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Isolated perfused udder model for transcriptome analysis in response to *Streptococcus agalactiae*

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Research Article

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Abstract

This study aimed to evaluate the transcriptional changes occurring in isolated perfused mammary alveolar tissue in response to inoculation with *S. agalactiae* and to identify the most affected biological functions and pathways after 3 h. Four udders taken at slaughter from cows with healthy mammary gland were perfused ex situ with warmed and gassed Tyrode's solution. Mammary alveolar tissue samples were taken from the left fore and rear quarters (IQ-inoculated quarters) before inoculation (hour 0) and at 3 h post inoculation (hpi) and at the same times from control right fore and rear quarters (not inoculated: NIQ). A total of 1756 differentially expressed genes (DEGs) were identified between IQ and NIQ at 3 hpi using edgeR package. Within this set of DEGs, 952 were up regulated and mainly involved with innate immune response and inflammatory response, e.g., *CD14*, *CCL5*, *TLR2*, *IL-8*, *SAA3*, as well as in transcriptional regulation such as *FOS*, *STAT3* and *NFKBIA*. Genes down-regulated (804) included those involved with lipid synthesis e.g., *APOC2*, *SCD*, *FABP3* and *FABP4*. The most affected pathways were chemokine signaling, Wnt signaling and complement and coagulation cascades, which likely reflects the early stage response of mammary tissue to *S. agalactiae* infection. No significant gene expression changes were detected by RNA-Seq in the others contrasts. Real time-PCR confirmed the increase in mRNA abundance of immune-related genes: *TLR2*, *TLR4*, *IL-1*β, and *IL-10* at 3 hpi between IQ and NIQ. The expression profiles of *Casp1* and *Bax* for any contrasts were unaffected whereas *Bcl2* was increased in IQ, which suggests no induction of apoptosis during the first hours after infection. Results provided novel information regarding the early functional pathways and gene network that orchestrate innate immune responses to *S. agalactiae* infection. This knowledge could contribute to new strategies to enhance resistance to this disease, such as genomic selection.

Bovine mastitis is one of the most expensive diseases impacting on dairy industry, both in terms of animal welfare and reduced milk yield and quality (Contreras and Rodríguez, 2011). The disease results from the inflammatory response of the mammary gland caused by metabolic and physiological changes, traumas, or more frequently environmental or contagious pathogenic microorganisms (Oviedo-Boyso *et al*., 2007). *Streptococcus agalactiae* is a major obligate pathogen of bovine mammary gland, which is transmitted contagiously within the herd, i.e. from one cow to another during milking (Keefe, 1997). Unlike other important mastitis-causing pathogens, which are generally associated with acute infections, *S. agalactiae* causes chronic subclinical infections (Keefe, 1997). The herd level prevalence is quite high, especially in countries with emerging dairy industries (Duarte *et al*., 2004). Current control strategies involve hygiene practices on the farm and antibiotic administration. However, treatments using antibiotics are coming under increasing public scrutiny due to the possible development of resistant pathogens (Wilson *et al*., 1999). Therefore, there is a need to develop new control strategies through selection of cows with better ability to resist udder disease. To achieve this goal our in-depth understanding of the events that occur during infection must be improved.

The response to intramammary infection (IMI) differs substantially between Gram-negative bacteria, such as *Escherichia coli* and Gram-positive bacteria such as *Streptococcus uberis* and *Staphylococcus aureus* (Petzl *et al*., 2008; Bannernam, 2009; Günther *et al*., 2011). These studies demonstrate the importance of dissecting the immune response of the mammary gland to different etiological agents, using transcriptomic profiling to understand the patho-physiology of mastitis and to develop appropriate control strategies. Nevertheless, despite the great significance of *S. agalactiae* as a causative agent, only a few studies using

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microarray technique have analyzed the changes in expression of cytokines and chemokines from milk samples during mastitis infection by this pathogen (Fonseca *et al.*, 2011, 2015). Furthermore, the majority of studies used infection models that measured the response relatively late after experimental inoculation (Bannernam *et al.*, 2004*a*, 2004*b*; Petzl *et al.*, 2008) or during acute naturally acquired clinical mastitis (Fonseca *et al.*, 2011) when significant necrotic damage may have been evident. However, little is known about the network of genes that orchestrate local mammary tissue responses at the earliest stages of infection by *S. agalactiae*.

An ethical and practical issue about the use of in vivo models represents an important limitation in animal studies and the *ex-vivo* or extracorporeal model approach is a potentially useful alternative. This isolated perfused bovine udder approach has been reported to be suitable in studies of mammary gland metabolism (Hardwick and Linzell, 1960) and pharmacokinetics of substances after intramammary administration (Kietzmann *et al.*, 1993, 2010) and recently to evaluate gene expression (Pinto *et al.*, 2017). However, this method has not been widely used to evaluate the transcriptional response of mammary gland against pathogens. In particular, the use of isolated perfused bovine udder could be highly advantageous to investigate the early stage response to mastitis infection. It does not require the live animal to be challenged, and may complement in vitro studies.

High-throughput technologies, such as RNA-Sequencing (RNA-Seq) are valuable tools to address complex biological questions, such as the host and bacterial pathogen responses, by simultaneously measuring the expression of the whole transcriptome within a tissue (Richards *et al.*, 2013). Capturing subtle transcriptomic changes can provide identification of candidate genes that can be used as markers for resistance to mastitis in dairy cattle breeding programs. Therefore, we applied the RNA-Seq technique aiming to evaluate the transcriptional profile of the isolated perfused bovine udder in response to challenge with *S. agalactiae* and to identify the most affected gene networks and pathways in mammary alveolar tissue 3 h post-inoculation.

Materials and methods

All animal care and handling procedures followed regulations approved by the animal research ethics committee of Embrapa Gado de Leite (license no. 11/2011).

Experimental animal

Six Girolando dairy cows scheduled for culling and without history of udder diseases were pre-selected from the herd of Campo Experimental José Henrique Bruschi of Embrapa Gado de Leite (Coronel Pacheco, Minas Gerais, Brazil). Before slaughter, the animals were submitted to clinical examination of the udder and the California Mastitis test after milking. At seven and three days prior slaughter, as well as on the day of slaughter, milk samples were collected and submitted to microbiological tests, performed according to NMC (1987) and only the cows that presented negative result to *S. agalactiae* were selected.

Isolated perfused bovine udder, inoculation, and collection of the samples

Within 10–15 min post slaughter, four udders were collected, cooled at 8 °C and blood clots in the vessels of the glands were cleared using heparinized Tyrode solution (137 mM NaCl; 2.70 mM KCl; 1.36 mM $CaCl_2{\cdot}2H_2O$; 0.50 mM $MgCl_2{\cdot}6H_2O$; 0.36 mM $NaH_2PO_4{\cdot}H_2O$; 11.90 mM $NaHCO_3$ and 5.50 mM glucose). The extracorporeal udder system was designed as described by Kietzmann *et al.* (1993) and Ehinger and Kietzmann (2000), using temperature of the perfusion fluid at 37 °C. Methodological details are in the online Supplementary File. The viability of the perfused udders was controlled using biochemical parameters such as lactate dehydrogenase (LDH)-activity, glucose consumption and lactate production in the perfusate (Ehinger and Kietzmann, 2000). Perfusate was sampled at 0 and 3 h post-inoculation (hpi) with *S. agalactiae* and indicated that the perfused bovine udders were viable throughout the experiment as reported by our research group before (Pinto *et al.*, 2017).

The left fore and rear quarters (IQ-inoculated quarters) were inoculated with the same strain of *S. agalactiae* used by Fonseca *et al.* (2015). To prepare the inoculum, the culture was resuspended in 1× PBS (2.7 mM KCl; 2 mM K_3PO_4; 137 mM NaCl; 10 mM NaH_2PO_4) to a final concentration of 100 CFU/ml determined by spectrophotometer. The bacterial suspension was infused into a final volume of 10 ml. The right fore and rear quarters were infused with sterile 1× PBS (NIQ-non-inoculated quarters) and used as control.

Alveolar tissue samples were collected aseptically by biopsy from all four quarters (two IQ and two NIQ) before inoculation (hour 0) and 3 h later using a Tru-Cut needle and stored in RNAlater® (Ambion, Austin, TX, USA) following the manufacturer's instructions at −20 °C until RNA extractions were carried out. The experimental unit was considered to be the individual quarter. Therefore, a total of 16 tissue samples were obtained for the RNA extraction, sequencing and Real-Time PCR analyses.

RNA sequencing

From total RNA, we used the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) for library preparation following the manufacturer's instructions. Libraries were multiplex, 8 libraries per lane, and paired-end sequenced with a HiSeq 2000 analyzer (Illumina Inc.). Sequences fragments were mapped to the annotated bovine reference genome UMD 3.1 using TopHat algorithm according to protocol described by Trapnell *et al.* (2012), following the default TopHat settings. Quality control analysis was performance using FastQC tool (version 0.65) available on Galaxy platform (https://usegalaxy.org/). In short for the assembly procedure, the individual assembly of the transcripts for each sample was performed using the Cufflinks program (Galaxy tool version 2.2.1.0) (Trapnell *et al.*, 2010).

Expression values were calculated by counting for each gene how many aligned reads overlap its exons using Htseq-count (Anders *et al.*, 2014). The significance of gene expression changes across time for a total of four contrasts (0 h IQ × 3 h IQ; 0 h IQ × 0 h NIQ; 3 h IQ × 3 h NIQ; 0 h NIQ × 3 h NIQ) were assessed using edgeR package (Galaxy tool version 0.0.2) (Robinson *et al.*, 2010).

Functional enrichment analyses

The list of differentially expressed genes (DEGs) and regulatory elements was used as target gene list to compare with a background gene list formed by all genes expressed in the mammary tissue. The target vs. background list comparison was

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performance by up-loading these list into DAVID, used to perform functional enrichment (Huang *et al.*, 2009). With DAVID genes were annotated in terms of their known function, gene ontology terms, and pathways. The output of DAVID analyses are overrepresented pathways or ontologies associated with target gene list. Significant results after Benjamini–Hochberg correction for multiple testing are reported. To identify regulatory elements in our data set, we used the AnimalTFDB bovine database (http://bioinfo.life.hust.edu.cn/AnimalTFDB/download_index?tr=Bos_taurus; accessed 10 October 2017), which includes classification and annotation of genome wide TF, transcription cofactors, and chromatin remodeling factors.

Real-time PCR and data analyses

To further investigate the immune response induced by *S. agalactiae*, we selected five genes for validation by real-time PCR because they perform important functions in the immune response (*Bax*—BCL2 associated X, apoptosis regulator; *Bcl2*—B-Cell CLL/Lymphoma 2; *TLR-2*—toll like receptor 2; *TLR-4*—toll like receptor 4; and *IL8*—interleukin 8). Beside these five genes, we evaluated the expression of three other genes not present on the list of DEGs by real-time qPCR based on their important role in inflammatory response to mastitis; (*IL1*β—interleukin β; *IL10*—interleukin 10; and *Casp1*- caspase 1). Methodological details are in the Supplementary File.

Results

RNAseq analysis of mammary alveolar tissue challenged with Streptococcus agalactiae

The number of sequences expressed in the mammary alveolar tissue was 27 404 of which 21 691 transcripts corresponded to annotated genes and 5821 as expressed sequences not yet described. Of note, the number of genes deemed expressed and its splice variants would vary with the use of newer and increasingly annotated versions of the bovine reference genome, which should be available in the future (FAANG Consortium *et al.*, 2015). Just over 6% of expressed genes were differentially expressed in the IQ *vs.* NIQ 3 h after inoculation with *S. agalactiae*. Of the 1756 DEGs, 952 were up-regulated and 804 were down-regulated between IQ and NIQ (online Supplementary Table S3). Table 1 shows the top 10 genes up-regulated (9.30 to 5.77-fold). These genes play roles in immune response during infection including *CD14* (CD14 molecule), *CCL5* (chemokine (C–C motif) ligand 5), *TLR2* (toll like receptor 2), *IL-8* (interleukin-8 (IL-8)), *SAA3* (serum amyloid A3), as well as in transcriptional regulation such as *FOS* (Fos proto-oncogene, AP-1 transcription factor subunit), *STAT3* signal (transducer and activator of transcription 3) and *NFKBIA* (NFKB inhibitor alpha). The most down-regulated genes (−8.90 to −6.19 fold) 3 h after IMI are also shown in Table 1. Most of these are engaged in lipid metabolism (Stearoyl-CoA desaturase—*SCD*, Lipin 1—*LPIN1*, Apolipoprotein C-II—*APOC2*, Fatty acid binding protein 3—*FABP3* and Fatty acid binding protein 4—*FABP4*). We did not detect differences in the 0 h IQ × 3 h IQ, 0 h IQ × 0 h NIQ, 0 h NIQ × 3 h NIQ contrasts.

The GO results for molecular function indicated a marked effect on DNA binding transcription factor activity (16 genes) and genes encoding cofactor zinc binding (41 genes; Table 2). The biological process most enriched identified by DAVID ($P < 0.01$) revealed overrepresentations of genes acting in inflammatory response, innate immune response, negative regulation of apoptosis process and cell proliferation (Table 2). GO analysis revealed that 28 genes including *CD14*, *CD46* (CD46 molecule), *ALOX5* (arachidonate 5-lipoxygenase), *CCL2* (chemokine (C-C motif) ligand 2), *CCL5*, *PTX3* (pentraxin 3) and *SAA3* were involved in inflammatory response and innate immune response (online Supplementary Table S2).

Pathway enrichment analysis with DAVID identified 31 DEGs participated in three KEGG pathways. Among them, 14 genes were enriched in the Wnt signaling pathway and 11 genes encoding complement and coagulation cascade pathway (Supplemental file 2). Chemokine signaling pathway was also overrepresented which included two top up-regulated genes such as *CCL5*, *IL-8* among seven genes belonging in this group (Table 2).

Validation of differentially expressed genes by real-time PCR

Both the RNAseq and real-time PCR analysis revealed variations in the expression of *TLR2* (Table 1 and Fig. 1) and *TLR4* (online Supplementary Table S2 and Fig. 1). There was an increase in the mRNA abundance of *TLR2* and *TLR4* at 3 hpi with *S. agalactiae* relative to 0 h.

As defined by the RNAseq analysis, all immune-associated genes tested in real-time PCR confirmed no differences in gene expression before inoculation of *S. agalactiae* (0 h) between IQ and NIQ. However, at 3 hpi we observed increase mRNA abundance of *Bcl2* ($P = 0.046$), *TLR2* ($P = 0.037$), *TLR4* ($P = 0.049$), *IL-1*β ($P = 0.010$) and *IL-10* ($P = 0.008$) in IQ compared to NIQ (Fig. 2). *IL-8* mRNA abundance was numerically greater in IQ, but this difference was not significant.

Discussion

The innate immune response is one of the first lines of host defense to infection as it has the capacity to immediately recognize and respond to earliest signs of infection (Bannerman, 2009). So far, the majority of experiments in vivo and in vitro have studied pathogen-specific induction of innate immune response after challenge by *E. coli* (Bannerman *et al.*, 2004*a*; Günther *et al.*, 2010, 2011), *S. aureus* (Petzl *et al.*, 2008) and *S. uberis* (Bannerman *et al.*, 2004*b*; Swanson *et al.*, 2009). In contrast, the transcriptional profile of bovine mammary tissue after challenge with *S. agalactiae* is poorly characterized. Only a few studies have investigated the changes in gene expression in milk samples after IMI with *S. agalactiae* (Fonseca *et al.*, 2011, 2015).

Transcriptome analysis clearly indicated that the mammary alveolar tissue 3 h after inoculation with *S. agalactiae* experienced a wide transcriptional response which encompassed more than 1700 genes, providing new data that complements the in vivo studies during the first 3 h post-inoculation. As expected, no differences in expression were observed between IQ and NIQ before inoculation of *S. agalactiae*. This confirmed that the experimental units (udder quarters) were under the same conditions at the beginning of the experiment.

Transcriptional profiling studies have demonstrated that cells implicated in immune defense (including epithelial cells) respond to bacterial stimuli with transcriptional activation program, which are interpreted as generic 'alarm signal' for infection. Variations on this common transcriptional theme result from pathogen-specific response (Jenner and Yong, 2005). Of the 10 genes highly up-regulated, three are implicated in sequence-specific DNA

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Table 1. List of top 10 up- and down-regulated genes in mammary alveolar tissue 3 h post- inoculation with *Streptococcus agalactiae* from inoculated quarters compared to non-inoculated quarters

Gene symbol	Fold change	*P*-value	Gene symbol	Fold change	*P*-value
Gene not annotated 1	9.30	2.17×10^{-5}	Gene not annotated 4	−8.90	7.20×10^{-7}
Gene not annotated 2	8.48	2.45×10^{-4}	Gene not annotated 5	−7.70	5.33×10^{-6}
FOS	8.08	8.17×10^{-5}	Gene not annotated 6	−7.29	1.23×10^{-4}
STAT3	8.06	4.93×10^{-5}	*SCD*	−7.45	8.66×10^{-6}
NFKBIA	7.80	2.00×10^{-4}	*LPIN1*	−7.23	3.88×10^{-4}
CD14	7.74	1.52×10^{-6}	*FABP4*	−7.18	2.08×10^{-4}
CCL5	7.66	3.67×10^{-4}	*APOC2*	−7.05	1.61×10^{-6}
IL-8	7.33	4.93×10^{-5}	*FABP3*	−6.39	1.56×10^{-5}
SAA3	7.62	1.43×10^{-5}	*DRAM1*	−6.38	1.19×10^{-5}
TLR2	5.77	5.00×10^{-5}	*CD46*	−6.19	7.20×10^{-6}

Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*), signal transducer and activator of transcription 3 (*STAT3*), NFKB inhibitor alpha (*NFKBIA*), CD14 molecule (*CD14*), chemokine (C-C motif) ligand 5 (*CCL5*), interleukin-8 (*IL-8*), serum amyloid A3 (*SAA3*), toll like receptor 2 (*TLR2*), stearoyl-CoA desaturase (*SCD*), lipin 1 (*LPIN1*), fatty acid binding protein 4 (*FABP4*), Apolipoprotein C-II (*APOC2*), fatty acid binding protein 3 (*FABP3*), DNA damage regulated autophagy modulator 1 (*DRAM1*), CD46 molecule (*CD46*).

Table 2. Description of the significant molecular function, biological processes and pathways among the 1756 DEGs between inoculated and non-inoculated quarters at 3 h post-inoculation with *Streptococcus agalactiae*, identified by GO enrichment analyses using *DAVID 6.8*

Category	Genes	*P*-value
Molecular function		
Sequence-specific DNA binding transcription factor activity	16	3.73×10^{-3}
Zinc ion binding	41	1.40×10^{-2}
Biological process		
Inflammatory response	20	3.57×10^{-4}
Innate immune response	10	2.44×10^{-4}
Negative regulation of apoptotic process	16	4.66×10^{-3}
Positive regulation of cell proliferation	13	4.78×10^{-3}
KEGG pathway		
Chemokine signaling pathway	7	3.07×10^{-3}
Wnt signaling pathway	14	4.57×10^{-2}
Complement and coagulation cascades	10	4.99×10^{-2}

DEGs, differentially expressed genes.

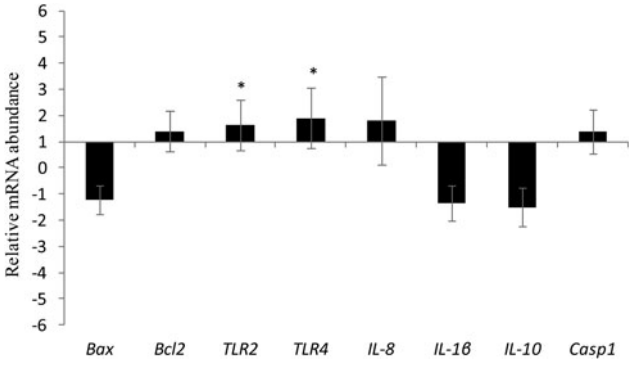


Fig. 1. Relative mRNA abundance of *Bax*, *Bcl2*, *TLR2*, *TLR4*, *IL8*, *IL1*β, *IL10*, *Casp1* genes 3 h post-inoculation with the *Streptococcus agalactiae* strain compared to 0 h (before inoculation) from inoculated quarter (*$P < 0.05$).

binding transcription factors; *NFKBIA*, *FOS* and *STAT3*. The up-regulation of these transcription factors may be underlying the transcriptional changes of the genes involved in innate immune response and inflammatory response. In agreement with our results, Lutzow *et al.* (2008) and Kosciuczuk *et al.* (2017) reported that *NFKBIA* and *FOS* are activated in *Staphylococcus* -induced mastitis whereas Moyes *et al.* (2009) noted the same effect when the infection was caused by *S. uberis*. The signal transducer and activator of transcription 3 (*STAT3*) is known not only as a mediator of acute-phase response but also in pro-inflammatory pathways (Ahyi *et al.*, 2013) and likely to underpin the observed increase in expression of genes encoding the acute-phase protein serum amyloid A3 (SAA3) in *S. agalactiae*-inoculated quarters. In vivo studies conducted by Swanson *et al.* (2009) also showed the induction expression of *STAT3* and *SAA3* genes by *S. uberis*, a Gram-positive bacterium (as is *S. agalactiae*). Our results indicate that these transcription factors have a critical role in the immune response in the udder to *S. agalactiae* at the earliest stage of IMI. Serum amyloid proteins have immunological properties and the SAA3 isoform (M-SAA3) has been reported as being specific to infected quarters compared to healthy quarters (Molenaar *et al.*, 2009). It is possible to suggest that the acute-phase protein SAA3 plays an important role in the local inflammatory response with antibacterial activity during *S. agalactiae* infection.

Perceiving the presence of a pathogen is the first and compulsory step in the innate immune response against an invading pathogen. In our study, a number of genes implicated in innate immune response category, and in particular with TLR signaling have been identified (Table 1 and Supplementary Table S2). This observation confirmed results from Fonseca *et al.* (2015) who observed increased expression of both *TLR2* and *TLR4* in milk samples after experimental in vivo infection with *S. agalactiae.* These findings indicate that *TLR2*, *TLR4* and *CD14* receptors are regulated jointly at the beginning of the innate immune response in mammary alveolar tissue.

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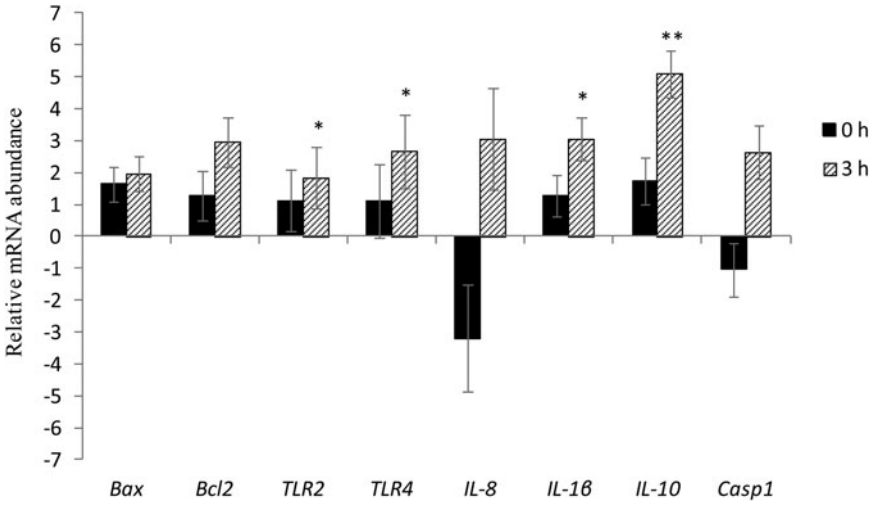


Fig. 2. Relative mRNA abundance of *Bax*, *Bcl2*, *TLR2*, *TLR4*, *IL8*, *IL1*β, *IL10*, *Casp1* genes at time 0 h and 3 h post-inoculation between inoculated quarter and non-inoculated quarter (*$P < 0.05$; **$P < 0.01$).

The activation of toll-like receptor signaling results in expression of several pro-inflammatory cytokines (*IL1*β—*interleukin 1 beta*, *IL-6*—*Interleukin 6*), chemokines (*IL-8*) and others mediators involved in the immune response, cell proliferation and apoptosis (Ibeagha-Awemu *et al*., 2008). Chemokine signaling pathway was one of the major pathways affected by IMI challenge with *S. agalactiae*. Three genes belonging to this pathway were up-regulated; *IL-8*, *CCL5* and *CCL2* (3.22-fold; Supplementary Table S2). IL-8 is a potent chemokine capable of initiating an inflammatory response and plays a primary role in the recruitment of neutrophils into the udder (Persson *et al*., 2003). In our present study, the *IL-8* gene was found up-regulated by RNAseq analysis whereas by real time PCR *IL-8* there was a numerical increase at 3 h post-inoculation. According to previous studies, IMI caused by Gram-negative bacteria provokes a much stronger increase in *IL-8* expression and secretion than Gram-positive pathogens, which tend to induce a weaker modulation and more delayed response (Bannernam *et al*., 2004*b*, 2009; Günther *et al*., 2011). Thus, further investigation will be require to verify the likely differential expression of *IL-8* in response to IMI by *S. agalactiae* within 4 or 6 h post-infection. CCL2 and CCL5 are essential chemokines for recruitment of leukocytes which comprise the second line of cellular immune defense (Schukken *et al*., 2011). Our results showed the importance of these chemokines in the earliest stages of the response to IMI infection by *S. agalactiae*. These results are in accordance with findings of Fonseca *et al*. (2015) who showed that *CCL2* was up-regulated in milk samples after inoculation with the same strain in the udders. Similarly to our results, only a few studies demonstrated that the chemokine CCL5 (also known as RANTES) is strongly expressed in the infected udder as well as in inflammation stimulated MEC (Günther *et al*., 2009, 2011).

IL-1β is known as a key pro-inflammatory cytokine that regulates not only the expression of several immune response genes including others cytokines (e.g. IL-8) and acute phase proteins, but also genes involved in cell proliferation and apoptosis (Lahouassa *et al*., 2007). Because IL-1β is a key component of the innate immune response, we expected induction of this cytokine in the first hours post-inoculation. Although we observed no changes in *IL-1*β expression by RNAseq technique, real-time PCR analysis showed that this gene had increased at 3 hpi in IQ compared to NIQ. This result confirms those found in the literature, as *IL-1* is critical in inflammation of the mammary gland of cows caused by *Staphylococci* (Lutzow *et al*., 2008; Kosciuczuk *et al*., 2017). The reasons why *IL1*β was not identified in the RNA-Seq analysis whereas it showed differential expression when measured by Real time PCR are not clear, but may relate to the differing sensitivities of the two technologies. Real-time PCR is considered to be a highly sequence-specific method for expression analysis (Costa *et al*., 2013).

Because *S. agalactiae* mastitis is usually characterized by a moderate inflammatory response of the udder, we investigated by real time-PCR if IL-10, one of the major anti-inflammatory cytokines, might participate in the modulation of the inflammation in the early stages of response to this pathogen. Despite a mild up-regulation of *IL1*β (3.05-fold) during IMI, the observed 5.10 fold up-regulation of *IL-10* probably hampered inflammatory activity. In agreement with our findings, previous in vivo experiments have found an increase in milk concentrations of IL-10 after IMI with *E. coli* and *S. uberis* (Bannerman *et al*., 2004*a*, 2004*b*) while *S. aureus* infection fails to induce this response in the host (Bannerman *et al*., 2004*a*). In addition, it is worth pointing out that in those cows with the greatest persistent concentrations of *S. aureus* or *S. uberis* in milk, induction of IL-10 is absent or delayed (Bannerman, 2009). Thus it is reasonable to suggest that earlier induction of IL-10 could be beneficial to the ability of the cows to limit bacterial growth and eradicate the pathogen without an exacerbated inflammatory response that can damage the epithelial lining of the mammary gland and lead to reduction in milk production.

The induction of apoptosis by bacterial pathogens is a well-established cellular process (Weinrauch and Zychlinsky, 1999). However, only a few studies have reported direct evidence for involvement of apoptosis during mastitis (Long *et al*., 2001). We found that several genes belonging to the apoptotic process were down-regulated at 3 h post-inoculation with *S. agalactiae*. *DRAM1* (DNA damage regulated autophagy modulator 1) was the most down-regulated followed by other apoptotic related genes such as *Bax* (−2.95-fold), *Casp6* (−2.15-fold), *Casp1* (−3.24-fold). Guan *et al*. (2015) reported that DRAM1 regulates apoptosis through increasing BAX levels. Given that the apoptosis process was among the enriched biological processes in our study, we investigated the expression of *Casp1*, *Bax* and *Bcl2* genes belonging to this group by real-time PCR. Only *Bcl2* gene was

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confirmed as being differentially expressed 3 h post-inoculation. Taken together, these results strongly support the idea that in our isolated perfused model there was no evidence for induction of apoptosis at the early stages of response to *S. agalactiae*- mastitis because no differences were found in the expression of pro-apoptotic genes (*Bax* and *Casp1*) for any contrast when measured by real-time PCR. In addition, the 2.95-fold increase in anti-apoptotic *Bcl2* expression 3 h post-inoculation between IQ and NIQ corroborates this hypothesis.

Our data revealed that the Wnt signaling pathway was affected by *S. agalactiae*-inoculation. We observed that 14 differentially expressed genes were associated with this pathway. Wnt signal transduction has been implicated in inflammation and innate immunity and homeostasis *via* Toll-like receptor and NF-κB pathways (Sun *et al.*, 2005). Recently, Noyes *et al.* (2011) identified potential candidate genes in the Wnt signaling pathway in response to *Trypanosoma congolense* infection in bovine liver, kidney and lymph nodes. Our findings revealed a role of the Wnt signaling pathway during the early immune response to *S. agalactiae*.

The complement and coagulation cascades pathway was among the three most affected pathways. Similarly to our results, a previous study has also found that complement and coagulation cascade pathway was the major affected pathway in mammary tissue naturally infected with *S. aureus* (Huang et al. 2014). The observed up-regulation of *C1QC* (complement C1q C chain) was similar to results from Moyes *et al.* (2009) who observed 1.37 fold—increase in *S. uberis*-infected mammary quarter. The component C1q is the first step in the initiation of the classical pathway of complement cascade which plays an important role in innate immunity (Rainard, 2003). CD46 plays a primary function in the host's response to pathogens by regulating complement activation which is a critical step in the early control of infection (Meiffren *et al.*, 2010). Previous reports showed that pathogen receptor CD46 on the cell surface can trigger cell autophagy to control infection by *Streptococcus* (Cattaneo, 2004; Joubert *et al.*, 2009). Moreover, Wang *et al.* (2014) recently identified a new variant splice designated CD46 transcript variant (*CD46-TV*) that was associated with resistance to mastitis caused by *Streptococcus* in dairy cows. In our study, *CD46* gene was one of the most down-regulated indicating the relevant role of this pathogen receptor in the risk of mastitis caused by *S. agalactiae*.

Another interest finding of the RNA-Seq analysis was that several genes involved with lipid metabolism such as, *SCD*, *LPIN1*, *APOC2*, *FABP3* and *FABP4* were down-regulated in mammary quarters inoculated with *S. agalactiae*. As in the present study, Swanson *et al.* (2009) also observed down-regulation of genes related with lipid metabolism (e.g. *LPIN1*, *APOC2*, *FABP3)* in mammary tissue after IMI with *S. uberis*. This is further supported by Günther *et al.* (2009) who observed marked decreases in mRNA expression of factors associated with lipid metabolism in bovine MEC infected by *E. coli*. The products of these genes are known to be involved in milk fat synthesis (Bionaz and Loor, 2008). Our findings highlighted the potential association between activation of inflammatory immune response and suppression lipid metabolism in *S. agalactiae*-inoculated mammary quarters. Taken together, the observed responses in inoculated mammary alveolar tissue likely reflect transcriptional changes in bovine MEC. Because the experiment has used an isolated perfused udder model, it is reasonable to suggest that the predominant response would be from the resident macrophages which serve both sentinel as well as effector functions of immune defense in healthy udder (Strandberg *et al.*, 2005).

In conclusion, our study demonstrated that the first 3 h after *S. agalactiae*- inoculation elicited a strong transcriptomic response leading to an overall up-regulation of genes involved in innate immune and inflammatory response and marked inhibition of lipid synthesis-related genes. The results provide comprehensive information about the early response factors associated with innate immune response in *S. agalactiae*-inoculated mammary quarter. We showed that *TLR2*, *TLR4*, *CD14* and *IL-1*β genes were highly up-regulated in inoculated-quarters which could be crucial stimulators of innate immunity. It is important to note that no apoptosis induction had occurred, either in non-inoculated or inoculated quarters at 3 h after the experimental inoculation, which validates the model to evaluate the early response to other mastitis-causing pathogens

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