously described (Filipe et al., 2018). We used bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a consistently expressed gene and bovine IL-1β as control. Bovine primers were used because they also specifically recognize goat sequences of GAPDH (GI:1062975189) and IL-1 β (GI:1834304), respectively. Primers were purchased from ThermoFisher Scientific (Carlsbad, CA, USA). The expression of IL-1β gene was normalized using the calculated GAPDH cDNA expression (mean) of the same sample and run.

2.7 Array hybridization and statistical analysis

The microarray design, used in this study, was previously described (Cremonesi et al., 2012). All the probes were synthesized in duplicate, along with negative and quality controls, on a 90K feature custom array from CombiMatrix (Seattle, WA). One μg of RNA was amplified and labelled with Cy5-ULS using the RNA Amplification and Labelling Kit from CombiMatrix (ampULSe Cat. no. GEA-022; Kreatech Biotechnology, Amsterdam, The Netherlands). All procedures were carried out according to the manufacturer's protocols. The purified labelled aRNA was quantified using a NanoDrop spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Four μg of labelled RNA were fragmented to a uniform size and hybridized to the custom array following the

Combimatrix CustomArray 90K Microarray Hybridization and Imaging Protocol. Arrays were stripped and re-hybridized using the protocol of the manufacturer. The hybridized arrays were scanned with a GenePix 4000B microarray scanner (Axon, Toronto, CA) and the images (TIF format) were exported to the CombiMatrix Microarray Imager Software, to perform quality checks of the hybridizations and the spots on the slide. Data were extracted and loaded into R software using the Limma analysis package from Bioconductor. A design matrix was created using Limma functions to describe the experimental samples and replicates. The raw intensities were processed using quantile normalization and data were then log2 transformed for statistical analysis. Limma performs a linear regression analysis on the hybridizations, using a group-means parameterization approach to compare the different conditions, and performs a false discovery rate adjustment with Benjamini-Hochberg correction for multiple testing (Smyth et al., 2004).

Differentially expressed genes were identified using an adjusted P-value cut off equal to 0.01 and 0.05. Each gene symbol of the DE genes identified with R was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Using lists of DE genes as input to the IPA library identified associated canonical pathways, biological functions and networks which were used to investigate the biological context. The IPA library items were ranked based on significance of association with the input list of genes. For the canonical pathways this significance was determined based on two parameters: (i) ratio of the number of genes from the input data set that map to the canonical pathway divided by the total number of genes in that pathway and (ii) p-values calculated using Fischer's exact test determining the probability that the association is explained by chance alone. For the biological functions and networks, the significance was linked to the p-value calculated by a right tailed Fisher's exact test. The p-values for the network analysis consider the number of affected genes in the network and the size of the network.

2.8 Statistical analysis

To compare the expression of *PTX3*, *IL-1R8* in the two experimental groups (HSCS and LSCS) in the histological and immunohistochemical analysis we used the Mann-Whitney test or for not normally distributed and low number of data a Shapiro-Wilk test, considering values of $p < 0.05$ to be statistically significant. To evaluate the significance of IL-1β RNA messenger expression in the two experimental groups (HSCS and LSCS), we used the Mann-Whitney test, considering $p < 0.05$ to be statistically significant. For the biological function and network analysis, the p-value significance calculated by right-tailed Fisher's exact test was used.

3. Results

3.1 HSCS goats present an increased PMN interlobular infiltration

In sham inoculated mammary glands the overall microscopic morphology was similar in HSCS and LSCS goats. Mild to moderate interstitial lymphoplasmacytic inflammation was evidenced in healthy mammary glands mostly in LSCS goats, mainly in perialveolar interstitium and interlobular interstitium (Figure 1A and 2A). Intra-alveolar macrophage infiltration was similar for all control and infected mammary tissues, both from LSCS and HSCS. Intra-alveolar neutrophils were absent in all control tissues (Supplementary table 1).

In *S. aureus* inoculated left half udders the level of intra-alveolar neutrophils was increased in both LSCS and HSCS goats (Figure 1A and 2A). Neutrophils were present in glandular tissue and in the interstitium. In *S. aureus* inoculated left half udders lymphoplasmacytic inflammation was more severe than un-infected udders and was observed in interlobular interstitium (mainly in LSCS) and in periductal interstitium (mainly in HSCS) (Figure 1B, 1C, 2B and 2C). In HSCS goats, ulceration of ductal epithelium with formation of interstitial micro-abscesses was common following infection (Figure 2C). Epithelial duct ulcerations and microabscesses were never observed in LSCS goats. The percentage of mammary alveoli affected did not differ between goat lines, while interstitial inflammation was more severe in HSCS goats (Figure 2B).

Gram stain revealed the presence of Gram-positive cocci in variable numbers mostly in the lumens of glands only in infected mammary udders of both lines (Figure 1D and 2D), with a higher number of bacterial aggregates seen in the HSCS line (Figure 2D and 2E). No central aggregation of bacteria inside the micro-abscesses was observed.

3.2 LSCS and HSCS present a different immunohistochemical pattern of expression of innate immune related molecules

In order to better understand the immune responses in the 2 goat lines, the expression of *IL-1R8*, a negative regulator of the inflammation, mediated by TLRs and ILRs and *PTX3*, a pentraxin molecule with antibacterial and immune response regulatory activity, were analysed (Riva et al. 2012; Mantovani et al. 2013) (Supplementary table 2). No significant differences in IL-18R protein expression were observed in healthy mammary tissue samples of LSCS and HSCS lines. Similarly, no differences were observed comparing superficial, mid, and deep tissue samples of control mammary glands. In infected mammary tissues, a generalized reduction in the number and staining intensity for IL-1R8 positive cells was detected for ductal epithelium, this reduction in numbers was more pronounced in LSCS goats. The intensity score of IL-1R8 was significantly lower in LSCS compared to HSCS for ductal epithelial cells (Figure 3A). In contrast, a significant increase of IL-1R8 staining was observed in lymphoplasmacytic inflammatory and macrophage infiltrates of

LSCS compared to HSCS goats (Figure 3B and 3C). The reduced expression of IL-1R8 in the inflammatory infiltrate could be associated with the (not significant) increased expression of IL-1β mRNA in infected udders of HSCS (Figure 3F).

Comparing mammary tissues from HSCS *vs* LSCS goats the only distinctive difference was observed in healthy udders of HSCS goats in which apical portion of ductal epithelium had lower staining intensity and percentage of PTX3 positive cells in the ductal epithelial of LSCS goats (Figure 3D and 3E). No other major differences in PTX3 expression were detected for in normal and infected mammary tissue samples of the two different goat lines. PTX3 staining intensity increased and the percentage of positive cells was similar for LSCS *vs* HSCS goat infected mammary tissues. No significant differences in PTX3 expression were observed in superficial or deep tissue samples.

3.3 LSCS and HSCS present a different gene expression profiling involved inflammatory response and lipid metabolism

The analysis of all the infected *vs* the healthy tissues revealed 21 genes to be differentially expressed (p-value < 0.05 and log2 fold change > 1.5). No genes showed a decrease in expression in infected vs healthy samples (Supplementary Table 3). The 8 top differentially expressed genes (DEGs) had an important role in chemotaxis of cells (EGR1, ICAM1) and in the inflammatory response (LTF, PTX3, ANXA2, S100A9, CD9, C3) (Figure 4). Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc.) identified a series of functional categories associated with the DE genes; the most representative was "Cellular movement" (p value = 7.48E-09) followed by "Cellular Growth and Proliferation" (p value = 1.43E-04), "Cellular Movement, Immune Cell Trafficking" (p value = 9.22E-10), "Inflammatory Response" (p value = 1,96E-07).

Comparison of HSCS *vs* LSCS infected tissues identified 20 DE genes (p-value < 0.05 and log2 fold change > 1.5). The top six up- and down-regulated genes were mainly involved in "Inflammatory response" and "Lipid metabolism", respectively (Table 1). *CXCL8* and *CXCL2*, were up regulated in the HSCS compared to the LSCS line (Figure 5). Both these chemokines are involved in neutrophil recruitment stimulating mobilization, emigration from blood vessels, infiltration, chemiotaxis of neutrophils and delay of neutrophil apoptosis. Moreover, CXCL2 and CXCL8 are involved in activation and movement of macrophages. But another up-regulated gene, *TNFRSF6B*, is responsible of inhibition in macrophages cell movement (Supplementary Figure 1).

LPL, which was upregulated in the LSCS line, induces the breakdown of fatty acid, hydrolysis and storage of cholesterol, accumulation of lipid, the uptake of cholesterol ester, synthesis of fatty acid

and the levels of polyunsaturated fatty acids. The comparison of healthy tissues between HSCS *vs* LSCS showed no differences in gene expression or pathways activated.

4. Discussion

In the present study the mammary gland tissues of two lines (HSCS and LSCS) of goats were analysed with the hypothesis that tissues from these lines would respond differently to *S. aureus* experimental infection. Earlier work reported that there is no difference in response between the lines in blood or milk somatic cells (Cremonesi et al., 2012). Divergent selection based on SCS in sheep and cattle have been performed with the goal of creating animals increased resistant to inter-mammary infections (Brand et al., 2011; Rupp et al., 2009), and indeed have shown a decrease in mastitis in the low SCS line. However, to date it the mechanisms that improve udder health are not understood.

In the present study the comparison of the HSCS and LSCS goat lines, showed an increase in the inflammatory response in the LSCS line, both at the histological level, in terms of leukocyte recruitment, presence of micro-abscesses, interalveolar macrophages and PMN, and the molecular level, in terms of *CXCL8*, *IL-6*, *NFKBIZ* and *IL-1β* mRNA expression. This increased inflammatory response could be due to the higher expression of *IL-1R8*, a negative regulator of the inflammation triggered by TLRs and ILRs (Riva et al. 2012), in the ductal epithelial cells. This increased inflammation is not able to control *S. aureus* infection, as demonstrated by the higher number of cocci in the infected udders of the HSCS compared to the LSCS goat line. Impaired bacterial clearance could be a consequence of the increase expression of IL-1R8 in the interstitial leukocytes combined with the lower levels of PTX3 in the ductal epithelial cells of HSCS compared to the LSCS. PTX3 is a soluble antimicrobial involved in the fight against the pathogens (Mantovani et al. 2013; Filipe et al. 2018). Our results suggest that HSCS goats developed a strong and persistent inflammatory response following *S. aureus* experimental infection that is not efficient in the clearance of the bacteria, and moreover contributes to severe tissue damage. These results are in line with previous observations that exaggerated local proinflammatory responses, with an excessive release of cytokines, can cause tissue damage and impair the immune response to the pathogens (Wall et al. 2009; Garlanda et al. 2007; Veliz-Rodriguez et al. 2012). The transcriptomic analysis of infected *versus* all healthy mammary tissues showed the eight top differentially expressed genes (*EGR1, ICAM1, LTF, PTX3, ANXA2, S100A9, CD9, C3*). These gene products are involved in immune and inflammatory response, in the regulation of innate resistance to pathogens and in the regulation of cell metabolism, as previously observed in milk somatic cells (Cremonesi et al., 2012). *PTX3* was among the most up-regulated genes in the present study and in both milk

somatic cells and white blood cells (Cremonesi et al., 2012) together with S100A9; PTX3 acts as anti-microbial agent. S100A9 and other members of the same protein family, LTF and CD9, are involved in the cellular immune response to pathogens. ICAM1 which had higher expression in the LSCS mammary tissue was also up regulated in milk somatic cells (Cremonesi et al., 2012). In the mammary gland tissue, it plays a role in trans-endothelial migration of leukocytes.

Histopathology revealed severe interstitial neutrophilic inflammation in HSCS goat line, compared to the minimal luminal neutrophilic inflammation in LSCS goats. A striking difference between the two goat lines was the presence of interstitial micro-abscesses and epithelial duct ulceration which was limited to the HSCS line. This is consistent and increased expression of IL-1β. IL-1β mRNA was increased in all infected samples but was significantly increased in HSCS line. Neutrophil recruitment has been linked to IL-1β production during *S. aureus* infection and IL-1β peaks 4 hours after *S. aureus* infection and this increase alone is enough for micro-abscess development in mice (Miller et al., 2007; Cho et al., 2012).

In this work the initial stages of *S. aureus* intrammamary infection were evaluated. At these stages neutrophil aggregation and abscess formation have been reported to be unrelated to bacterial load (Cheng et al., 2011). This finding derives from the observation that neutrophils but not monocytes/macrophages or other MHCII-expressing antigen presenting cells are the predominant source of IL-1β at sites of *S. aureus* infection (Cho et al., 2012). In summary, *S. aureus* releases lipoproteins actively with recognition by TLR with rapid activation of pro-inflammatory signals that recruit inflammatory cells to the site of infection. Neutrophils can express pattern recognition receptors including TLR2, NOD2, FPR1 and the ASC/NLRP3 inflammasome that are activated by *S. aureus* lipopetides and lipoteichoic acid in an a-toxin-dependent mechanism TLR. Neutrophils at the site of infection produce increasing levels of IL-1β that has proven necessary for neutrophil concentration. This mechanism represents a loop where the progressive increase of neutrophils increases the concentration of IL-1β potentiating local inflammation, chemotaxis and accumulation of neutrophils leading to micro-abscesses development and tissue damage (Cho et al., 2012).

Micro-abscess formation occurs when neutrophils accumulate at sites of tissue damage and especially at sites of damaged epithelia (Cheng et al., 2011). Epithelial lesions could contribute to microabscess formation in HSCS line by two ways, facilitating *S. aureus* penetration in mammary interstitium following ulceration and by production of proinflammatory mediators including IL-8 (Russo et al., 2014) and IL1β (Cheng et al., 2011).

A reduction of the local immune defence of the duct epithelial cell lining in HSCS goats was likely to be involved in the more severe lesions observed in HSCS infected udders. Comparing LSCS and HSCS mammary ducts, *PTX-3* expression was reduced in ductal epithelial cell of HSCS goats.

PTX-3 is thought to be a first line of defense in goat udders (Filipe et al., 2018) and was found to be up-regulated in mammary tissue following *S. aureus* infection, confirming previous reports (Mantovani et al., 2013). The striking reduction of PTX-3 expression in epithelial cells of mammary ducts of HSCS goats, revealed by immunohistochemistry, may indicate a reduction of PTX-3 epithelial protective mechanisms facilitating *S. aureus* adhesion, toxin induced tissue damage, ulceration and penetration into the mammary interstitial tissue. Higher levels of PTX-3 in LSCS goat normal tissues and in the interstitial inflammation may be indicative of the protective and modulating role of PTX-3 in this selection line.

IL-1R8 was more highly expressed in ductal epithelial cells of LSCS possibly contributing to protection of epithelial cells from *S. aureus* damage, whereas the reduction of IL-1R8 expression in the HSCS line observed by immunohistochemistry may represent be involved in epithelial damage. IL-1R8, also known as TIR8 or SIGIRR, is a member of Interleukin-1 receptor family with negative regulatory activity on TLRs and ILRs with anti-inflammatory functions (Molgora et al., 2016 and 2018). Previously, IL-1R8 has been reported to be down-regulated in epithelial mammary cells following *S. aureus* infection. However, as previously reported (Filipe et al., 2019), a general up-regulation in leukocytes infiltrating the infected mammary tissues has been observed suggesting the IL1-R8 may be a target of *S. aureus* immune invasion. Abscess maturation and organization around large *S. aureus* colonies occur 4 to 5 days post infection (Cheng et al., 2011). In this work central bacterial aggregation in micro-abscesses were not observed in inflamed tissue of either line or is most likely due to early analysing of the animals. However, a higher bacterial tissue load was seen in HSCS goat tissues, which could be related to the inherent predisposition of HSCS goats to *S. aureus* infection and to increased inflammatory response. The level of both bacterial load and immune response are associated with increased synthesis of acute inflammatory mediators and contemporarily to reduced protective mechanisms at the site of bacterial entry, such the epithelial lining. Infected mammary glands from LSCS goats had a higher percentage of macrophages compared to the corresponding LSCS non-infected gland.

In conclusion. our data suggest that the two genetic lines developed different inflammatory responses. Specifically, HSCS seemed to respond with a more severe inflammation compared to LSCS to *S. aureus* infection. These results explain, to some extent the difference between the lines and are a starting point for further studies to unravel the genetic basis of differences in mastitis incidence.

References

Banos, G., Bramis, G., Bush, S.J., Clark, E.L., McCulloch, M.E.B., Smith, J., Schulze, G., Arsenos, G., Hume, D.A., Psifidi, A., 2017. The genomic architecture of mastitis resistance in dairy sheep. BMC Genomics. 18, 624.

Bonnefont, C.M., Rainard, P., Cunha, P., Gilbert, F.B., Toufeer, M., Aurel, M.R., Rupp, R., Foucras, G., 2012. Genetic susceptibility to *S. aureus* mastitis in sheep: differential expression of mammary epithelial cells in response to live bacteria or supernatant. Physiol Genomics. 44, 403-16.

Bonnefont, C.M., Toufeer, M., Caubet, C., Foulon, E., Tasca, C., Aurel, M.R., Bergonier, D., Boullier, S., Robert-Granié, C., Foucras, G., Rupp, R., 2011. Transcriptomic analysis of milk somatic cells in mastitis resistant and susceptible sheep upon challenge with *Staphylococcus epidermidis* and *Staphylococcus aureus*. BMC Genomics. 12, 208.

Brand, B., Hartmann, A., Repsilber, D., Griesbeck-Zilch, B., Wellnitz, O., Kühn, C., Ponsuksili, S., Meyer, H.H., Schwerin, M., 2011. Comparative expression profiling of *E. coli* and *S. aureus* inoculated primary mammary gland cells sampled from cows with different genetic predispositions for somatic cell score. Genet. Sel. Evol. 43, 24.

Brenaut, P., Lefèvre, L., Rau, A., Laloë, D., Pisoni, G., Moroni, P., Bevilacqua, C., Martin, P., 2014. Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to *Staphylococcus aureus*. Vet Res. 45, 16.

Cheng, A.G., DeDent, A.C., Schneewind, O., Missiakas, D., 2011. A play in four acts: *Staphylococcus aureus* abscess formation. Trends Microbiol. 19(5):225-32.

Cho, J.S., Guo, Y., Ramos, R.I., Hebroni, F., Plaisier, S.B., Xuan, C., Granick, J.L., Matsushima, H., Takashima, A., Iwakura, Y., Cheung, A.L., Cheng, G., Lee, D.J., Simon, S.I., Miller, L.S., 2012. Neutrophil-derived IL-1β is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. PLoS Pathog. 8(11):e1003047.

Cremonesi, P., Capoferri, R., Pisoni, G., Del Corvo, M., Strozzi, F., Rupp, R., Caillat, H., Modesto, P., Moroni, P., Williams, J.L., Castiglioni, B., Stella, A., 2012. Response of the goat mammary gland to infection with *Staphylococcus aureus* revealed by gene expression profiling in milk somatic and white blood cells. BMC Genomics. 13, 540.

Filipe, J., Bronzo, V., Curone, G., Castiglioni, B., Vigo, D., Smith, B., Roccabianca, P., Moroni, P., Riva, F., 2019. *Staphylococcus aureus* intra-mammary infection affects the expression pattern of IL-R8 in goat. Comp Immunol, Microbiol Infectious Dis. JUL;30;66:101339. DOI: 10.1016/j.cimid.2019.101339.

Filipe, J., Curone, G., Bronzo, V., Pisoni, G., Cremonesi, P., Pollera, C., Turin, L., Vigo, D., Roccabianca, P., Caniatti, M., Moroni, P., Riva, F., 2018. Pentraxin 3 is up-regulated in

epithelial mammary cells during *Staphylococcus aureus* intra-mammary infection in goat. Comparative immunology, microbiology and infectious diseases. 59, 8-16.

Garlanda, C., Di Liberto, D., Vecchi, A., La Manna, M.P., Buracchi, C., Caccamo, N., Salerno, A., Dieli, F., Mantovani, A., 2007. Damping excessive inflammation and tissue damage in *Mycobacterium tuberculosis* infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. J. Immunol. 179, 3119-3125.

Heringstad, B., Klemetsdal, G., Ruane, J., 2000. Review article: Selection for mastitis resistance in dairy cattle: a review with focus on the situation in the Nordic countries. Lives. Prod. Sci. 64, 95-106.

Johnson, L.R., Clarke, H.E., Bannasch, M.J., De Cock, H.E.V., 2004. Correlation of rhinoscopic signs of inflammation with histologic findings in nasal biopsy specimens of cats with or without upper respiratory tract disease. J. Am. Vet. Med. Assoc. 225, 395–400.

Lutzow, Y.C., Donaldson, L., Gray, C.P., Vuocolo, T., Pearson, R.D., Reverter, A., Byrne, K.A., Sheehy, P.A., Windon, R., Tellam, R.L., 2008. Identification of immune genes and proteins involved in the response of bovine mammary tissue to *Staphylococcus aureus* infection. BMC Vet Res. 4,18.

Mantovani, A., Valentino, S., Gentile, S., Inforzato, A., Bottazzi, B., Garlanda, C., 2013. The long pentraxin PTX3: a paradigm for humoral pattern recognition molecules. Ann. N. Y. Acad. Sci. 1285, 1-14.

Miller, L.S., Pietras, E.M., Uricchio, L.H., Hirano, K., Rao, S., Lin, H., O'Connell, R.M., Iwakura, Y., Cheung, A.L., Cheng, G., Modlin, R.L., 2007. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. J. Immunol. 179(10):6933-42.

Molgora, M., Barajon, I., Mantovani, A., Garlanda, C., 2016. Regulatory Role of IL-1R8 in Immunity and Disease. Front. Immunol. 7:149.

Molgora, M., Supino, D., Mantovani, A., Garlanda, C., 2018. Tuning inflammation and immunity by the negative regulators IL-1R2 and IL-1R8. Immunol. Rev. 281(1):233-247.

Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: structure and function in blood and milk. Vet. Res. 34, 597-627.

Pighetti, G.M., Elliott, A.A., 2011. Gene polymorphisms: the keys for marker assisted selection and unraveling core regulatory pathways for mastitis resistance. J. Mammary Gland Biol. Neoplasia. 16, 421-32.

Riva, F., Bonavita, E., Barbati, E., Muzio, M., Mantovani, A., Garlanda, C., 2012. TIR8/SIGIRR is an Interleukin-1 Receptor/Toll Like Receptor Family Member with Regulatory Functions in Inflammation and Immunity. Front. Immunol. 3, 322.

Rupp, R., Beaudeau, F., Boichard, D., 2000. Relationship between milk somatic cell counts in the first lactation and clinical mastitis occurrence in the second lactation of French Holstein cows. Prev. Vet. Med. 46, 99-111.

Rupp, R., Bergonier, D., Dion, S., Hygonenq, M.C., Aurel, M.R., Robert-Granié, C., Foucras, G. 2009. Response to somatic cell count-based selection for mastitis resistance in a divergent selection experiment sheep. J Dairy Sci. 92, 1203-1219.

Rupp, R., Boichard, D., 2003. Genetics of resistance to mastitis in dairy cattle. Vet. Res. 34, 671-688.

Rupp, R., Clément, V., Piacere, A., Robert-Granié, C., Manfredi, E., 2011. Genetic parameters for milk somatic cell score and relationship with production and udder type traits in dairy Alpine and Saanen primiparous goats. J Dairy Sci. 94, 3629-3634.

Russo, R.C., Garcia, C.C., Teixeira, M.M., Amaral, F.A., 2014. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert. Rev. Clin. Immunol. 10:593-619.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3.

Sordillo, L.M., 2005. Factors affecting mammary gland immunity and mastitis susceptibility. Lives. Produc. Sci. 98, 89-99.

Sørensen, L.P., Mark, T., Madsen, P., Lund, M.S., 2009. Genetic correlations between pathogen-specific mastitis and somatic cell count in Danish Holsteins. J Dairy Sci. 92, 3457–3471.

Véliz Rodriguez, T., Moalli, F., Polentarutti, N., Paroni, M., Bonavita, E., Anselmo, A., Nebuloni, M., Mantero, S., Jaillon, S., Bragonzi, A., Mantovani, A., Riva, F., Garlanda, C., 2012. Role of Toll interleukin-1 receptor (IL-1R) 8, a negative regulator of IL-1R/Toll-like receptor signaling, in resistance to acute *Pseudomonas aeruginosa* lung infection. Infect. Immun. 80, 100-109.

Vimercati, C., Cremonesi, P., Castiglioni, B., Pisoni, G., Boettcher, P.J., Stella, A., Vicenzoni, G., Moroni, P., 2006. Molecular typing of *Staphylococcus aureus* isolated from cows, goats and sheep with intramammary infections on the basis of gene polymorphisms and toxins genes. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 53, 423–428.

Wall, R., Powell, A., Sohn, E., Foster-Frey, J., Bannerman, D., Paape, M., 2009. Enhanced Host Immune Recognition of Mastitis Causing *Escherchia coli* in CD-14 Trans. Mice. Animal Biotech. 1, 1-14.

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Table 1: Differentially expressed genes after comparison between HSCS *vs* LSCS goat in infected tissues.

Infected tissues	LogFC	Gene name	Gene function
CXCL8	2.659	C-X-C motif chemokine ligand 8	One of the major mediators of the inflammatory response; secreted by several cell types; acts as a chemoattractant, and a potent angiogenic factor.
SLC6A14	2.548	Solute carrier family 6 member 14	Encodes a member of the solute carrier family 6. Members of this family are sodium and chloride dependent neurotransmitter transporters.
NFKBIZ	2.397	NFKB inhibitor zeta	Member of the ankyrin-repeat family, induced by lipopolysaccharide (LPS) that has a role in inflammatory responses by their interaction with NF-B proteins through ankyrin-repeat domains
CXCL2	2.261	Chemokine (C-X-C motif) ligand 2	Antimicrobial gene, part of a chemokine superfamily that encodes secreted proteins involved in immunoregulatory and inflammatory processes
SDC4	1.581	Syndecan 4	receptor in intracellular signaling
TNFRSF6B	1.511	TNF receptor superfamily member 6b	The encoded protein is postulated to play a regulatory role in suppressing FasL- and LIGHT-mediated cell death
SYNE4	-1.721	Spectrin repeat containing nuclear envelope family member 4	Member of the nesprin family of genes
SLC36A4	-1.654	Solute carrier family 36 member 4	Symporter activity and amino acid transmembrane transporter activity
BTN1A1	-1.642	Butyrophilin subfamily 1 member A1	Major protein associated with fat droplets in the milk; member of the immunoglobulin superfamily
SUPT3H	-1.564	SPT3 homolog, SAGA and STAGA complex	Probable transcriptional activator

		component	
LPL	-1.521	Lipoprotein lipase	Encodes lipoprotein lipase
GCNT4	-1.519	Glucosaminyl (N-acetyl) transferase 4, core 2	Mucin-type biosynthesis

Figure legend

Figure 1. Histological analysis of LSCS goats.

A) Representative image of control right half udder: Occasional intra-alveolar macrophages, mild interstitial accumulation of low number of small mature lymphocytes and plasmacells. Haematoxylin and Eosin stain (original magnification 10X). B) Representative image of left inoculated half udder: Inflammation is mostly evidenced as a variable intra-alveolar accumulation of non-degenerated neutrophils; interstitial inflammation is mild. Haematoxylin and Eosin stain (original magnification 20X). C) Representative image of left inoculated half udder. Moderate, diffuse interstitial lymphoplasmacytic inflammation. Haematoxylin and Eosin stain (original magnification 10X). D) Representative image of left inoculated half udder: Presence of low numbers of intra-alveolar Gram positive coccoid bacterial aggregates. Gram stain (original magnication 400X).

Figure 2. Histological analysis of HSCS goats.

A) Representative image of control right half udder: Mild interstitial fibrosis with minimal interstitial lymphocytes and plasmacells. Haematoxylin and Eosin stain (original magnification 20X). B) Representative image of left inoculated half udder: Severely inflamed mammary tissue characterized by ductal epithelial ulceration, luminal to interstitial severe neutrophilic inflammation. Upper right presence of an interstitial microabscess. Haematoxylin and Eosin stain (original magnification 20X). C) Representative image of left inoculated half udder: large interstitial microabscess composed by a prevalence of degenerated neutrophils. Haematoxylin and Eosin stain (original magnification 20X). D) Representative image of left inoculated half udder: Presence of elevated numbers of intra-alveolar Gram positive coccoid bacterial aggregates (a). Haematoxylin and Eosin stain (original magnification 20X); Elevated numbers of coccoid Gram-positive bacteria admixed with necrotic debris in the lumen of an ulcerated and inflamed mammary gland duct (b). Gram stain (original magnification 400X).

Figure 3. Different pattern of expression of innate immune related molecules.

A, B, C, D, E) Immunohistochemical analysis of IL-1R8 and PTX3 expression in control and infected udders of the two goat lines (Results are expressed as mean ± standard deviation; Mann-Whitney test). F) IL-1b mRNA expression was analyzed by Real Time PCR in control and infected udders of the two goat lines. The gene expression level of the target gene was normalized to GAPDH and the results are presented as Arbitrary Units (2^Delta Ct x 10000). (Results are expressed as mean ± standard deviation; Mann-Whitney test).

Figure 4. Differentially expressed genes involved in Inflammatory response obtained by the analysis of all the infected *vs* the healthy tissues (IPA; Ingenuity Systems, Inc.).

Figure 5. Involvement of CXCL8 and CXCL2 genes in inflammatory response with effects on neutrophils in infected tissues comparing HSCS *vs* LSCS lines.

Supplementary material.

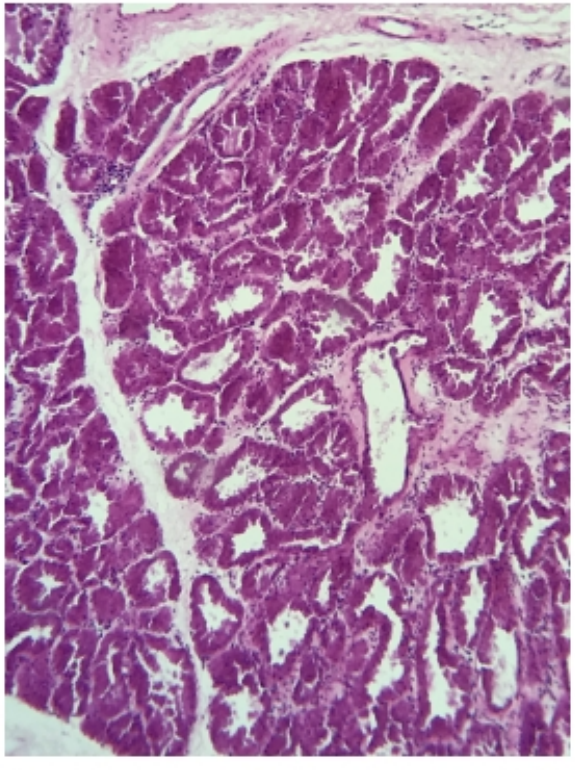
Supplementary Table 1. Histological evaluation of control and infected mammary gland tissue samples. (Legend: Mean of three sections evaluated from 3 different depths of sampling. LPC= lymphoplasmacytic inflammation. PMN= neutrophilic inflammation. *Tissue score of severity of inflammation: - = 0<5%, +/- 5-10%, + 10-25%, ++ 25-50%, +++>50-75%, ++++>75%; **Score of severity of bacterial infiltration: - absent, + mild, ++ moderate, +++severe)

Supplementary Table 2. TIR8 and PTX3 Immunohistochemical expression in control and infected mammary tissues (Legend: D= mammary duct epithelium; G: mammary gland acinar epithelium; S: secretory material in lumens; LPC: interstitial lymphoplasmacytic inflammation M: macrophages; PMN: neutrophils. Positivity score: – Negative, +/- < 25%, ++ 25-50%, ++ 50-75%, +++ > 75%; Intensity score: – Negative, +/- < weak++, mild, ++ moderate, +++ strong).

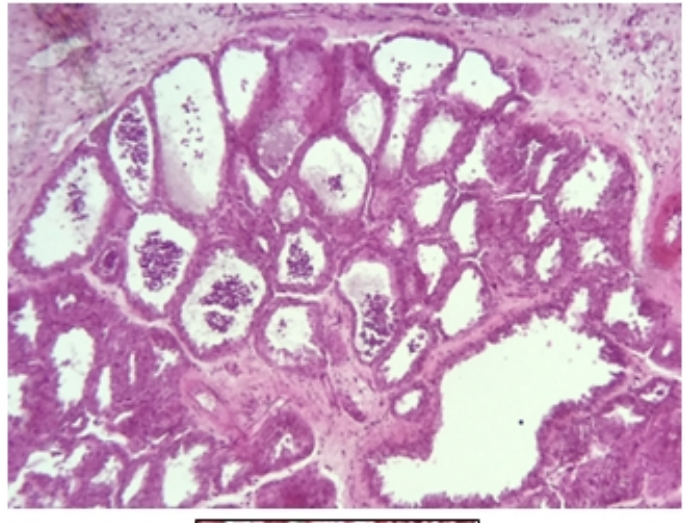
Supplementary Table 3. Differentially expressed genes obtained comparing all the infected *vs* all the healthy tissues.

Supplementary Figure 1. Effect of CXCL2 and CXCL8 genes on macrophages in infected tissues comparing HSCS *vs* LSCS lines.

A



B



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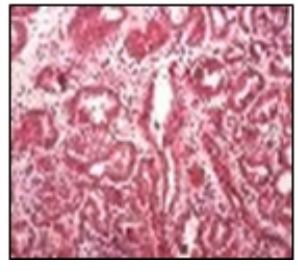

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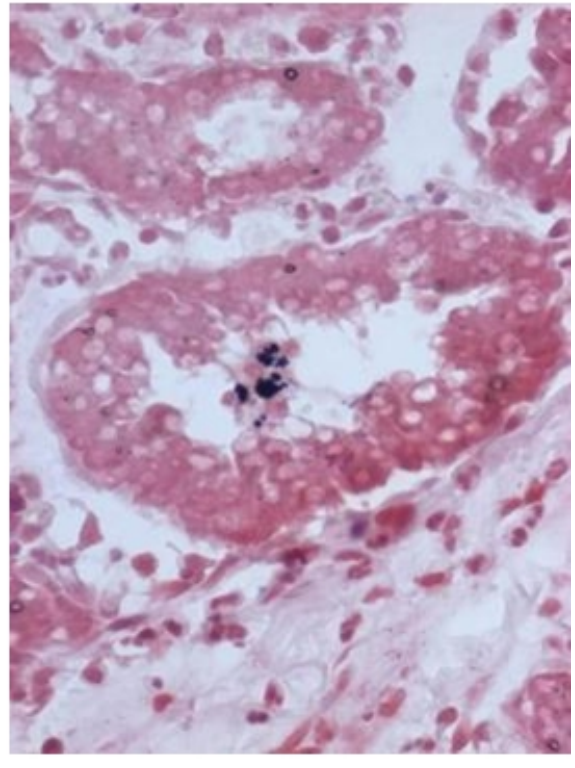

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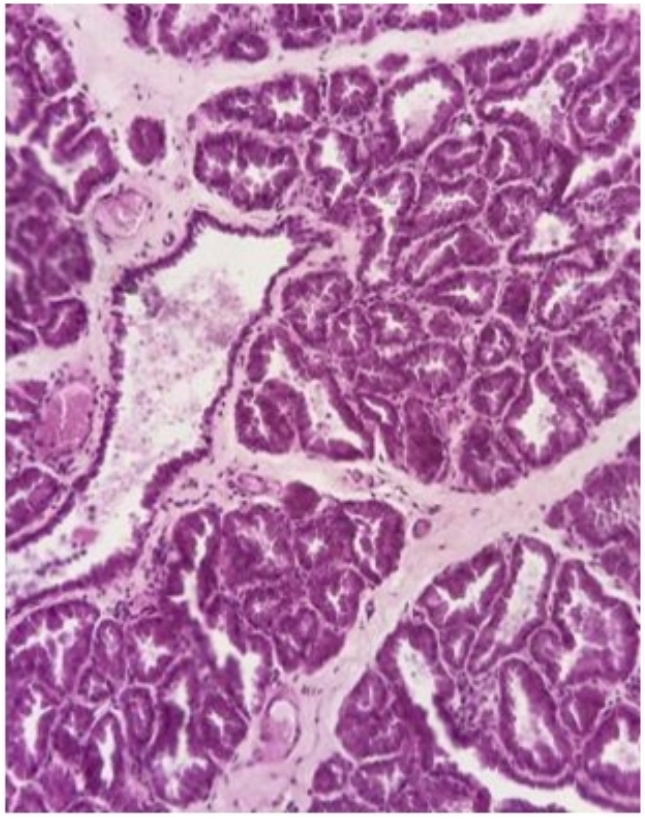

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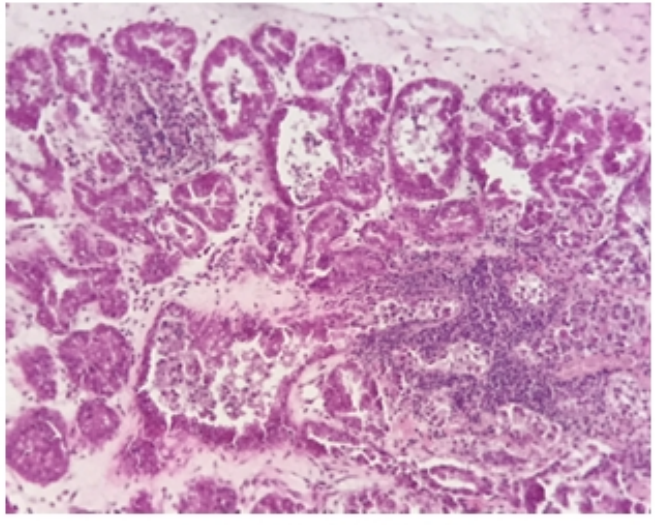

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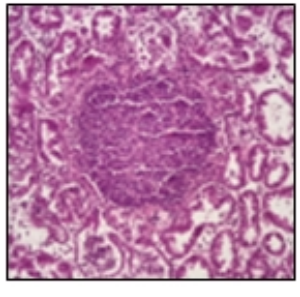

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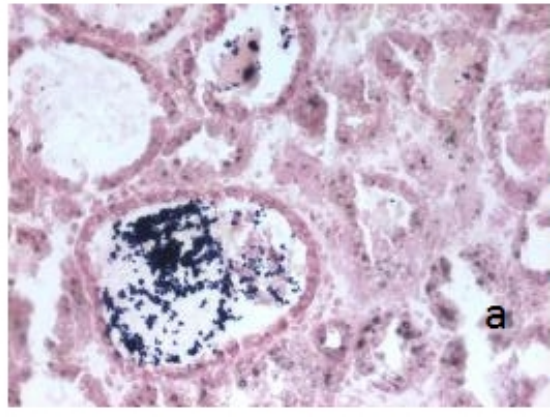

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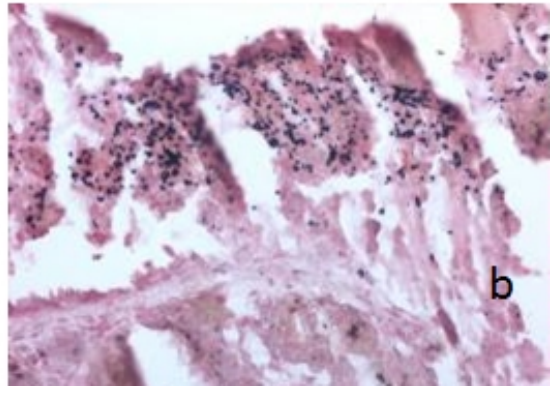

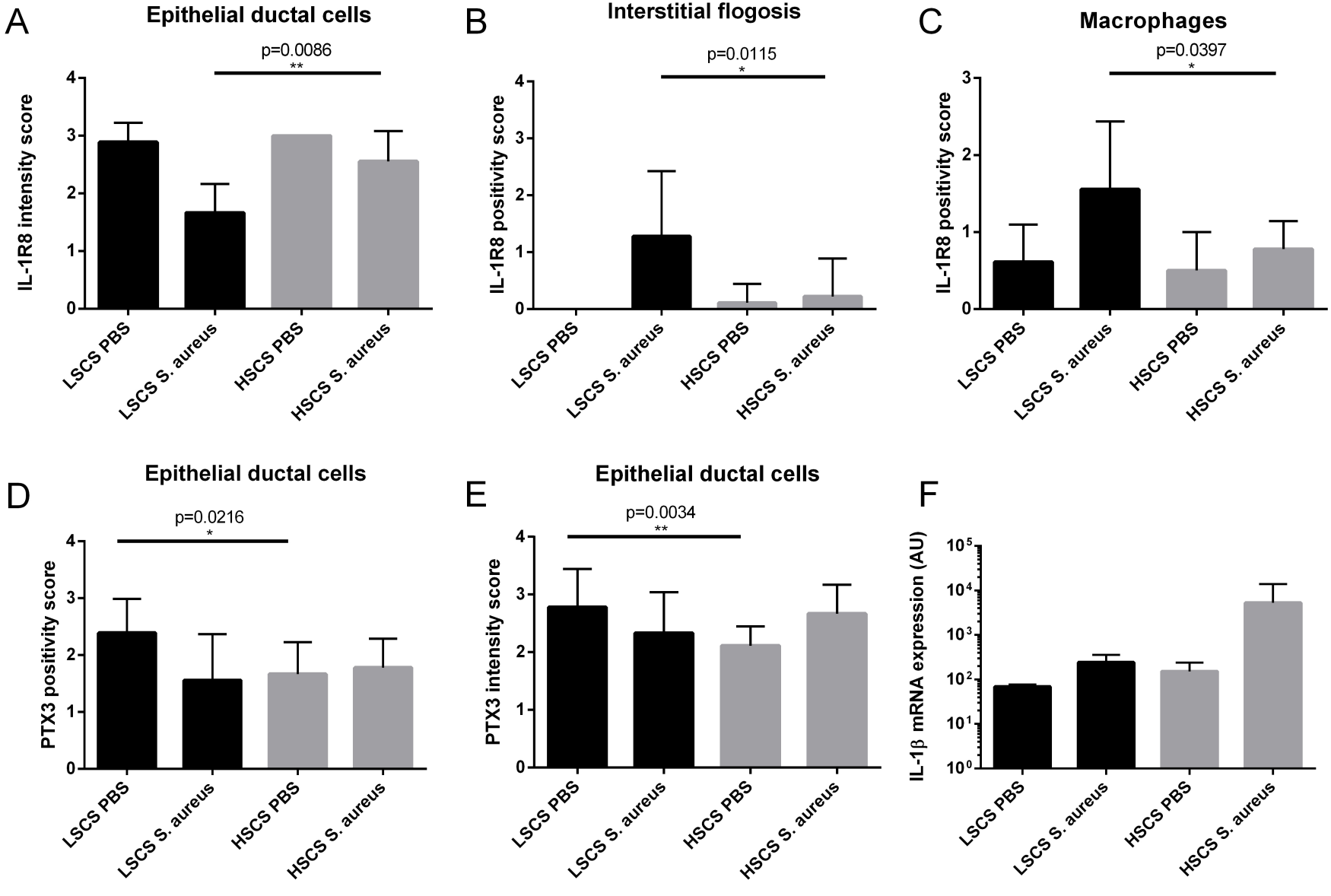
A
Epithelial ductal cells
p=0.0086
**
IL-1R8 intensity score
0
1
2
3
4
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus
B
Interstitial flogosis
p=0.0115
*
IL-1R8 positivity score
0
1
2
3
4
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus
C
Macrophages
p=0.0397
*
IL-1R8 positivity score
0
1
2
3
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus
D
Epithelial ductal cells
p=0.0216
*
PTX3 positivity score
0
1
2
3
4
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus
E
Epithelial ductal cells
p=0.0034
**
PTX3 intensity score
0
1
2
3
4
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus
F
IL-1β mRNA expression (AU)
10^0
10^1
10^2
10^3
10^4
10^5
LSCS PBS
LSCS S. aureus
HSCS PBS
HSCS S. aureus