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Going further post-RNA-seq: *In silico* functional analyses revealing candidate genes and regulatory elements related to mastitis in dairy cattle

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Research Article

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Abstract

This study aimed to obtain a better understanding of the regulatory genes and molecules involved in the development of mastitis. For this purpose, the transcription factors (TF) and MicroRNAs (miRNA) related to differentially expressed genes previously found in extracorporeal udders infected with *Streptococcus agalactiae* were investigated. The Gene-TF network highlighted *LOC515333*, *SAA3*, *CD14*, *NFKBIA*, *APOC2* and *LOC100335608* and genes that encode the most representative transcription factors *STAT3*, *PPARG*, *EGR1* and *NFKB1* for infected udders. In addition, it was possible to highlight, through the analysis of the gene-miRNA network, genes that could be post-transcriptionally regulated by miRNAs, such as the relationship between the *CCL5* gene and the miRNA bta-miR-363. Overall, our data demonstrated genes and regulatory elements (TF and miRNA) that can play an important role in mastitis resistance. The results provide new insights into the first functional pathways and the network of genes that orchestrate the innate immune responses to infection by *Streptococcus agalactiae*. Our results will increase the general knowledge about the gene networks, transcription factors and miRNAs involved in fighting intramammary infection and maintaining tissue during infection and thus enable a better understanding of the pathophysiology of mastitis.

Bovine mastitis is an inflammatory response of the mammary gland caused by metabolic and physiological changes, trauma or, more often, environmental or contagious pathogenic microorganisms (Oviedo-Boyso *et al.*, 2007) that is responsible for significant economic losses in dairy cattle (Contreras and Rodríguez, 2011). There is a wide range of pathogens that cause mastitis, including Gram-negative and Gram-positive bacteria, mycoplasmas and algae (Zadoks *et al.*, 2011). *Streptococcus agalactiae* is an important Gram-positive bacterium that causes contagiously transmitted chronic subclinical infections in cows (Keefe, 1997; Zadoks *et al.*, 2011). The prevalence of this pathogen in dairy herds is quite high, especially in countries with emerging dairy industries (Duarte *et al.*, 2004) and current control strategies involve farm management practices and antibiotic administration promoting the possible emergence of resistant pathogens (Guterbock *et al.*, 1993; Wilson *et al.*, 1999). Therefore, it is necessary to develop new methodologies to control *S. agalactiae*, including breeding more resistant cattle through enhanced genomic selection. Genomics and transcriptomic data have elucidated gene networks and physiological cellular processes important in the response to *S. agalactiae* (Sbardella *et al.*, 2019; Weller *et al.*, 2019). In our laboratory, bovine extracorporeal udders have proved to be a successful tool for ex situ transcriptomic analysis of the innate response triggered by *S. agalactiae* in mammary tissue and display metabolic pathways associated with the inflammatory response (Pinto *et al.*, 2017; Sbardella *et al.*, 2019; Weller *et al.*, 2019). In order to contribute to a better knowledge of the genes involved in mastitis development, this study aimed to investigate transcription factors and MicroRNAs related to differentially expressed genes (DEGs) previously found in these bovine extracorporeal udders infected with *S. agalactiae*.

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Materials and methods

RNA-seq data

RNA-sequence (RNA-seq) data were obtained from previous studies of our group (Sbardella *et al.*, 2019; Weller *et al.*, 2019). Briefly, four perfused udders were inoculated with a strain of *S. agalactiae* (FSL S3-026). For each udder, two quarters were inoculated (left anterior and posterior) and two were used as control (right anterior and posterior). Samples of alveolar tissue were collected at 0 and 3 h times after inoculation and the RNA sequenced by HiSeq 2000 analyzer (Illumina Inc.). Expression values were calculated by counting for each gene how many aligned reads overlapped its exons using Htseq-count (Anders *et al.*, 2014). The significance of gene expression changes contrasts were assessed using edgeR package (Galaxy tool version 0.0.2) (Robinson *et al.*, 2010; Sbardella *et al.*, 2019; Weller *et al.*, 2019).

DEG selection for enrichment and metabolic pathway analysis

The ClueGO Cytoscape application was used to correlate the groups of differentially expressed genes (DEGs) with biological processes (Bindea *et al.*, 2009) and highlight the roles and terms of gene ontology based on hypergeometric test and Bonferroni correction establishing edges between genes and the chosen term (biological process, cellular component or molecular function). As input, we used the most differentially expressed genes (top 5% of each up and down-regulated genes) previously identified by Sbardella *et al.* (2019) and Weller *et al.* (2019) (online Supplementary Table S1). Thus, we were able to obtain gene networks highlighting biological processes and compare the groups of genes (up and down-regulated) visualizing their functional differences or similarities.

Identification of transcription factors associated with inflammatory response

The search for promoter sequences was carried out using the current assembly of the bovine genome taking positions of 3.000 bp upstream and 300 bp downstream to the position of each 5% DEGs list. The generated data were used as input in the TFM-Explorer software (http://bioinfo.lifl.fr/tfm-explorer/form.php) which uses weight matrices from the JASPAR database (Sandelin *et al.*, 2004) to detect all potential transcription factor (TF) binding sites from a set of gene sequences by calculating a score function with a threshold (*P*-value) equal or greater than 10^{-3} for each position and each sequence, such as described in Touzet and Varré (2007).

Construction of the Gene-TF network

The list of TFs generated by TFM-Explorer was used as an input file in Cytoscape (Shannon *et al.*, 2003) using the Biological Networks Gene Ontology tool (BiNGO) (Maere *et al.*, 2005). Thus, it was possible to determine which biological processes were significantly overrepresented assuming Bonferroni correction patterns and hypergeometric statistical test used to estimate the proportion of genes associated to a particular biological process. TF presenting biological processes associated with inflammatory responses were selected, and a literature review was performed to confirm the relationship between TFs and inflammatory response. In this way, we selected key-TF for the inflammatory response. In order to identify which genes were most connected to each key-TF, the NetworkAnalyzer tool was used in Cytoscape. According to the number of TF binding sites present in the promoter regions of the genes, it was possible to determine the gene-TF network highlighting candidate genes/TF for inflammatory response in mammary gland infected with *S. agalactiae*.

Real-time PCR and data analyses

Among the enriched genes in the gene-TF network, we selected five for validation by real-time PCR because they perform important functions in the immune response (*NFKBIA* – NFKB Inhibitor Alpha, *SAA3* – Serum Amyloid A3, *CD14* – Cluster of Differentiation 14, *STAT3* – Signal Transducer and Activator of Transcription 3 e *SCD* – Stearoyl-CoA Desaturase). Methodological details are in the Supplementary File, primers are given in online Supplementary Table S2.

Identification of miRNAs and construction of the gene-miRNAs network

To establish the gene-miRNAs network, we first searched for miRNAs differentially expressed in RNA-seq data. After identifying the differentially expressed miRNAs, we did a literature review aiming to select miRNAs related to inflammatory response. From these miRNAs, we used the online miRWalk® software (http://mirwalk.umm.uni-heidelberg.de/) to identify possible target genes. Only target genes that were also differentially expressed in our database were selected for subsequently analyses. Thus, in order to identify which genes are the most linked to each miRNA, NetworkAnalyzer tool in Cytoscape® were used. In this way, and according to the number of binding sites between genes and miRNAs, it was possible to determine the most enriched genes and miRNAs through the gene-miRNAs network.

Results

Gene–biological processes network

Some of the most enriched biological processes in the gene–biological processes network were cellular response to bacterial lipoprotein and cellular response to the triacyl bacterial lipopeptide. The *TLR2* and *CD14* genes, both up-regulated, shared these biological processes. The genes *CXCL8* and *CCL5* were also observed in the network sharing cellular response by interleukin 1 and cellular response to molecules of bacterial origin processes (Fig. 1).

Gene–TF network

Twenty candidate TF were identified for up-regulated gene group and 22 for the down-regulated group (one Supplementary Table S3). Those TF were used as input to the BiNGO app from Cytoscape software to search for biological processes related to inflammatory response. According to the enriched biological processes and a literature review, it was possible to select four main key-TF related with immune response and/or inflammatory response (Table 1). Based on the key-TF, a gene-transcription factors network was constructed (Fig. 2). This network provided an overview of shared key-TF among candidate genes, as well as highlighting the most connected genes within each group for inflammatory response (*LOC515333*, *SAA3*, *NFKBIA*, *IL8*, *CD14*, *APOC2*, and *LOC100335608*).

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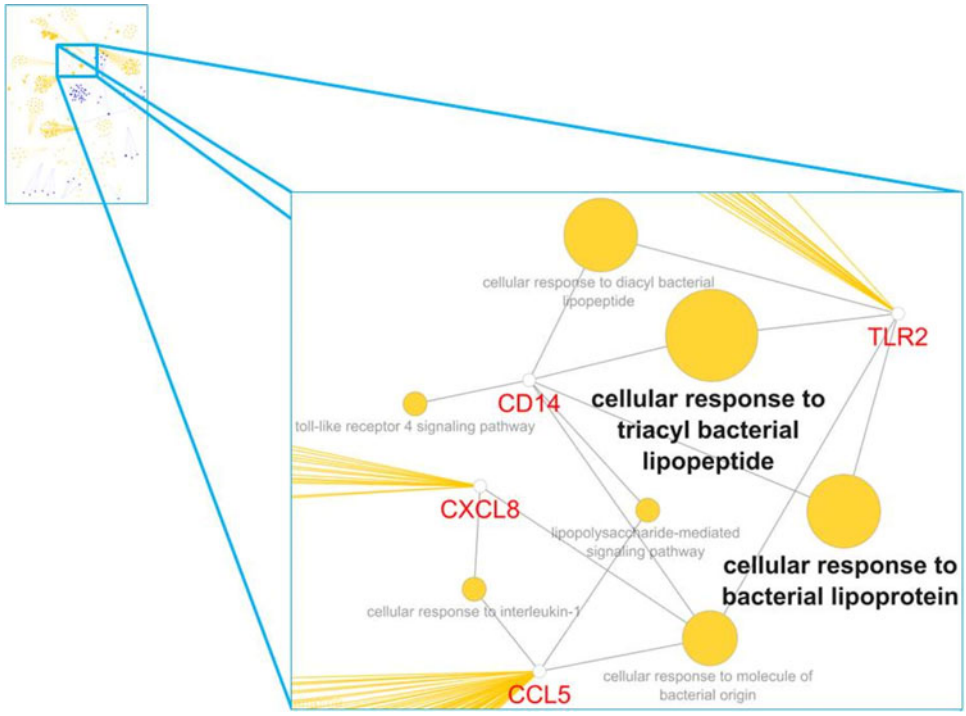

Fig. 1. Network of main biological processes from differentially expressed genes in response to *Streptococcus agalactiae*. Functional group network, a zoom in the main biological terms (yellow nodes) and genes (white nodes labeled in red). Yellow biological processes are linked to up-regulated genes. White nodes with yellow edges represent the up-regulated genes. The size of the biological processes node corresponds to the enrichment of the ClueGO app.

Table 1. Main transcription factors associated with top 5% up and down-regulated genes in alveolar mammary tissue 3 h post-inoculation with *Streptococcus agalactiae* from inoculated quarters compared to not inoculated quarters, their biological process and literature evidences to inflammatory response

TF	Gene group	Biological process (GO)	Reference[a]
STAT3	Up and down-regulated	IL-6 mediated signaling pathways/ Inflammatory response/ Cytokine stimulus response.	Tegowski *et al.* (2018)
EGR1	Down-regulated	Development of immune system/ Differentiation of T cells, lymphocyte and leukocyte.	Kosciuczuk *et al.* (2017)
NFKB1	Up and down-regulated	Regulation of cytokine production/ Cell differentiation.	Wang *et al.* (2017)
PPARG	Down-regulated	Differentiation of leukocytes and monocytes/ Development of the immune system.	Wang *et al.* (2017)

[a]Cited references are just a sample of a vast literature.

Validation of differentially expressed genes by real-time PCR

To further investigate the immune response induced by *S. agalactiae*, we selected five genes to be confirmed by real-time PCR aiming to substantiate the involvement of the identified key biological processes. As defined by RNA-seq analysis, all immune-associated genes tested in real-time PCR confirmed differences in gene expression prior to inoculation of *S. agalactiae* (0 h) compared to inoculated udders. Furthermore, we observed an increase in *CD14* (expression = 5.117), *NFKBIA* (expression = 2.645), STAT3 (expression = 2.281), *SAA3* (expression = 2.618) expression and decreased *SCD* gene expression (expression = 0.130) at 3 h after inoculation with *S. agalactiae* between inoculated and uninoculated samples (Table 2).

Gene–miRNAs network

28 candidate miRNAs (14 up-regulated miRNAs and 14 down-regulated) were analyzed (online Supplementary Table S4). Based on a literature review, it was possible to select the major miRNAs related with immune/inflammatory response (bta-miR-193a, bta-miR-363, bta-miR-148b, bta-miR-205 and bta-let-7e), which were used to assemble the gene-MicroRNA (gene-miRNA) network (Fig. 3). Therefore, the observations in the current study highlight which genes had the highest number of binding sites for the selected miRNAs (e.g. *SCD*, *LPIN1*, *RPS26* and *MPP6*), which may play a role in regulate those protein expression.

Discussion

The host's first line of defense against infection is the innate immune response, as it has the ability to recognize and respond quickly to the first signs of infection (Bannerman, 2009). It is known that the innate immunity response occurs after challenge by *E. coli* (Bannerman *et al.*, 2004*a*; Günther *et al.*, 2016), *S. aureus* (Petzl *et al.*, 2008) and *S. uberis* (Bannerman *et al.*, 2004*b*;

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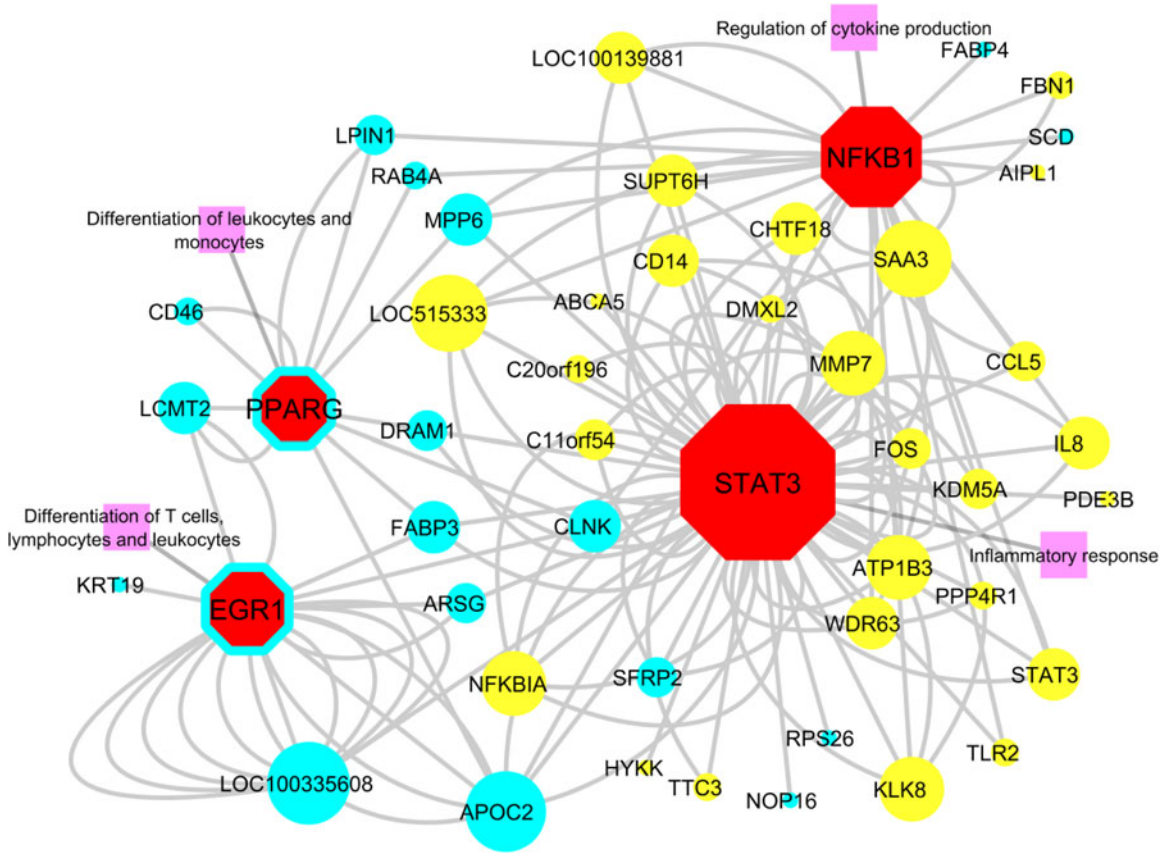

Fig. 2. Gene-transcription factor network. Red colored octagonal nodes represent the key-TFs associated with inflammatory response. Circular nodes represent differentially expressed genes, being up-regulated (yellow) and down-regulated (blue). The node size corresponds to the network analysis of Cytoscape, where nodes with larger sizes have a greater number of transcription factor binding sites. Red nodes with blue borders are TFs that showed binding site only to down-regulated genes. Pink squares represent the biological processes related to TFs.

Swanson *et al*., 2009). Other studies have investigated changes in gene expression in milk samples after intramammary infection with *S. agalactiae* (Fonseca *et al*., 2015). Recently, the transcriptional profile of bovine mammary tissue was investigated after challenge with *S. agalactiae* (Weller *et al*., 2019; Sbardella *et al*., 2019), however, a better understanding of the regulatory elements of gene expression, such as transcription factors and MicroRNAs, is needed.

In our study, genes related to the innate immune response were enriched in the biological process network, highlighting the main biological roles (such as cellular response to interleukin-1 and cellular response to molecule of bacterial origin) and connections between DEGs such as the Toll-like receptor 2 (*TLR2*), Cluster of Differentiation 14 (*CD14*), CC Motif Ligand 5 (*CCL5*) and CXC Motif 8 (*CXCL8*) (Fig. 1). Among its signaling pathways, *CD14*, as an adapter molecule of the TLR signaling pathway, plays an important role in bacterial infection as a high-affinity lipopolysaccharide receptor, which activates intracellular signaling pathways, leading to the release of cytokines (Shin *et al*., 2015). In our study, the *CD14* gene was highlighted in the analysis of the gene-biological process network. Like in our study, Thorgersen *et al*. (2010) associated *CD14* with early inflammatory and hemostatic responses in a Gram-negative sepsis model as an important innate immunity molecule in pigs.

The presence of *TLR2* in the network corroborates the results obtained by Fonseca *et al*. (2015) who observed increased expression of *TLR2* and *TLR4* in milk samples after in vivo infection with *S. agalactiae*. These results suggest that these genes are regulated together at the beginning of the immune response in mammary alveolar tissue. The *CCL5* and *CXCL8* genes are important mediators of the inflammatory response. Among their functions, they include orchestrating the migration of monocytes and T cells to injured or infected sites (Gao *et al*., 2016) and recruiting polymorphonuclear neutrophils to the site of infection (Rosales *et al*., 2017). In our results, these genes were enriched with important roles at the time of infection, highlighting the importance of these chemokines in these stages of the innate immune response to intramammary infection by *S. agalactiae*. As in our findings, Fonseca *et al*. (2015) demonstrated that *CCL5* is positively regulated in milk samples. Other studies have suggested this gene as a biomarker of *Mycobacterium* infection in bovine cells, in the sense of presenting a difference in expression against infection of the pathogen (Shin *et al*., 2015). In addition, Günther *et al*. (2016) evaluated the immune response to different pathogens in uterine cell types and observed the differential expression of *CXCL8* against *E. coli* infection.

Considering such evidence besides their over-expression in bovine extracorporeal udders infected with *S. agalactiae*, it is suggested that *TLR2*, *CD14*, *CCL5* and *CXCL8* might play a role in the inflammatory response under this scenario. In the present study, *TLR2* and *CD14* were linked to the cellular response to bacterial lipoprotein. The *CCL5* was related to the positive regulation

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Table 2. Relative expression of genes enriched in the gene–TF network

Gene	Type	Reaction efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
STAT3	TRG	0.8778	2.281	0.357–14.337	0.092–63.742	0.032	UP
CD14	TRG	0.9749	5.117	0.479–47.087	0.064–1.458.216	0.003	UP
NFKBIA	TRG	0.8442	2.645	0.429–15.529	0.114–285.232	0.017	UP
SAA3	TRG	0.9078	2.618	0.276–28.717	0.068–220.190	0.045	UP
SCD	TRG	0.8427	0.130	0.004–6.973	0.001–473.513	0.007	DOWN
B-ACTINA	REF	0.9938	1.331				
HPRT	REF	0.8789	0.752				

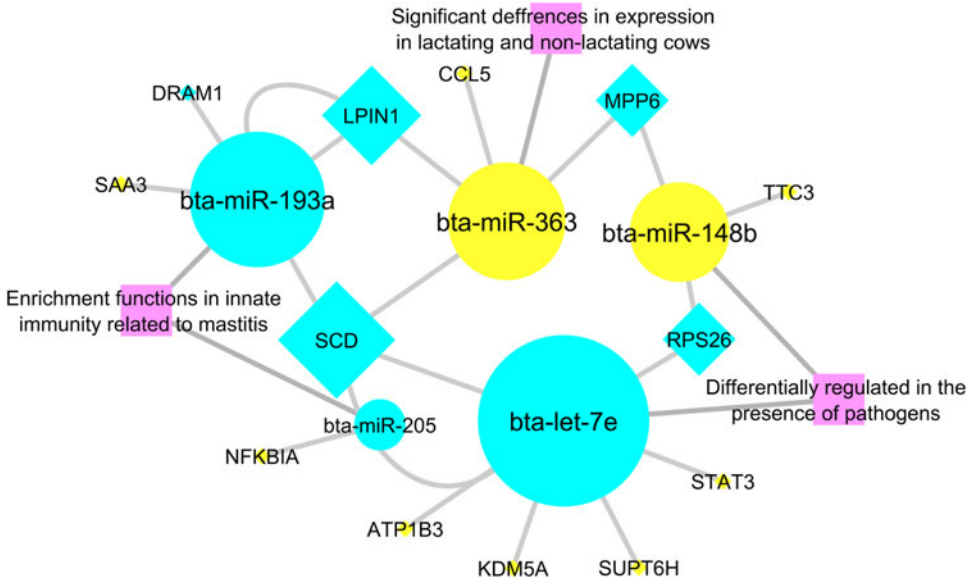

Fig. 3. Gene-miRNA network. Blue circular nodes are down-regulated miRNAs; yellow circular nodes are up-regulated miRNAs. Blue diamond-shaped nodes are down-regulated genes; yellow diamond-shaped nodes are up-regulated genes. The pink square nodes represent evidence in the literature relating the miRNAs to inflammatory response.

of injury response, positive regulation of inflammatory response and positive regulation of lipase activity. Also, in the biological processes network presented in this study, the *CXCL8* gene was related to the cellular response by *IL1* and cellular response to the molecule of bacterial origin. Therefore, it is reasonable to suggest the investigation of these molecules as candidates for biomarkers of inflammatory response under *S. agalactiae* infection.

Besides these candidate genes highlighted *via* biological processes, we identified other TF (*STAT3*, *EGR1*, *NFKB1* and *PPARG*) that are expected to play a role in *S. agalactiae* mastitis and built a gene-TF network. This network draws attention to genes also involved in the inflammatory response (*LOC515333*, *SAA3*, *NFKBIA*, *IL8*, *CD14*, *APOC2* and *LOC100335608*).

The serum amyloid A3 gene (*SAA3*) belongs to the up-regulated group and was one of the most enriched genes in the network, according to the number of connections to the major TFs. Similar to our findings, Molenaar *et al.* (2009) showed that the *SAA3* gene is highly expressed during bovine mastitis, being differentially expressed between infected and uninfected quarters and with minimal or undetectable expression in healthy quarters (Eckersall *et al.*, 2001, 2006). Alpha NFKB Inhibitor (*NFKBIA*) is a member of the gene family encoding proteins that interact with Rel dimers to inhibit the NF-kappa-B/Rel complexes that are involved in inflammatory responses. According to our results, the positive regulation of this gene may be involved with the connection with *STAT3* and *NF-kappa-B* TFs. Lutzow *et al.* (2008) reported that *NFKBIA* was activated in mastitis induced by *S. uberis*. Also, Moyes *et al.* (2009) observed the same behavior when the infection was caused by *S. aureus*. These findings support our results, whereas this gene was also over-represented in the gene-TF network.

Another two highlighted genes in this study, *CD14* and *TLR2,* have *STAT3* binding sites, which mediate cellular responses to interleukins such as interleukin-6 (*IL-6*) that are identified in the promoters of various acute phase protein genes. *IL-6* also acts as a regulator of the inflammatory response by regulating the differentiation of naive CD4 (+) T cells into Th17 helper or regulatory T cells (Lee, 2018). As in our results, Fonseca *et al.* (2015) observed increased expression of *TLR2*, *TLR4* and *CD14* in milk samples after experimental in vivo infection with *S. agalactiae*. In the study of Weller *et al.* (2019), the *CD14* gene was highly regulated three hours after *S. agalactiae* inoculation in bovine extracorporeal udder.

Once selected key-TF had been shown to be associated with the inflammatory response, higher up-regulated gene enrichment

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was expected in the gene-TF network. However, two down-regulated genes were highlighted (*APOC2* and *LOC100335608*). The gene *LOC100335608* corresponds to the Rabphilin 3A Like in *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*. In humans, the protein encoded by this gene (rabphilin 3A) plays a direct regulatory role in calcium ion-dependent exocytosis in endocrine and exocrine cells and plays a key role in pancreatic cell secretion of insulin (Matsunaga *et al*., 2017). In addition, this gene is described as a tumor suppressor in humans (Putcha *et al*., 2015). In cattle, the gene *LOC100335608* encodes the protein type 3A and can be associated with fatty acids in milk of Dutch cattle (Li *et al*., 2014). RNA-seq analysis of bovine extracorporeal mammary gland revealed several genes involved in lipid metabolism, such as down-regulated *APOC2*, *FABP3* and *FABP4* (Weller *et al*., 2019). In addition, Swanson *et al*. (2009) studying gene regulation profiles in mammary tissue after *S. uberis* infection also observed the downregulation of genes related to lipid metabolism, such as *LPIN1*, *FABP3* and *APOC2*. In this study, the genes *APOC2* and *LOC100335608* were enriched in the TF gene network with binding to key-TFs *PPARG* and *EGR1*. Although *PPARG* and *EGR1* TFs were related to biological processes involved in inflammatory response, they were also associated with lipid metabolism (*EGR1*) and lipid storage negative regulation (*PPARG*).

Furthermore, among the key-TFs, the signal transducer and activator of transcription 3 (*STAT3*) was the most represented. Shin *et al*. (2015) analyzed transcriptional profiles of bovine cells infected by *Mycobacterium avium* subsp. *paratuberculosis* and observed differences in expression pattern between *STAT3* target genes, suggesting that they may be used as biomarkers for *Mycobacterium* infection. In this study, all enriched genes cited above had binding sites for this TF.

All genes selected from gene-TF network analysis had their expression levels confirmed by real-time PCR (Table 1). As in our results, Lutzow *et al*. (2008) observed high expression of *NFKBIA* and *CD14* during intramammary infection with *S. aureus*. In vivo studies by Swanson *et al*. (2009) also showed high expression of *STAT3* and *SAA3* genes in the challenge of infection by *S. uberis*, a Gram-positive bacterium, as well as *S. agalactiae*. Our results indicate that these genes play a critical role in the immune response in the *S. agalactiae* udder during early stage of intramammary infection. Another finding of interest while combining RNA-seq and gene network analysis was that several genes involved with lipid metabolism, such as *SCD*, *LPIN1*, *APOC2*, *FABP3*, and *FABP4*, were suppressed in quarters inoculated with *S. agalactiae* and shared TF involved also with inflammatory response in accordance to Swanson *et al*. (2009). From the most enriched genes in the gene-TF network, and those that were evidenced in biological processes network (*TLR2*, *CD14* and *CXCL8*), most of them were not enriched in the gene-miRNAs network, with the exception of *SAA3* and *CCL5*. The *SAA3* gene had a binding site for bta-miR-193a, which was down-regulated in mammary alveolar tissue 3 h after inoculation with *S. agalactiae*. In this case, bta-miR-193a should not be exerting relevant action on the *SAA3* gene during this period in the alveolar tissue as it is down-regulated in the RNA-seq data. On the other hand, *CCL5* had a binding site for bta-miR-363, which was up-regulated in mammary alveolar tissue 3 h after inoculation with *S. agalactiae*. Thus, their role in the inflammatory response of mammary gland is uncertain, in terms that miRNAs generally act to inhibit translation, but also, as previously described, may positively act on gene transcription (Portnoy *et al*., 2011). Our results indicate that the *CCL5* gene was highly expressed in mammary alveolar tissue infected with *S. agalactiae* presenting biological processes related to the inflammatory response and enriched in gene-TF network.

In conclusion, our results point to a number of genes that are prominent in biological processes and gene-TF networks, and which have not been linked to up-regulated miRNAs, but which are likely candidate genes for the inflammatory response markers in mammary glands, with possible relation to mastitis resistance trait. The gene-TF network highlighted genes that may act at the time of infection by being up or down-regulated have been discussed. In addition the gene-miRNA network indicated which genes could be negatively or positively regulated by translation inhibition, mRNA degradation or transcription activation by miRNAs. These genes are deserving of more intensive study. In this way, using post-RNA-seq analyses, we can propose the most likely candidate genes (*LOC515333* and *CD14*), transcription factors (*STAT3* and *NFKB1*) and miRNAs (bta-miR-193a and bta-miR-363) with possible roles in the inflammatory response of mammary glands under *S. agalactiae* infection.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000571

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