

Research Article

Cite this article: Shangraw EM, McFadden TB (2024) Early and gland-specific changes in gene expression in response to LPS-induced mastitis in a single quarter of Holstein cows. *Animal Nutriomics* 1, e9, 1–12. <https://doi.org/10.1017/anr.2024.15>

Received: 25 May 2024
Revised: 12 July 2024
Accepted: 27 July 2024

Keywords:

lactation; endotoxin; acute inflammation; RNA from milk fat; noninvasive sampling

Corresponding author:

Thomas B. McFadden;
Email: mcfaddent@missouri.edu

Early and gland-specific changes in gene expression in response to LPS-induced mastitis in a single quarter of Holstein cows

Erin M. Shangraw  and Thomas B. McFadden 

Division of Animal Sciences, University of Missouri, Columbia, MO, USA

Abstract

We previously reported changes in gene expression in mammary tissue from non-inflamed mammary glands adjacent to an inflamed gland challenged with lipopolysaccharide (LPS). We determined if changes in the expression of selected genes in non-inflamed glands would be replicated in RNA isolated from milk fat. Cows were milked twice daily prior to experiment. Per cow, one mammary gland (Q_{LPS}) was randomly assigned to receive an intramammary infusion of 50 μ g LPS immediately after morning milking on d-0. The ipsilateral (Q_I) and contralateral (Q_C) mammary glands adjacent to Q_{LPS} remained untreated. Quarter foremilk samples from all mammary glands were collected on d-1 and d-0 for milk composition and isolation of RNA for quantification of selected genes via quantitative polymerase chain reaction. Symptoms of clinical mastitis developed only in Q_{LPS} and were apparent within 3 h post-challenge. In Q_I and Q_C , lactose percentages were lower at 12 h post-challenge compared to d-1, but milk fat and protein contents were not different. For gene expression, 7 of 13 selected genes were differentially regulated in non-inflamed glands. In Q_I but not Q_C , *LALBA* expression was lower at 12 h post-challenge than on d-1. One gene of interest, *LPIN1*, was significantly upregulated in Q_I and Q_C but downregulated in Q_{LPS} at 12 h post-challenge. Five additional immune or stress-related genes were significantly upregulated in Q_{LPS} and, to a lesser but significant degree, in Q_I and Q_C compared to d-1. Notably, expression of two immune genes (*NFKBIA*, *PTX3*) was significantly greater in Q_I than Q_C despite Q_I having a numerically lower somatic cell count. Minor changes in the composition of milk secreted by non-inflamed mammary glands were linked to several immune and stress responses in those glands. Further, individual non-inflamed mammary glands responded differently depending on their position relative to the mastitic gland.

Introduction

Milk production losses caused by mastitis remain an economic burden for the dairy industry. During acute clinical mastitis, typically one mammary gland is affected with observably abnormal milk and the four cardinal signs of inflammation: heat, redness, edema, and pain. However, a fifth common symptom of inflammation, reduced function, can be observed in all mammary glands whether the other cardinal signs are present or absent in a particular gland. Thus, decreased milk production that develops in the mastitic gland is a local effect, i.e. it occurs in the same mammary gland, whereas decreased milk production in non-inflamed glands adjacent to the mastitic gland is a systemic effect (Mitterhuemer et al. 2010; Shangraw et al. 2020; Shuster et al. 1991a). Milk composition is likewise affected in adjacent glands, although an inflamed mammary gland will typically produce less milk with a higher somatic cell count (SCC) and lower lactose concentration than those remaining non-inflamed (Shangraw et al. 2020; Shuster et al. 1991b). At the transcriptional level, the local effect of mastitis in the inflamed gland is well-studied and shows a predictable immune response (Mitterhuemer et al. 2010; Shangraw et al. 2021). In contrast, little is understood of the transcriptional changes in non-inflamed glands neighboring the inflamed gland, which involve comparably fewer genes with lower magnitude changes in expression.

Our efforts to determine the mechanisms regulating mammary function in non-inflamed neighboring glands supported previous evidence that different milk components are differentially regulated. In agreement with Shuster et al. (1991a), we found that milk fat was the earliest component affected in neighboring glands, such that fat concentrations were significantly lower in foremilk strippings from both mastitic and adjacent glands in lipopolysaccharide (LPS)-challenged cows than control glands in untreated cows 3 h after milking (Shangraw et al. 2020). On the other hand, lactose concentrations declined more gradually in the adjacent gland than in the LPS-challenged gland (Shangraw et al. 2020). This decline in lactose may partially explain the reduced milk yields because lactose is the major osmolyte in milk and a key driver

of milk volume. However, small changes in milk fat and lactose concentrations are still poorly explained. For example, a change in lactose content could reflect decreasing secretory rates (Shuster et al. 1991b) or increasing tight junction permeability (Stelwagen et al. 1999). One way to determine how milk fat, lactose, and other milk components are regulated in non-inflamed glands adjacent to an inflamed mammary gland is by studying mammary gene expression.

In our previous experiments, we identified several systemic factors that appear to play a role in regulating gene expression and reducing milk production in adjacent glands. The earliest response, 3 h after an immune challenge, implicated pro-inflammatory cytokines as mediators of the effects on adjacent glands. Pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α) and IL-1 β , are reported to affect the expression of genes in the lactose synthesis pathway and induce degradation of glucose transporters (Kobayashi et al. 2016). By 12 h, additional gene signatures in our previous study suggested that glucocorticoids and oxidative stress could also be involved in the reduced milk yields (Shangraw et al. 2021). Both of these mediators are immunomodulatory and could influence lactation; however, it remains debatable whether these observed changes in gene expression are the direct effect of systemic mediators on secretory tissue or the result of an influx of activated immune cells to all glands (Mitterhuemer et al. 2010). To our knowledge, there have been no investigations involving LPS-induced mastitis challenge that assessed gene expression in the adjacent mammary glands earlier than 3 h and prior to changes in milk composition. Thus, to obtain a more detailed timeline of early events without invasive sampling that could alter responses, we decided to isolate RNA from milk fat rather than mammary tissue.

Milk fat presents a unique and noninvasive source of RNA for studying mammary gene expression. Secreted milk fat globules from the mammary epithelial cells (MECs) often contain a cytoplasmic crescent formed when the membrane is pinched off and released from the cell. These milk fat globules contain RNA that is representative of the MEC transcriptome in healthy goats (Brenaut et al. 2012), cows (Cánovas et al. 2014), and water buffaloes (Sharma et al. 2018). Moreover, milk fat RNA can be compared against the milk it was isolated from, allowing subsequent analyses to be examined in the context of simultaneous changes in milk yield and composition, including potential effects of infiltrating immune cells (Brenaut et al. 2014). Thus, milk fat presents an opportunity for more frequent sampling of RNA without compromising mammary function in contrast to the collection of RNA from multiple mammary tissue biopsies.

The anatomical position of an individual mammary gland in relation to an inflamed gland in the udder might also affect the response of the non-inflamed gland. For example, infusing a single gland with *Escherichia coli* resulted in increased blood flow to both glands on the challenged side but not to the glands of the control side (Potapow et al. 2010). Moreover, Kimura et al. (2005) reported that zymosan-activated lymphocytes can migrate out of one mammary gland into blood and lymph and be subsequently recovered from a different mammary gland. Thus, increased blood flow coupled with the interchange of activated immune cells and release of proinflammatory cytokines from the inflamed gland might be expected to expose an ipsilateral mammary gland to more inflammatory mediators than those to which a contralateral gland is exposed. More broadly, incidence rates of intramammary infections and high SCC can also be correlated within cows by mammary gland position, with higher rates in rear than front quarters

and in right than left quarters (Adkinson et al. 1993; Barkema et al. 1997). In this way, an ipsilateral mammary gland might be more affected than a contralateral gland. If so, this would hold serious implications for assuming independence of individual mammary glands in experimental designs.

We hypothesized that genes we previously identified as being differentially expressed in mammary tissue biopsies of neighboring glands in an LPS challenge model (Shangraw et al. 2020) would be differentially expressed in RNA isolated from milk fat using a similar experimental design. These genes are associated with milk synthesis, immune, and stress pathways, and may be causally involved in mediating the systemic effects under study. Alternatively, they may serve as useful biomarkers. We further hypothesized that the non-inflamed gland ipsilateral to the mastitic gland would show greater changes in milk composition and gene expression than the contralateral gland. The objectives of this experiment were to (1) determine if genes previously identified as differentially expressed in mammary tissue are similarly differentially expressed in milk fat, (2) confirm and extend the temporal response of non-inflamed glands to localized mastitis via more frequent sampling than our previous study, and (3) determine if the position of a non-inflamed gland in relation to the mastitic gland causes a different response in milk composition and gene expression.

Materials and methods

Animals

All procedures involving animals were approved by the University of Missouri Institutional Animal Care and Use Committee (Protocol #9283). Pregnant Holstein cows ($n = 8$) were selected based on low cow-level SCC <200,000 cells/mL. Parity ranged from one to three lactations (1.75 ± 0.25 ; mean $\pm$ SEM). All cows were in late lactation (297 ± 21 DIM) averaging 27.1 ± 1.6 kg milk/d. Cows were moved from free-stall housing to a shaving-bedded pack barn with free access to water and a total mixed ration formulated to meet or exceed lactational requirements. All cows were milked in the parlor twice daily at 0630 and 1830.

Design

Three days prior to the experiment, milk SCC was determined per quarter. If all quarters had an SCC <100,000 cells/mL, the mammary gland (Q_{LPS}) to be challenged with an intramammary infusion of LPS was randomly assigned. However, for three cows with a greater SCC (range: 293–583,000 cells/mL) in one quarter, Q_{LPS} was assigned to the quarter diagonal to the high SCC quarter. Mammary glands contralateral (Q_C), ipsilateral (Q_I), and diagonal (Q_D) to Q_{LPS} remained unchallenged (Fig. 1). Each mammary gland position (left front, left rear, right front, right rear) was selected for LPS challenge in two of the eight cows.

Milk sampling and challenge

Total milk yields were recorded per cow the day before (d-1) and day of (d-0) the LPS challenge. On d-1, immediately after the a.m. milking (0 h), at 1, 3, and 6 h, and immediately before the p.m. milking (12 h), a strip milk sample (10–30 mL) was collected from all mammary glands for determination of milk composition. An additional 20–40 mL strip sample of foremilk was collected for isolation of RNA from milk fat from only Q_{LPS} at 1, 3, and 12 h.

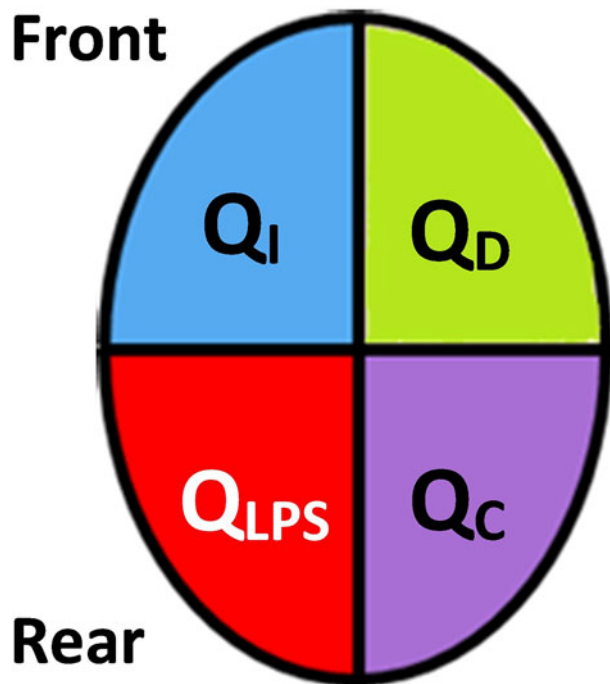


Figure 1. Example layout for mammary gland designations. One mammary gland was randomly assigned to receive an intramammary injection of 50 μ g LPS (Q_{LPS}). The ipsilateral (Q_I), contralateral (Q_C), and diagonal (Q_D) mammary glands remained unchallenged. The gland diagonal to Q_{LPS} was not analyzed for gene expression.

On d-0, immediately after a.m. milking, a 0 h strip sample of hindmilk was collected from all mammary glands for milk composition. Then, the Q_{LPS} teat was scrubbed twice with 70% ethanol prior to inserting a sterile teat cannula and injecting 50 μ g LPS (*E. coli* serotype O55:B5, L6529, Sigma-Aldrich) in 5 mL 0.9% saline. Injections for all cows were completed within 11 min. Strip samples of foremilk for milk composition (all quarters) and for RNA isolation (Q_{LPS} , Q_C , and Q_I) were collected at 1, 3, 6, and 12 h post-challenge. After collecting 12 h samples, cows were milked. Additional strip samples were collected from all mammary glands for milk composition immediately after p.m. milking (12.5 h) and of foremilk just prior to a.m. milking (24 h) the next day. Our milk sampling schedule was designed to optimize milk fat sampling for RNA. Differences in milk composition between fore and hindmilk have been described (Bruckmaier et al. 2004). All milk composition samples were preserved with bronopol and stored at 4°C until shipment to Mid-South Dairy Records (Springfield, MO) for determination of fat, total protein, lactose, and SCC. Rectal temperatures were taken on d-0 at 1, 3, 6, 12, and 24 h post-challenge.

Milk fat RNA isolation and extraction

Methods for RNA isolation from milk fat were adapted from Brenaut et al. (2012). Within 5 min of collection, milk was centrifuged at 2,500 g for 15 min at 4°C to separate milk fat. Then, 4–800 mg of the cream layer was transferred into a 5-mL sterile tube containing 2 mL TRIzol solution (Invitrogen, Life Technologies, Carlsbad, CA) using a clean metal spatula, whereupon the mixture was vortexed thoroughly to disrupt and mix samples, then immediately placed on dry ice before storage at –80°C. Most fat samples from Q_{LPS} on d-0 contained stringy clumps and

did not fully mix despite prolonged vortexing. Sample processing was completed within 1 h after milk collection.

Total RNA was extracted from milk fat using a two-step TRIzol isolation and column purification process. Samples were thawed on ice if homogeneous or, if clots were evident, homogenized while frozen using a Tissue Tearor homogenizer (BioSpec, Bartlesville, OK). Next, samples were vortexed for 1 min, then centrifuged at 12,000 g for 10 min at 4°C to separate lipids. Avoiding the lipid layer and any cell debris, the clear pink infranatant was aspirated and divided equally into two RNase-free 2-mL microfuge tubes (~1 mL supernatant each) and left for 5 min at room temperature. Next, after adding 200 μ L chloroform to infranatant, tubes were vigorously shaken for 10 s, incubated on ice for 2–3 min, and centrifuged for 12 min at 12,000 g at 4°C. For each sample, the clear aqueous layers were pooled in a new RNase-free tube, to which an equal volume of 70% ethanol was added. RNA was purified from this mixture using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions. RNA was eluted from the final column with 30 μ L of nuclease-free water and stored at –80°C.

cDNA synthesis

RNA concentration was determined by NanoDrop (Thermo Scientific, Waltham, MA). Samples were diluted to 200 ng/ μ L, if necessary, prior to DNase treatment. Per 20 μ L reaction, 17 μ L sample was mixed with 1 μ L DNase I and 2 μ L 10 $\times$ buffer (Ambion, Austin, TX). After incubating at 37°C for 30 min, 2 μ L 110 mM EDTA was added and the enzyme was inactivated by heat treatment at 75°C for 10 min. After DNase treatment, the A260/A280 ratio averaged 2.08 ± 0.03 for all samples. In addition, because we anticipated some dilution of RNA from milk fat with RNA from infiltrating immune cells, we assessed a single representative sample of RNA from an untreated and an LPS challenged gland from one cow by Fragment Analyzer (Advanced Analytical Technologies Inc., Ankeny, IA); the RINs were 1.6 and 6.5 for the untreated and challenged glands, respectively. Treated RNA was stored at –80°C.

For cDNA synthesis, samples were diluted to 50 ng/ μ L with RNase-free water. Each 40 μ L reaction contained 20 μ L sample (1 μ g RNA) and 20 μ L master mix (High Capacity cDNA, Applied Biosystems, Foster City, CA): 4 μ L 10 $\times$ buffer, 1.6 μ L 25 $\times$ dNTPs, 4 μ L random primers, 2 μ L MultiScribe reverse transcriptase, and 8.4 μ L nuclease-free water. The reaction was performed in a T100 thermal cycler (BioRad Laboratories, Inc., Hercules, CA), programmed to 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min, then held at 4°C. After synthesis, 30 μ L cDNA was diluted with 120 μ L 10 mM Tris for storage at –20°C.

Quantitative PCR

Genes of interest and reference genes were selected based on results from our previous study (Shangraw et al. 2021) along with *CD18* and *CD68*, which are markers of neutrophils and macrophages, respectively (Brenaut et al. 2012). Primers were designed across exon–exon junctions using Primer-BLAST (Ye et al. 2012), as summarized in Table 1, and were purchased from Integrated DNA Technologies (Coralville, IA). Primer efficiency was validated using a 5-point (5 ng–5 pg) dilution series of a cDNA pool from all samples. A no template control was run as a negative control.

For real-time quantitative polymerase chain reaction (PCR), samples were diluted 1:2 (final dilution 1:10) with RNase-free water. Per gene, all samples were analyzed in duplicate against a standard curve made from the pooled cDNA, and plated in

Table 1. Primer list for genes expressed in milk fat

Gene name ^a	Forward primer (F) 5' → 3'	Reverse primer (R) 5' → 3'	GenBank accession number	Product size, bp	Primer concentration, nM
<i>FASN</i>	CATTCAGGTACGTGAGGAAGAG	CCAGTGATGATGTAGCTCTTGT	NM_001012669.1	114	400
<i>CSN2</i>	CCTGGTGAGATTGTGGAAG	GGAAGGGATAGACTAGAGACTG	NM_181008.2	163	800
<i>LALBA</i>	TCCTGGTAGGCATCCTATTC	ACGTGGTACAGACCCATT	NM_174378.2	126	800
<i>NFKBIA</i>	GAGACTCGTTCCTGCACTTAG	AGCGATTCTGGCTGGTTAG	NM_001045868.1	161	200
<i>PTX3</i>	GCAGGTTGTGAAACAGCCAT	CCCAAATGCAGGCACTGAAA	NM_001076259.2	109	200
<i>MTHFD2</i>	GCGTGGGAATCAACAGTGAGA	GAACAAGGAGGCCGTCTACA	NM_001075755.2	114	200
<i>LPIN1</i>	GTACAAGCAAGTGGGAGTGT	CTCACATACGAGGAGATGTTGG	NM_001206156.2	102	200
<i>FKBP5</i>	CAGATCTCCATGTGCCAGAAA	TTACTGGCCTCTTCTTTGC	NM_001192862.1	113	400
<i>HK1</i>	CCGAAGTGAAGGATGACCAAG	AAATCTCCCTTTCTGAGCCA	NM_001012668.2	205	400
<i>FOLR1</i>	GCTGTGCCTTTTAGTGTGTGTG	TGGGCTTCTATGCTGGTGTT	NM_001206532.1	183	400
<i>TSC22D3</i>	GCTCTTCTCCACAGTGCCCT	GTTCTTCTCCACAGCTCCC	NM_001103342.1	154	400
<i>RPL4</i>	AGCGCTGGTCATGTCTAAAG	CTCATTCGCTGAGAGGCATAG	NM_001014894.1	168	200
<i>RPS23</i>	GTGTGTCAGGGTTCAGCTAAT	ACTCCAGGAATGTACCAAC	NM_001034690.2	150	200
<i>CD18</i>	CAGATCAACGTCCCGATCAC	CGCAGTTCTTCCCGATGTAG	NM_175781.1	235	400
<i>CD68</i>	GATTCAAGCAGGATCCACTG	GGCAGCAAGATGGACTTATC	NM_001045902.1	292	400

^a*FASN*: fatty acid synthase; *CSN2*: beta casein; *LALBA*: alpha lactalbumin; *NFKBIA*: nuclear factor- κ B inhibitor alpha; *PTX3*: pentraxin 3; *MTHFD2*: methylenetetrahydrofolate dehydrogenase 2; *LPIN1*: lipin 1; *FKBP5*: FK506 binding protein 51; *HK1*: hexokinase 1; *FOLR1*: folate receptor alpha; *TSC22D3*: TSC22 domain family protein 3; *RPL4*: ribosomal protein L4; *RPS23*: ribosomal protein S23; *CD18*: integrin beta chain-2; *CD68*: cluster of differentiation 68.

a 384-well plate. Each amplification reaction contained 5 μ L PerfeCTa SYBR Green SuperMix (QuantaBio, Beverly MA), 2 μ L 50:50 mix of forward and reverse primers with an optimized final primer concentration of 200–800 nM (see Table 1), 1 μ L water, and 2 μ L standard or sample. Plates were run in a C1000 Touch thermal cycler (BioRad Laboratories, Inc., Hercules, CA) using the following cycling protocol: polymerase activation at 95°C for 3 min, then 40 cycles of 95°C for 15 s (denaturation), 60°C for 20 s (annealing), and 68°C for 20 s (extension), followed by a melting curve from 65°C to 95°C using an incremental temperature increase of 0.5°C every 10 s. Primer efficiency values ranged from 0.93 to 1.15 with standard curve R^2 ranging from 0.98 to 0.99. Quantification cycle values (C_q) were imported into Microsoft Excel for further analyses. Arbitrary cDNA concentrations were determined from the C_q of each sample and the slope of the standard curve by the following equation: $10^{((C_q - b)/m)}$, where b is the intercept and m is the slope. To normalize expression, the arbitrary amount of the target gene was divided by the geometric mean amount of two reference genes, *RPL4* and *RPS23*, in the same sample.

Statistics

All statistics were run using SAS 9.4 (SAS Institute Inc., Cary, NC). For each variable, data were tested for normality using the PROC UNIVARIATE procedure. Rectal temperature was analyzed as a one-way analysis of variance (ANOVA) using the PROC GLIMMIX procedure with repeated measures, with time as the fixed effect and cow the subject of repeated measures. Milk yield was analyzed as a two-way ANOVA using the PROC GLIMMIX procedure with repeated measures, with time and day as the fixed effects and cow the subject of repeated measures.

Milk composition data were analyzed as a three-way ANOVA using the PROC GLIMMIX procedure. SCC data were $\log_{10}$ transformed for analysis. The statistical model included the fixed

effects of day, treatment, time, and their interactions. Quarter within cow was the subject of repeated measures. For variables with missing data, degrees of freedom were calculated using the Kenward–Roger approximation option.

For PCR gene expression, data were analyzed as a two-way ANOVA model using the PROC GLIMMIX procedure with repeated measures to determine the main effects of time, treatment, and their interaction, where quarter within cow was the subject of repeated measures. Where necessary, gene expression data were $\log_{10}$ - or square root- transformed to achieve a normal distribution. For d-1 only, statistics were run on the missing 6 h samples using the average of 3 and 12 h for each cow. For this reason, no datapoints are presented for samples at 6 h on d-1. When the treatment effect or interaction was significant, preplanned comparisons of interest were (1) Q_{LPS} against d-1, Q_I or Q_C , (2) Q_I or Q_C against d-1 expression, and (3) Q_I against Q_C . For genes expressed by Q_{LPS} at magnitudes far greater than all other treatments (*NFKBIA*, *PTX3*, *FKBP5*, *MTHFD2*), a second dataset excluding Q_{LPS} was run to compare Q_I and Q_C against each other and to d-1 expression. All variables were modeled using the covariance structure that resulted in the smallest Akaike's information criterion. Degrees of freedom were calculated using the Kenward–Roger approximation option and P -values adjusted using the adjdfe = row option. Data for *MTHFD2* were non-normal even after transformation and the pre-planned comparisons stated above were run using the Wilcoxon signed-rank test.

For all variables, treatment and interaction effects were considered significant at $P \leq 0.05$ and results are reported as untransformed LSmeans and SEM unless specified.

Results

Cows displayed signs of clinical mastitis (fever, sickness behaviors, altered milk) 3 h after intramammary LPS infusion. Rectal

temperature peaked at 6 h ($40.2 \pm 0.18^\circ\text{C}$; $P < 0.001$), returned to pre-challenge temperatures at 12 h, and was lower than pre-challenge at 24 h ($37.5 \pm 0.18^\circ\text{C}$; $P < 0.01$). Average combined milk yield of all quarters was significantly lower post-challenge compared to the a.m. yield just prior to challenge: at 12 h post-challenge, milk yield was $71 \pm 8\%$ ($P < 0.01$) of 0 h yields and partially recovered by 24 h to $82 \pm 6\%$ ($P = 0.03$).

Milk composition

Milk fat concentration post-challenge was significantly lower only in Q_{LPS} compared to d-1 ($P < 0.05$). Relative to d-1 samples, milk fat concentrations in Q_{LPS} were significantly lower at 3 and 6 h post-challenge but were not different immediately before the 12 h milking (Fig. 2A). Fat content of Q_{LPS} was also significantly lower than that of Q_C and Q_I at 3 h (Fig. 2I; $P < 0.05$) and immediately after the 12 h milking ($P < 0.001$). There was no difference in milk fat concentration over time in Q_C or Q_I post-challenge compared to d-1, Q_D , or to each other (Fig. 2C, E and I).

Milk total protein concentration post-challenge was significantly greater in Q_{LPS} compared to d-1 (Fig. 2B; $P < 0.001$). Relative to d-1, Q_{LPS} showed two peaks, the first apparent at 3 h and plateauing to 6 h, then the second peak after the 12 h p.m. milking post-challenge which persisted to 24 h (Fig. 2B). There was no difference in total protein concentration over time in Q_C or Q_I post-challenge compared to d-1, Q_D , or to each other (Fig. 2D, F and J; $P > 0.20$).

Lactose concentration post-challenge declined significantly in Q_{LPS} by 3 h compared to d-1 and remained low thereafter (Fig. 3A). Relative to d-1, lactose concentration in Q_I was not different except at 12 h just before the p.m. milking, when lactose was lower in Q_I (Fig. 3C; $P < 0.05$). Lactose was also numerically lower in Q_C compared to d-1 at 3, 6, and 12 h before p.m. milking, though only significantly different at 6 h (Fig. 3E; $P = 0.05$). Q_C and Q_I were not different from each other or Q_D (Fig. 3I).

Milk SCC post-challenge increased significantly at 3 h in Q_{LPS} compared to d-1 (Fig. 3B; $P < 0.05$). Cells increased exponentially in Q_{LPS} , reaching the maximum detection limit of 10 million cells/mL at 6 h. Except at 12 h before p.m. milking, Q_{LPS} remained significantly greater than all other quarters thereafter (Fig. 3J). There was also a significant but smaller magnitude increase in the SCC of Q_C at 6 h compared to d-1 (Fig. 3F; $P < 0.01$). At 12 h, the SCC of both Q_I and Q_D , but not Q_C , were significantly greater than d-1 (Fig. 3D, F and H). Both Q_I and Q_D followed a similar pattern after the 12 h p.m. milking, with significantly lower SCC immediately after milking compared to Q_C (Fig. 3J; $P < 0.001$).

Milk fat RNA

Yields of RNA isolated from milk fat of Q_I and Q_C glands were mostly low ($<5 \mu\text{g}$ RNA/g of milk fat) and displayed the characteristics of exosomal RNA (Fig. 4A). However, milk fat from Q_{LPS} , particularly at 6 and 12 h post-challenge, yielded up to 10 times more RNA per gram of milk fat with an RNA profile resembling that of somatic cells (Fig. 4B), suggesting possible contamination of the RNA in milk fat with RNA from leukocytes infiltrating the gland. To assess this, we measured gene expression of the immune cell markers *CD18* and *CD68*. Relative expression of both markers was low and stable over 12 h on d-1 before the LPS challenge, then increased significantly in Q_{LPS} at 6 and 12 h post-challenge compared to d-1 (Fig. 5, $P < 0.01$). Additionally, the mean expression of these genes over time was greater in Q_I than either d-1 or

Q_C . However, only the difference between Q_I and d-1 approached statistical significance ($P = 0.08$).

Genes selected as markers for synthesis of milk fat (*FASN*), casein (*CSN2*), and lactose (*LALBA*) were significantly downregulated in Q_{LPS} compared to d-1 and both Q_C and Q_I (Fig. 5). In Q_{LPS} , compared to d-1, all milk synthesis markers were significantly downregulated at 6 h post-challenge ($P < 0.05$) and continued to decline in expression at 12 h. For *FASN* expression, Q_I and Q_C were not significantly different than d-1. There were significant differences in *CSN2* expression at 1 h post-challenge in Q_I and Q_C compared to d-1; however, this appears to be driven by unaccountably higher expression of *CSN2* at 1 h on d-1 in relation to all other timepoints on d-1. Otherwise, *CSN2* expression in Q_I and Q_C was not significantly different compared to d-1. Expression of *LALBA* in Q_I , but not Q_C , was significantly lower than d-1 at 12 h post-challenge ($P < 0.05$). Lastly, the expression of all these genes related to milk component synthesis was not significantly different between Q_I and Q_C .

Several genes of interest were differentially expressed in Q_{LPS} , Q_C , and Q_I , with most revealing a significant time $\times$ treatment interaction. For *LPIN1*, expression in Q_{LPS} was downregulated compared to d-1 at 12 h post-challenge (Fig. 6; $P < 0.05$). Intriguingly, *LPIN1* expression in both Q_C and Q_I was upregulated at 12 h post-challenge compared to Q_{LPS} ($P < 0.001$). Q_C also expressed significantly greater amounts of *LPIN1* relative to d-1 at 12 h post-challenge ($P < 0.05$) but was not different compared to Q_I . The expression of *HK1* was significantly higher in Q_{LPS} than either Q_C or Q_I at 6 and 12 h post-challenge (Fig. 6; $P < 0.05$). Further, both Q_C and Q_I expressed significantly greater levels of *HK1* compared to d-1 at 12 h post-challenge ($P < 0.05$) but were not different from each other ($P > 0.25$).

Both immune-regulatory genes, *NFKBIA* and *PTX3*, were most highly expressed in Q_{LPS} (see Fig. 6 insets), being more than 2 and 4 orders of magnitude higher than all other glands at 6 h post-challenge, respectively. *NFKBIA* was more highly expressed overall in Q_I compared to d-1 and to Q_C ($P < 0.01$). Although the time $\times$ treatment interaction for *NFKBIA* was not significant ($P = 0.26$), expression in both Q_C and Q_I increased over time, being 20 and 50 times more highly expressed at 12 h compared to the same time pre-challenge, respectively. *PTX3* was expressed more highly in Q_I and Q_C compared to d-1 at 3, 6 and 12 h post-challenge ($P < 0.001$). Although minor compared to the response in Q_{LPS} , Q_I and Q_C expressed 32 and 270 times more *PTX3* at 12 h compared to the same time pre-challenge, respectively. Additionally, *PTX3* expression was significantly upregulated in Q_I compared to Q_C at 6 h post-challenge ($P < 0.05$) and continued increasing to 12 h.

The final four genes related to two pathways of interest – glucocorticoid response (*FKBP5*, *TSC22D3*) and one-carbon metabolism (*MTHFD2*, *FOLR1*). Similar to the immune-regulatory genes, expression of *FKBP5* and *MTHFD2* significantly peaked in Q_{LPS} at 6 h compared to d-1 and both Q_C and Q_I (see Fig. 6 insets). Compared to d-1, *FKBP5* expression at 3 h was slightly but significantly greater in both Q_C ($P < 0.05$) and Q_I ($P = 0.06$), then increased markedly and plateaued between 6 and 12 h, respectively ($P < 0.001$). There was no difference between Q_C and Q_I . *MTHFD2* was also significantly upregulated in both Q_C and Q_I at 12 h compared to d-1 ($P < 0.05$). On the other hand, not all genes in these pathways showed differential gene expression in Q_C and Q_I . For *TSC22D3*, only Q_{LPS} was significantly downregulated compared to Q_C and Q_I ($P < 0.01$). The expression pattern of *FOLR1* appeared remarkably similar to

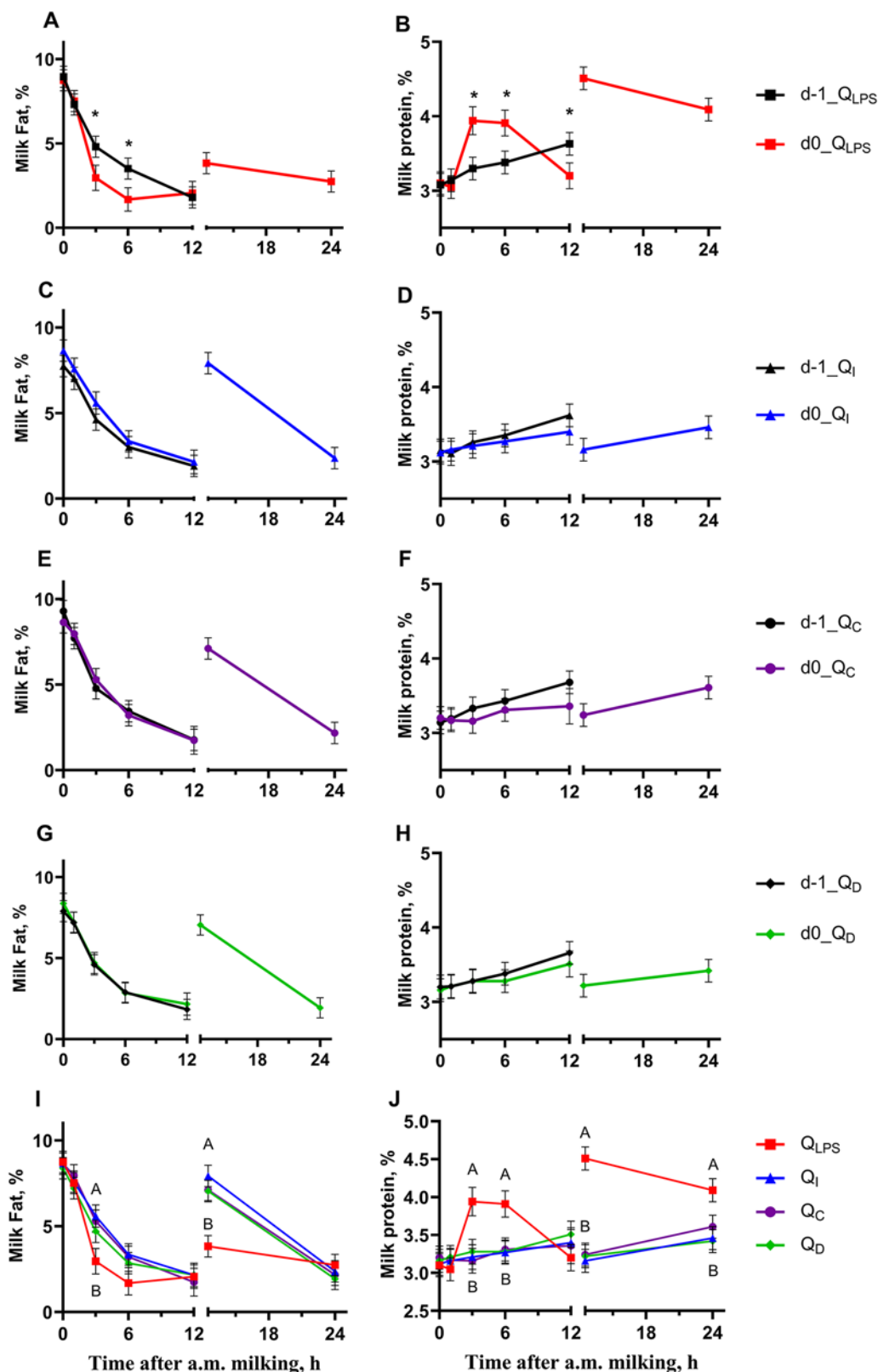


Figure 2. Milk fat and protein composition of LPS-treated and untreated adjacent mammary quarters. Strip milk samples collected on d-1 (pre-challenge) and d-0 (LPS challenge) immediately after a.m. milking, at 1, 3, and 6 h relative to a.m. milking, at 12 h relative to a.m. milking, which was immediately before p.m. milking, immediately after p.m. milking, and before d-1 a.m. milking (24 h). The break on the x-axis represents the 12 h milking. Legend represents: Q_{LPS} , injected with 50 μ g LPS at 0 h on d-0; Q_I , untreated ipsilateral gland to Q_{LPS} ; Q_C , untreated contralateral gland to Q_{LPS} ; Q_D , untreated mammary gland diagonal to Q_{LPS} . Percentages of milk fat (A, C, E, G, I) and total protein (B, D, F, H, J). Rows A–H: * = significant difference within time between d-1 and d-0. Rows I and J: A, B = significant treatment difference within time between Q_{LPS} and either Q_C or Q_I . LSmeans $\pm$ SEM reported and significance determined at $P \leq 0.05$.

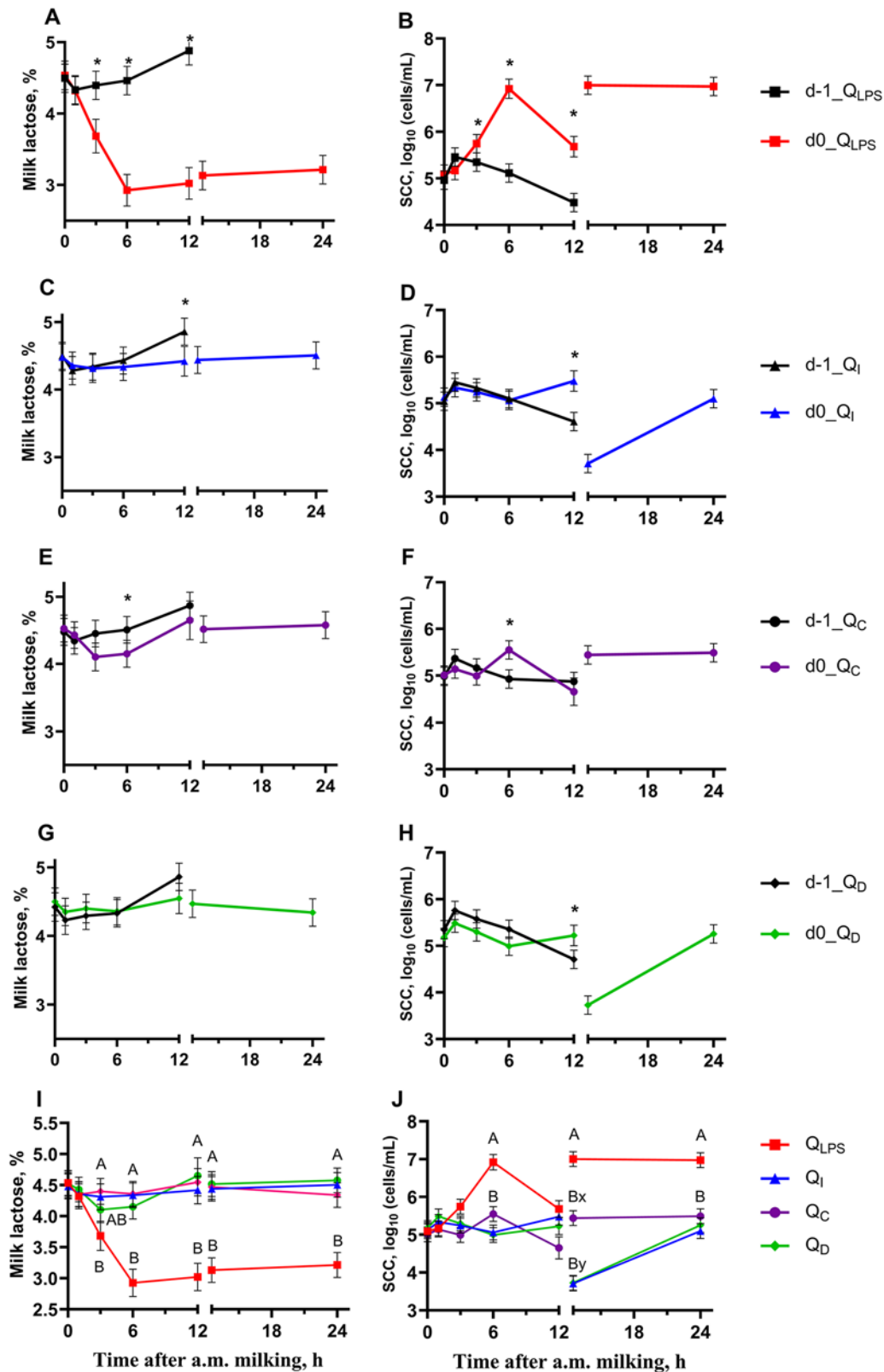
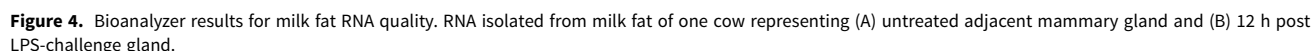


Figure 3. Milk lactose composition and SCC of LPS-treated and untreated adjacent mammary quarters. Strip milk samples collected on d-1 (pre-challenge) and d-0 (LPS challenge) immediately after a.m. milking, at 1, 3, and 6 h relative to a.m. milking, at 12 h relative to a.m. milking, which was immediately before p.m. milking, immediately after p.m. milking, and before d-1 a.m. milking (24 h). The break on the x-axis represents the 12 h milking. Legend represents: Q_{LPS}, injected with 50 μ g LPS at 0 h on d-0; Q_I, untreated ipsilateral gland to Q_{LPS}; Q_C, untreated contralateral gland to Q_{LPS}; Q_D, untreated mammary gland diagonal to Q_{LPS}. Milk lactose percentage (A, C, E, G, I) and log₁₀-transformed SCC (B, D, F, H, J). Rows A–H: * = significant difference within time between d-1 and d-0. Rows I and J: A, B = significant treatment difference within time between Q_{LPS} and either Q_C or Q_I; x, y = significant difference within time between Q_C and Q_I. LSmeans $\pm$ SEM reported and significance determined at $P \leq 0.05$.



The differential expression of *NFKBIA*, *PTX3*, and *CD18* supported our second hypothesis concerning the location of an adjacent gland relative to a mastitic gland. As predicted, the ipsilateral gland showed significantly greater expression of these immune-related genes compared to both the contralateral and pre-challenge samples. NF- κ B inhibitor α (*NFKBIA*) acts as a negative regulator of pro-inflammatory signaling (Kearns et al. 2006) while

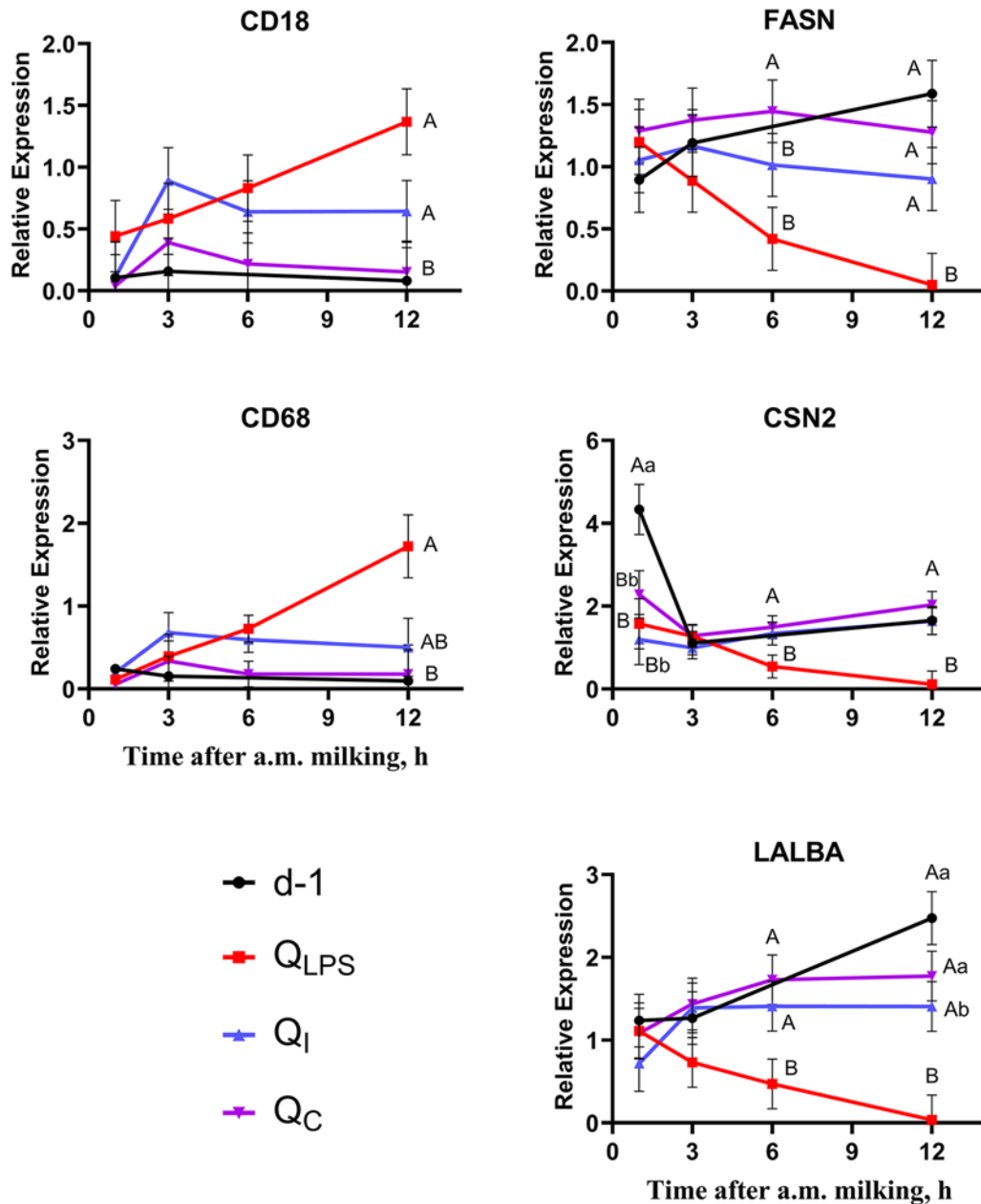


Figure 5. Relative expression of immune cell and milk enzyme genes in LPS-treated and untreated adjacent mammary glands. RNA isolated from milk fat of strip milk samples was collected at the indicated times relative to a.m. milking on d-1 and d-0. Expression of each gene was normalized to the geometric mean expression of two reference genes. Legend: d-1, pre-challenge control from gland designated to receive LPS (Q_{LPS}); Q_{LPS}, gland injected with 50 µg LPS at 0 h on d-0; Q_I, untreated gland ipsilateral to Q_{LPS}; Q_C, untreated gland contralateral to Q_{LPS}. A,B = significant treatment difference within time between Q_{LPS} and either d-1, Q_C or Q_I. a,b = significant difference within time between d-1 and either Q_C or Q_I. No datapoint is shown for missing data on d-1 at 6 h, but comparisons against that datapoint were run using the average of data for d-1 at 3 and 12 h. LSmeans ± SEM reported and significance determined at $P \leq 0.05$. CD18: integrin beta chain-2; CD68: cluster of differentiation 68; FASN: fatty acid synthase; CSN2: beta casein; LALBA: alpha lactalbumin.

pentraxin 3 (PTX3) is an acute phase protein released by activated leukocytes that dampens the immune response by binding P-selectin to prevent neutrophil recruitment (Deban et al. 2010). Higher expression of these genes, in addition to the higher expression of the immune cell markers CD18 and CD68, indicates that ipsilateral glands were either exposed to more pro-inflammatory mediators or were infiltrated by more activated leukocytes than contralateral glands. Notably, the SCC in ipsilateral glands was lower or not different than in contralateral glands at all timepoints post-challenge. Thus, the position of the gland and not the greater

concentration of somatic cells determined the expression of these genes. We note, however, that these differences in gene expression between ipsi- and contralateral mammary glands did not result in differences in milk composition between the two gland positions. During naturally occurring cases of clinical mastitis, gland position did not affect milk composition (Paixão et al. 2017) or differential leukocyte proportions (Schwarz et al. 2011) in milk from non-inflamed mammary glands. Whether or not anatomical position of a mammary gland affects milk, our data further highlight that non-inflamed glands cannot be considered independent when

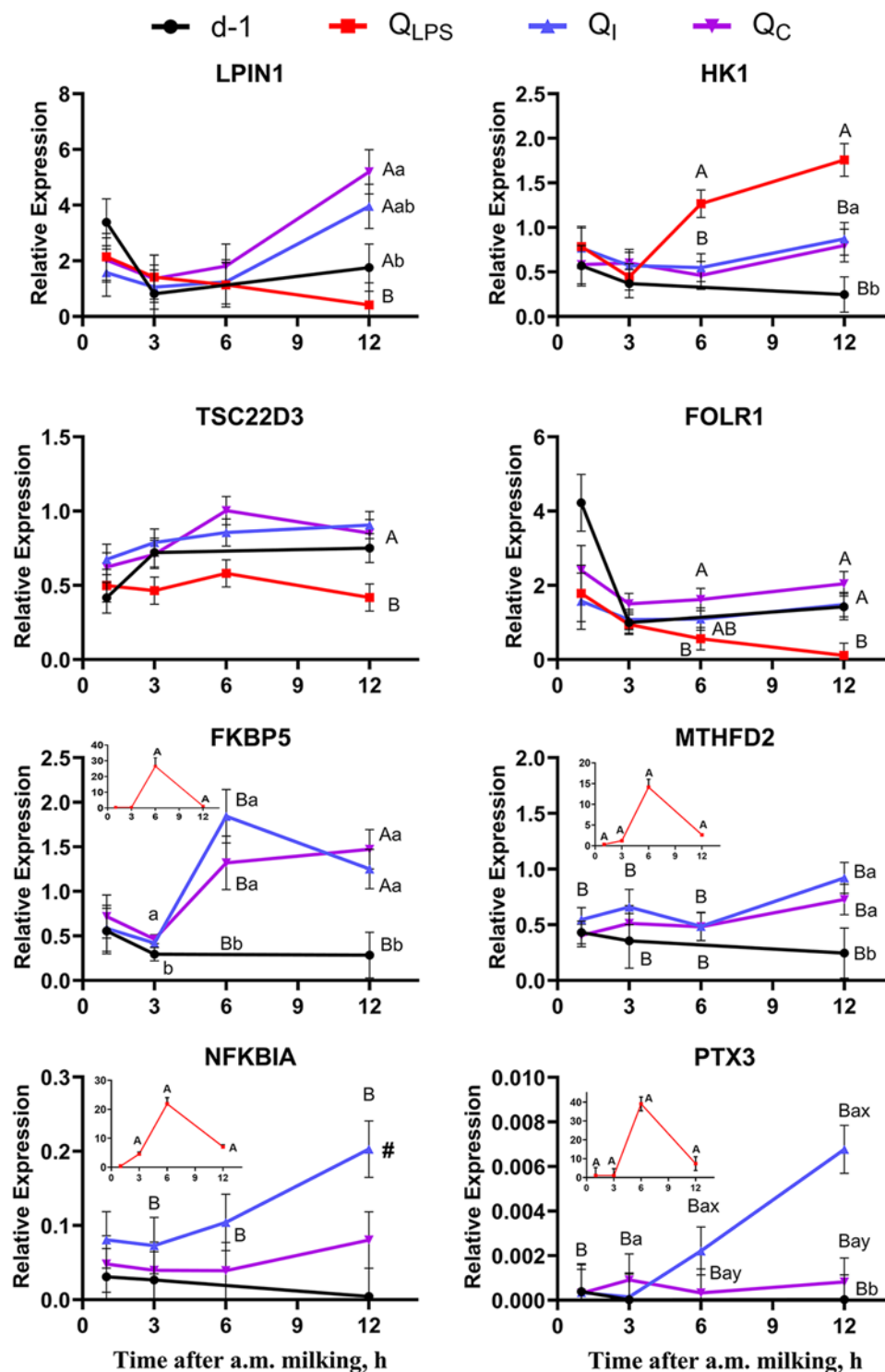


Figure 6. Relative expression of selected genes in LPS-treated and untreated adjacent mammary glands. RNA isolated from milk fat of strip milk samples was collected at the indicated times relative to a.m. milking on d-1 and d-0. Expression of each gene was normalized to the geometric mean expression of two reference genes. Legend: d-1, pre-challenge control from gland designated to receive LPS (Q_{LPS}); Q_{LPS}, gland injected with 50 µg LPS at 0 h on d-0; Q_I, untreated gland ipsilateral to Q_{LPS}; Q_C, untreated gland contralateral to Q_{LPS}. A,B = significant treatment difference within time between Q_{LPS} and either d-1, Q_C or Q_I. a,b = significant difference within time between d-1 and either Q_C or Q_I. x, y = significant difference within time between Q_C and Q_I. # = significant main effect between Q_C and Q_I treatments. Genes with an extreme Q_{LPS} response are graphed with an inset. No datapoint is shown for missing data on d-1 at 6 h but comparisons against that datapoint were run using the average of data for d-1 at 3 and 12 h. LSmeans ± SEM reported and significance determined at $P \leq 0.05$. *LPIN1*: lipin 1; *HK1*: hexokinase 1; *NFKBIA*: nuclear factor-κB inhibitor alpha; *PTX3*: pentraxin 3; *FKBP5*: FK506 binding protein 51; *MTHFD2*: methylenetetrahydrofolate dehydrogenase 2; *TSC22D3*: TSC22 domain family protein 3; *FOLR1*: folate receptor alpha.

- Brenaut P, Lefèvre L, Rau A *et al.* (2014) Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to *Staphylococcus aureus*. *Veterinary Research* **45**, 16.
- Bruckmaier RM, Ontsouka CE and Blum JW (2004) Fractionized milk composition in dairy cows with subclinical mastitis. *Veterinariai Medicina Czecho-slovakia* **49**, 283–290.
- Cánovas A, Rincón G, Bevilacqua C *et al.* (2014) Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-Sequencing. *Scientific Reports* **4**, 5297.
- Deban L, Russo RC, Sironi M *et al.* (2010) Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nature Immunology* **11**, 328–334.
- Ducker GS and Rabinowitz JD (2017) One-carbon metabolism in health and disease. *Cell Metabolism* **25**, 27–42.
- Han B, Yuan Y, Liang R *et al.* (2019) Genetic effects of LPIN1 polymorphisms on milk production traits in dairy cattle. *Genes* **10**, 265.
- Igoshin AV, Mishakova TM, Aitnazarov RB *et al.* (2024) Association of three single nucleotide polymorphisms in the LPIN1 gene with milk production traits in cows of the Yaroslavl breed. *Vavilovskii Zhurnal Genetiki i Selektzii* **28**, 117–125.
- Jensen K, Günther J, Talbot R *et al.* (2013) *Escherichia coli*- and *Staphylococcus aureus*-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters. *BMC Genomics* **14**, 36.
- Kearns JD, Basak S, Werner SL *et al.* (2006) I κ B ϵ provides negative feedback to control NF- κ B oscillations, signaling dynamics, and inflammatory gene expression. *Journal of Cell Biology* **173**, 659–664.
- Kimura K, Harp JA, Goff JP *et al.* (2005) Lymphocytes from one side of the bovine mammary gland migrate to the contra lateral gland and lymph node tissue. *Veterinary Immunology and Immunopathology* **108**, 409–415.
- Kobayashi K, Kuki C, Oyama S *et al.* (2016) Pro-inflammatory cytokine TNF- α is a key inhibitory factor for lactose synthesis pathway in lactating mammary epithelial cells. *Experimental Cell Research* **340**, 295–304.
- Kushibiki S, Shingu H, Komatsu T *et al.* (2006) Effect of recombinant bovine tumor necrosis factor- α on hormone release in lactating cows. *Animal Science Journal* **77**, 603–612.
- Li R, Dudemaine P-L, Zhao X *et al.* (2016) Comparative analysis of the miRNome of bovine milk fat, whey and cells. *PLOS ONE* **11**, e0154129.
- Mitterhuemer S, Petzl W, Krebs S *et al.* (2010) *Escherichia coli* infection induces distinct local and systemic transcriptome responses in the mammary gland. *BMC Genomics* **11**, 138.
- Paixão MG, Abreu LR, Richert R *et al.* (2017) Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis. *Journal of Dairy Science* **100**, 7522–7533.
- Potapow A, Sauter-Louis C, Schmauder S *et al.* (2010) Investigation of mammary blood flow changes by transrectal colour Doppler sonography in an *Escherichia coli* mastitis model. *Journal of Dairy Research* **77**, 205–212.
- Romano S, Xiao Y, Nakaya M *et al.* (2015) FKBP51 employs both scaffold and isomerase functions to promote NF- κ B activation in melanoma. *Nucleic Acids Research* **43**, 6983–6993.
- Schwarz D, Diesterbeck US, König S *et al.* (2011) Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *Journal of Dairy Research* **78**, 448–455.
- Shangraw EM, Rodrigues RO, Choudhary RK *et al.* (2021) Hypogalactia in mammary quarters adjacent to lipopolysaccharide-infused quarters is associated with transcriptional changes in immune genes. *Journal of Dairy Science* **104**, 9276–9286.
- Shangraw EM, Rodrigues RO, Witzke MC *et al.* (2020) Intramammary lipopolysaccharide infusion induces local and systemic effects on milk components in lactating bovine mammary glands. *Journal of Dairy Science* **103**, 7487–7497.
- Sharma A, Girdhar Y, Kaur M *et al.* (2018) Evaluation of milk fat as an alternative source of RNA from buffalo milk. *Buffalo Bulletin* **37**, 151–156.
- Shuster DE, Harmon RJ, Jackson JA *et al.* (1991a) Endotoxin mastitis in cows milked four times daily. *Journal of Dairy Science* **74**, 1527–1538.
- Shuster DE, Harmon RJ, Jackson JA *et al.* (1991b) Reduced lactational performance following intravenous endotoxin administration to dairy cows. *Journal of Dairy Science* **74**, 3407–3411.
- Shuster DE, Harmon RJ, Jackson JA *et al.* (1991c) Suppression of milk production during endotoxin-induced mastitis. *Journal of Dairy Science* **74**, 3763–3774.
- Stelwagen K, Farr VC and Mcfadden HA (1999) Alteration of the sodium to potassium ratio in milk and the effect on milk secretion in goats. *Journal of Dairy Science* **82**, 52–59.
- Tavakoli S, Zamora D, Ullevig S *et al.* (2013) Bioenergetic profiles diverge during macrophage polarization: Implications for the interpretation of ¹⁸F-FDG PET imaging of atherosclerosis. *Journal of Nuclear Medicine* **54**, 1661–1667.
- Ye J, Coulouris G, Zaretskaya I *et al.* (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134.