ISABELA FONSECA

GENE EXPRESSION PROFILE IN GYR AND CROSSBREED DAIRY COWS WITH MASTITIS

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Genetics and Breeding Graduate Program for the attainment of the title *Doctor Scientiae.*

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I dedicate this thesis to my son, Pedro

Summary

ABSTRACT

FONSECA, Isabela Fonseca, D.Sc., Universidade Federal de Viçosa, February, 2014. **Gene expression profile in gyr and crossbreed dairy cows with mastitis.** Adviser: Simone Eliza Facioni Guimarães. Co-advisers: Marta Fonseca Martins and Fernando Flores Cardoso.

Among the potential public health problems of animal production, infectious-contagious diseases stand out. Mastitis is among the main diseases affecting dairy cattle. It is an inflammatory response in the mammary gland caused by an influx of somatic cells, composed mainly of neutrophils, macrophages and lymphocytes. The speed and efficacy of the host's immune response to the invasive pathogen affects the establishment, persistence and severity of the infection. To characterize the gene expression and response mechanism to mastitis in crossbreed dairy cows and Gyr breed, we carried out a transcriptome study of the cells present in the milk from 20 crossbreed dairy cows with natural infection and 17 Gyr cows artificially infected with *Streptococcus agalactiae*. The group of crossbreed animals was composed of 10 animals free of infection and 10 with clinical mastitis. This group we quantified the relative expression of the genes *IL-2*, *IL-6*, *IL-8*, *IL-12*, *IFN-γ*, *TNF-α*, *TLR-2*, *SEMA5A*, and *FEZL* using the real-time PCR method. Among these genes, the *TLR-2* was express 2.5 times more in cows with mastitis ($P < 0.05$). In turn, the samples of Gyr cows group composed of 17 animals in lactation were evaluated by microarray and validated by real-time PCR technique. For this, we collected milk samples before inoculation (hour 0) and 4, 9 and 24 hours after inoculation of the bacteria on one of the quarters and at 0 and 24 hours from one of the quarters not inoculated. The microarray technique revealed the existence of 32 differentially expressed genes between inoculation and 4 hours afterward. The validation of these results by real-time PCR was done for the genes *AATK*, *CCL2*, *CCL20*, *CD40*, *CSF2*, *IL-1β*, *INHBA* and *NOS2A*. Besides these eight genes, the expression of six others was evaluated by real-time PCR even though they did not present a significant difference by the microarray technique (*IL-12*, *IL-17*, *TLR-2*, *TLR-4*, *GRO-α* and *TGF-β1*). Of the 14 genes analyzed by real-time PCR, all showed a significant difference in expression for at least one of the comparisons between times. This analysis indicated an increase in the expression of all the genes that presented a significant difference in relation to hour 0, with most of them presenting maximum expression 24 hours after inoculation

of the pathogen. The *IL-1β* gene showed peak expression 4 hours after inoculation, while the *CD40* and *INHBA* genes maintained their expression throughout the experimental period. The *IL-12* and *IL-17* genes showed the highest expression rates 24 hours after inoculation in relation to hour 0, with an increase in the level of expression by more than a factor of 20. Comparison of the gene expression between the inoculated and non-inoculated quarters showed greater expression in nine genes in the inoculated quarters. Analyses of gene networks revealed three modules with distinct characteristics 24 hours after inoculation and showed that some mechanisms are altered in Gyr dairy cows after infection of the mammary gland by *Strep. agalactiae*. However, it is important to emphasize that these results can not be directly compared because the mode of infection, the causative micro-organisms of mastitis and the races were different in the two studies. But these results suggest that the genes that exhibited significant differences in expression may play an important role in combating intramammary infection, particularly the *TLR-2*, which showed differential expression in both studies.

RESUMO

FONSECA, Isabela Fonseca, D.Sc., Universidade Federal de Viçosa, Fevereiro, 2014. **Perfil de expressão gênica em vacas gir e mestiças com mastite.** Orientadora: Simone Eliza Facioni Guimarães. Co-orientadores: Marta Fonseca Martins e Fernando Flores Cardoso.

Dentre os problemas sanitários na produção animal, as doenças infectocontagiosas são as que mais se destacam, sendo a mastite uma das principais doenças em gado de leite. Esta se caracteriza por uma resposta inflamatória na glândula mamária na qual ocorre um influxo de células somáticas compostas principalmente por neutrófilos, macrófagos e linfócitos. A rapidez e a eficácia da resposta imune do hospedeiro contra o patógeno invasor afeta o estabelecimento, a persistência e a gravidade da infecção. Com o objetivo de caracterizar a expressão gênica em animais mestiços e Gir Leiteiro em resposta à mastite foi realizado um estudo de transcriptoma de células presentes no leite de 20 animais mestiços com infecção natural e de 17 vacas Gir infectadas artificialmente com *Streptococcus agalactiae*. O grupo de animais mestiços era composto por 10 vacas hígidas e 10 vacas com mastite clínica. Neste grupo foi avaliada a expressão relativa dos genes *IL-2*, *IL-6*, *IL-8*, *IL-12*, *IFN-γ*, *TNF-α*, *TLR-2*, *SEMA5A* e *FEZL* por meio da metodologia de PCR em Tempo Real. Dentre estes genes, o *TLR-2* foi 2,5 vezes mais expresso nos animais com mastite ($P < 0,05$). Já as amostras do grupo de animais Gir, composto por 17 vacas em lactação, tiveram a expressão avaliada pela técnica de microarranjo e validada por PCR em Tempo Real. Neste caso foram coletadas amostras de leite antes da inoculação (tempo 0), 4, 9 e 24 horas após a inoculação da bactéria em um dos quartos e nos tempos 0 e 24 horas em um dos quartos não inoculado. Foram observados 32 genes diferencialmente expressos entre os tempos 0 e 4 horas após a inoculação pela técnica de microarranjo. A validação destes resultados por PCR em Tempo Real foi feita para os genes *AATK*, *CCL2*, *CCL20*, *CD40*, *CSF2*, *IL-1β*, *INHBA* e *NOS2A*. Além destes oitos genes, outros seis tiveram sua expressão avaliada por PCR em Tempo Real, apesar de não terem apresentado diferença significativa pela técnica de microarranjo (*IL-12*, *IL-17*, *TLR-2*, *TLR-4*, *GRO-α* e *TGF-β1*). Dos 14 genes analisados por PCR em Tempo Real, todos apresentaram diferença de expressão significativa em pelo menos um dos contrastes realizados entre os tempos. Esta análise indicou aumento de expressão de todos os genes que apresentaram diferença

significativa em relação ao tempo 0, sendo que a maior parte apresentou expressão máxima 24 horas após a inoculação do patógeno. O gene *IL-1β* apresentou pico de expressão 4 horas após a inoculação, enquanto que os genes *CD40* e *INHBA* mantiveram sua expressão alta por todo o período do experimento. Os genes *IL-12* e *IL-17* foram os que apresentaram maiores taxas de expressão 24 horas após a inoculação em relação ao tempo 0, com aumento no nível de expressão em mais de 20 vezes. Quando comparada a expressão dos genes entre os quartos inoculados e não inoculados no tempo 24, foi observada maior expressão em nove genes nos quartos inoculados. Análises de redes gênicas identificaram três módulos com características bem distintas entre os tempos 0 e 24 horas e mostraram que alguns mecanismos são alterados em vacas Gir Leiteiro após a infecção da glândula mamária por *S. agalactiae*. No entanto, é importante ressaltar que estes resultados não podem ser diretamente comparados porque o modo de infecção, os micro-organismos causadores da mastite e as raças foram diferentes nos dois estudos. Mas estes resultados sugerem que os genes que apresentaram diferenças significativas de expressão possivelmente desempenham funções importantes no combate à infecção intramamária, com destaque para o gene *TLR-2*, o qual apresentou diferença de expressão nos dois estudos.

GENERAL INTRODUCTION

Dairy farming is one of Brazil's most important agribusiness activities. In recent years, with the improvement in economic conditions and expansion of the middle class, the agricultural sector has shown strong growth, especially the dairy segment. Between 2000 and 2011, output of milk in the country grew 62.4%, surpassing the mark of 32 billion liters per year. According to the latest figures, Brazil is the world's fourth leading producer of milk, only behind the United States, India and China. Besides this, it is also in fourth place in consumption of fluid milk, at 10.9 billion liters in 2012 (http://www.cileite.com.br/content/panorama-do-leite). Even with the strong domestic consumption of milk and other dairy products, since 2004 Brazil has been a net exporter of these products, attesting to the productive capacity of its dairy farmers.

Despite the statistics showing the constant evolution of the milk production chain, in recent years the nation's dairy sector has been facing new competitive challenges and reduced profit margins, requiring increasingly efficient production systems. The effort to increase the productivity of herds mainly involves genetic improvement and adequate nutrition. The adaptation of animals for diverse environments has been pursued by crossing different breeds or subspecies (*Bos taurus* and *Bos indicus)*, to obtain animals with good productivity that are also adapted to the country's range of climate conditions. Zebu breeds (*B. indicus)* and their crosses are extremely important in the composition of Brazil's dairy cattle herds, accounting for about 80% of the total. The Gyr Dairy breed stands out in this context for incorporating rusticity, productivity and docility, besides being efficient at producing milk at low cost (Ferreira et al., 2007). The importance of this breed has been increasing in Brazil and other countries of Latin America, as well as in Africa and Asia, because it is widely used for crossing, mainly with the Holstein breed, besides being a good alternative for milk production as a pure breed (Vercesi Filho et al., 2007). Data from the Brazilian Artificial Insemination Association show that in 2012 the number of semen doses sold from Gyr Dairy sires was the second largest among dairy breeds, behind only Holstein bulls, having grown by 3% between 2010 and 2012. In the case of the Girolando breed, a cross between the Gyr Dairy and Holstein breeds, the number of doses sold more than doubled in the same period, the best performance among the 14 breeds evaluated. These statistics give an idea of

how much these breeds have been gaining importance for the Brazilian dairy industry.

However, it is not enough just to increase the milk production to maintain the sector's productivity. Three is also a need to improve installations, worker training, zootechnical and genetic methods, public policies and animal health. On the matter of animal health, mastitis is the main disease in economic terms. Besides reducing milk yield and quality (in terms of nutritional parameters and contamination by pathogens and antibiotic residues), it increases costs for labor and treatment and requires discarding substantial quantities of milk. Beyond the economic impact, mastitis is also of great concern in terms of animal welfare, because it causes fever, swelling of the udder and even locomotion difficulties (Contreras & Rodríguez, 2011).

According to Cassol (2010), the losses attributed to this disease amount to approximately US$ 200 for each affected cow, 70% of which is due to reduced production of mammary quarters with subclinical mastitis; 14% caused by devaluation of animals due to functional reduction of the afflicted quarters or early retirement or death of the animal; 8% due to the milk that must be discarded because of alterations and/or presence of antibiotic residues after treatment; and 8% from treatment costs, among them veterinarians' fees and the cost of drugs. Therefore, a better understanding of this disease is important to find solutions to these problems. Due to the high prevalence of mastitis in national herds, in Brazil it is estimated that lost production could occur between 12 and 15%, which means a total of 4.8 billion liters/year compared to annual production of 32 billion liters (Pritchett et al, 2005; Kelton 2006; FAO, 2013).

Along with adequate herd management and sanitary care, the selection of animals that are resistant to diseases and the incorporation of this trait in herds is a promising alternative to reduce the problems caused by infectious-contagious diseases. Besides this, the current trend of heightened consumer awareness regarding the quality/origin of food and of the potentially negative effects of administering antibiotics to producing animals increases the importance of improving the disease resistance of herds. The use of animals that are resistant to a determined disease reduces the need to apply drugs, with consequent decrease in the levels of contamination of products and the environment. Therefore, the incorporation of

genes that impart resistance through the selection of more resistant animals is a practice that should be encouraged.

One of the techniques employed to select resistant animals is the use of molecular markers in genetic improvement programs. Various strategies can be applied to identify these markers, among which studies of gene expression stand out. These studies generate knowledge about the biochemical and genetic mechanisms of resistance by clarifying the actions of the respective genes. Therefore, it is important to identify and characterize the gene expression in animals with mastitis for subsequent use of this information to aid the search for genes that can be tested and validated as markers of such physiological conditions. Research groups in Brazil and elsewhere in the world have been using molecular genetic methods for prospection and isolation of genes associated with resistance to diseases and better quality of bovine products (Denis et al., 2006; Lahouassa et al., 2007; Petzl et al., 2008). In recent years, new methods to indentify genes of interest by means of gene networks have become available (Chen et al., 2008; Reverter & Fortes, 2013). However, all these studies to characterize gene expression in cows with mastitis have used taurine animals. Thus, the use of zebu and crossbreed animals is important, since the existing references are scarce and limited in scope.

The objective of this study was to assess the gene expression in response to mastitis in crossbred (Holstein x Gyr) and Gyr Dairy cows.

References

Cassol, D.M.S.; Sandoval, G.A.F.; Pericole, J.J.; Gil, P.C.N.; Marson, F.A. Introdução Agentes da Mastite Diagnóstico e Tratamento. A Hora Veterinária, Ano 29, n° 175, maio /junho 2010.

Chen Y, Jun Zhu J, Lum PY, Yang X, Pinto S, MacNeil DJ, Zhang C, Lamb J, Edwards S, Sieberts SK, Leonardson A, Castellini LW, Wang S, Champy MF, Zhang B, Emilsson V, Doss S, Ghazalpour A, Horvath S, Drake TA, Lusis AJ, Schadt EE. Variations in DNA elucidate molecular networks that cause disease. Nature, v. 452, n. 7186, p. 42-435, 2008.

Contreras, G.A.; Rodríguez, J.M. Mastitis: Comparative Etiology and Epidemiology. Journal of Mammary Gland Biology and Neoplasia, v. 16, p. 339-56, 2011.

Denis, M.; Parlane, N.A.; Lacyhulbert, S.J.; Summers, E.L.; Buddle, B.M.; Wedlock, D.N. Bactericidal activity of macrophages against *Streptococcus uberis* is

different in mammary gland secretions of lactating and drying off cows. Veterinary Immunology and Immunopathology, v. 114, p. 111-120, 2006.

FAO (2013). Disponível em: http://www.cileite.com.br/sites/default/files/2013_02_PanoramaLeite.pdf. Acesso em 14 fev. de 2014.

Ferreira, M.B.D.; Lopes, B.C.; Ledic, I.L.; Fernandes, L.O.; Ribeiro, S.A. Características reprodutivas de touros da raça Gir. Revista Gir Leiteiro, n.7, p.30-38, 2007.

Kelton, D.F. Epidemiology: a foundation for dairy production medicine. Veterinary Clinics of North America: Food Animal Practice, v. 22, p. 21-33, 2006

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

Panorama do Leite: boletim eletrônico mensal. Coordenadores: Kennya Beatriz Siqueira e Rosangela Zoccal. Juiz de Fora: Embrapa Gado de Leite, v. 6, n. 78, maio 2013. Disponível em: <http://www.cileite.com.br/sites/default/files/2013_05_PanoramaLeite_0.pdf>. Acesso em: 16 jan. de 2014.

Petzl, W.; Zerbe, H.; Gunther, J.; Yang, W.; Seyfert, H.; Nurnberg, G.; Schuberth. H. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Veterinary Research, v. 39, p. 18, 2008.

Pritchett, J.; Thilmany, D.; Johnson, K. Animal Disease Economic Impacts: A Survey of Literature and Typology of Research Approaches. International Food and Agribusiness Management Review, v. 8, p. 23-45, 2005.

Reverter, A.; Fortes, M.R.S. Building single nucleotide polymorphism-derived gene regulatory networks: Towards functional genome-wide association studies. Journal of Animal Science, v. 91, p. 530-6, 2013.

Vercesi Filho, A.E.; Faro, L.E.; Paz, C.C.P.; Cardoso, V.L.; Silva, D.A.L.; Junqueira, F.S. Fatores que influenciam o preço do sêmen de touros da raça Gir Leiteiro. Revista Gir Leiteiro, n.7, p.78-81, 2007.

CHAPTER 1

Gene expression profile in zebu dairy cows infected with *Streptococcus agalactiae*

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Abstract

Mastitis is an inflammatory response in the mammary gland caused by an influx of somatic cells, composed mainly of neutrophils, macrophages and lymphocytes. The speed and efficacy of the host's immune response to the invasive pathogen affects the establishment, persistence and severity of the infection. To characterize the gene expression and response mechanism to infection by *Streptococcus agalactiae* in zebu dairy cows of the Gyr breed, we carried out a transcriptome study of the cells present in the milk from 17 animals. Milk samples were collected before inoculation (hour 0) and 4, 9 and 24 hours after inoculation of the bacteria into one of the quarters and at 0 and 24 hours from one of the quarters not inoculated. The transcriptome analysis was done by the microarray and real-time PCR techniques. The microarray technique revealed the existence of 32 differentially expressed genes between inoculation and 4 hours afterward. The validation of these results by real-time PCR was done for eight genes. Besides these eight genes, the expression of six others was evaluated by real-time PCR even though they did not present a significant difference by the microarray

technique. Of the 14 genes analyzed by real-time PCR, all showed a significant difference in expression for at least one of the comparisons between times. This analysis indicated an increase in the expression of all the genes that presented a significant difference in relation to hour 0, with most of them presenting maximum expression 24 hours after inoculation of the pathogen. The *IL-1β* (*interleukin 1β*) gene showed peak expression 4 hours after inoculation, while the *CD40* (*CD40 molecule*) and *INHBA* (*inhibin beta A*) genes maintained their expression throughout the experimental period. The *IL-12* (*interleukin 12*) and *IL-17* (*interleukin 17*) genes showed the highest expression rates 24 hours after inoculation in relation to hour 0, with an increase in the level of expression by more than a factor of 20. Comparison of the gene expression between the inoculated and non-inoculated quarters showed greater expression in nine genes in the inoculated quarters. Analyses of gene networks revealed three modules with distinct characteristics 24 hours after inoculation and showed that some mechanisms are altered in Gyr dairy cows after infection of the mammary gland by *Strep. agalactiae*.

Keywords: Gyr, immune response, mammary, mastitis

Introduction

Mastitis is an inflammatory response of the mammary gland caused by metabolic and physiological changes, traumas, or more frequently by environmental or contagious pathogenic microorganisms (Oviedo-Boyso et al., 2007), including gram-negative and gram-positive bacteria, mycoplasmas and algae (Zadoks et al., 2011). This inflammatory response is characterized by an influx of somatic cells composed mainly of neutrophils, macrophages and lymphocytes (Rainard and Riollet 2003), in which the speed and efficacy of the host's immune response against the invasive pathogen affects the establishment, persistence and severity of the infection (Bannerman 2009). Besides the economic impact of this disease, due to alterations in the milk composition, reduced milk production and treatment costs, among others, it also is cause for concern regarding animal welfare and human health (Contreras and Rodríguez 2011).

In bovines, mastitis is the only disease associated with infection by *Streptococcus agalactiae*, a gram-positive bacterium that is only transmitted contagiously within the herd, i.e., from one cow to another (Neave et al., 1969; Keefe

1997). Because of the great importance as a pathogen causing mastitis, in 2011 the first sequence was published of the complete genome of a strain of *Strep. agalactiae* (FSL S3-026) isolated from a cow with clinical mastitis (Richards et al., 2011). Nevertheless, although in recent years a large variety of immunological profiles and associated responses have been described after infection of the mammary gland with gram-positive (Alluwaimi et al., 2003; Denis et al., 2006) and gram-negative bacteria (Lahouassa et al., 2007; Petzl et al., 2008), no article has specifically analyzed the response profile of zebu animals (*Bos taurus indicus*) when infected by *Strep. agalactiae*.

Among the zebuine breeds, the Gyr breed is particularly well adapted to Brazilian environmental conditions. For this reason, it has been intensely used in crosses, and is the preferred breed for the formation of crossbred dairy herds in Brazil, responsible for roughly 70% of the country's milk production (Embrapa Gado de Leite 2005). Data from the Brazilian Association of Artificial Insemination show that in 2011 the number of semen doses sold from Gyr Dairy sires was second among dairy breeds in Brazil, only behind the Holstein breed, with this number having increased 52% between 2009 and 2011. Nevertheless, despite this breed's importance to the dairy industry, few studies have been published identifying genes capable of conferring resistance to mastitis in animals other than taurine breeds (*Bos taurus taurus*). There are indications of the existence of differences in the response profile to clinical mastitis among Gyr, Holstein and mestizo animals (Fonseca et al., 2009, 2011). Besides this, one of the main results of the Bovine HapMap Consortium 2009 was confirmation that cattle are divided into three main groups: European, Indian and African. For this reason, the response profile to various diseases in dairy cattle should be studied among different groups and breeds, so that these responses can be adequately compared to verify the similarities, differences, response mechanisms and possible particularities of prognosis and treatment.

Analyses of the overall profile of gene expression by means of the microarray technique have revealed the expression of various cytokines in infected udders (Lutzow et al., 2008; Günther et al., 2009, 2010). Other studies with gram-positive bacteria also have described a greater influx of leukocytes in the mammary gland and the expression of a large variety of cytokines and inflammatory mediators (Alluwaimi et al., 2003; Bannerman et al., 2004a, b). But all of these studies involve cattle of the Holstein breed. Based on these studies, the genes involved in the

immune response have been indicated as likely candidates for understanding resistance and susceptibility to mastitis (Alluwaimi et al., 2003; Oviedo-Boyso et al., 2007; Fonseca et al., 2009, 2011). An ample analysis of the orchestrated interaction of cytokines and chemokines during mastitis caused by a specific pathogen can help to better understand the regulation of the immune response in the udder to this pathogen (Schukken et al., 2011). Therefore, the identification of factors that contribute to the predisposition of the mammary gland to mastitis will facilitate the development of new strategies to control this disease, such as identification of genes that can be used as markers for resistance to mastitis in animal breeding programs.

To contribute to this effort, we applied the microarray and real-time PCR techniques for the main purpose of characterizing the gene expression in cells present in the milk of Gyr cows artificially infected with *Strep. agalactiae*. The aim of this characterization is to identify the genes responsible for conferring resistance to mastitis and understanding its regulation, for a better comprehension of the physiopathology of this disease. This knowledge can be used for more efficient treatment, especially in *B. taurus indicus* animals, where the references are scarce and limited in scope.

Material and Methods

Experimental animals

To select the animals of the experimental group, 105 Gyr dairy cows from the Getulio Vargas experimental farm of the Minas Gerais Agricultural Research Company, located in Uberaba (Minas Gerais, Brazil), were assessed regarding their history of mastitis between 2003 and 2007, order of calving and somatic cell count (SCC). Besides this, the animals were submitted to a series of three microbiological exams, and only the cows that presented a negative result to *Strep. agalactiae* on all three tests were selected, for a total of 17 animals, with different calving orders and at least 80 days of lactation.

The project was approved by the animal research ethics committee of the Embrapa Dairy Cattle Research Unit (Embrapa Gado de Leite), following the guidelines of Law 11,794/2008 and of Normative Resolution 1/2010 from the National Council for Control of Animal Experimentation of the Ministry of Science and Technology.

Inoculation and collection of the samples

Before experimentally induced intramammary infection, the 17 cows were submitted to clinical examination of the udder and the strip cup test after the first milking (procedure modified from Grönlund et al., 2006 and Lee et al., 2006). The cows were inoculated with strain 8137 of *Strep. agalactiae*, isolated in a pure culture from the milk of a cow with subclinical mastitis sensitive to β-lactam antibiotics, belonging to the microorganism collection of Embrapa Gado de Leite. The bacteria were seeded in plates containing blood agar medium, prepared with 5% defibrinated sheep blood, and afterward were transferred to brain heart infusion broth (BHI). To prepare the inoculum, the culture was diluted in 1X PBS (2.7 m*M* KCl; 2 m*M* KH_2PO_4; 137 m*M* NaCl; 10 m*M* Na_2HPO_4 pH 7.4) to a final concentration of 100 CFU/mL. The bacterial suspension was inoculated only in one mammary quarter of each cow (IQ-inoculated quarter). The other quarters were inoculated with sterile 1X PBS (NIQ-non-inoculated quarter). Milk samples were taken from the IQ before inoculation (time 0) and 24 hours after inoculation to perform the SCC and microbiological examination, to confirm the infection, and at 0, 4, 9 and 24 hours for extraction of RNA. Milk samples were also collected from the NIQ at 0 and 24 hours from each animal for RNA extraction. The milk samples were placed in isothermal boxes with ice for transport to the laboratory.

To minimize the adverse effects and assure the welfare of the animals, they were examined by a veterinarian before, during and after the experiment. Just after the last collection (24 hours after infection), a specific intramammary antibiotic to which the strain was sensitive was administered. From the start of the experiment and after the antibiotic treatment, the milk was discarded to comply with the waiting period. Besides this, the cows were kept isolated from the others on the farm and were only incorporated into the herd after confirmation of a cure by clinical and microbiological examination, which was repeated three times (30, 40 and 55 days post-infection). All the animals showed negative results for *Strep. agalactiae* after the third microbiological examination after antibiotic treatment. The microbiological and clinical examinations confirmed that the symptoms presented by the animals were from infection by *Strep. agalactiae*.

Isolation of RNA and quality control

The total RNA was extracted from the lysed milk cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), like as Leutenegger et al. (2000) and Lee et al. (2006). Aliquots of 15 mL of milk were centrifuged at 6,000 g for 10 min at room temperature. The pellets were washed twice in 7.5 mL PBS, ressuspended and lysated with 350 mL of lysis buffer according to the manufacturer's recommendations. After this step, the lysates were kept at -80°C until RNA extraction. Digestion with DNase I (Qiagen) was performed to remove any contamination with genomic DNA. The concentrations of the samples were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality of the total RNA was evaluated with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). As a procedure for standardization of RNA quality control, the average value of RIN (RNA integrity number) was 8. All the samples were stored at -80 ºC until use.

Large-scale gene expression experiments

Aliquots of the total RNA quantified and evaluated to assure integrity were hybridized with an Affymetrix GeneChip Bovine Genome Array chip (Affymetrix, Santa Clara, CA, USA). This chip contains over 23,000 bovine transcripts (the complete list can be accessed at http://www.affymetrix.com/support/technical/byproduct.affx?product=bovine). A GeneChip 3000 7G scanner (Affymetrix) was used to capture the images corresponding to the signal intensity from the hybridization, operated with the GeneChip Command Console software, and the Expression Console software (Affymetrix) was used to determine the hybridization quality. Computational packages from R (R Development Core Team, 2012) were used to search for differentially expressed genes. The *affy*, *simpleaffy* and *affyQCReport* packages, part of the Bioconductor project (Gentleman et al., 2004), available at http://bioconductor.org/biocLite.R, were used for quality control of each chip. Besides this, to achieve greater quality control and for pre-processing (transformation, background correction and summarization), of the data from the GeneChip, the RMAExpress freeware was used, available at http://rmaexpress.bmbolstad.com.

The chips considered to be of low quality (those that did not present a uniform pattern of RNA integrity, background value or mean fluorescence intensity,

as well as those that presented low correlation of the level of expression with other arrangements of the same experimental treatment) were eliminated from the analysis. Tests were performed to compare the level of gene expression of the animals at each sampling time, for a total of six contrasts for the IQ (0 h x 4 h; 0 h x 9 h; 0 h x 24 h; 4 h x 9 h; 4 h x 24 h; 9 h x 24 h) and the interaction between treatment and expression of a determined gene was analyzed using the Maanova package (Wu et al., 2003). These analyses relied on the mixed model approach in two steps proposed by Wolfinger et al. (2001), with array and sample as random effects and treatment (time after infection), gene and gene by treatment interaction as fixed effects. The significance tests were based on a t-test regularized by applying the James-Stein concept (Cui et al., 2005) with critical values defined by 500 permutations of the data. The p-values were then adjusted to control the rate of false discoveries by the method of Storey (2002).

Finally, to visualize the gene expression profiles of the genes differentially expressed over time (0, 4, 9 and 24 hours), we constructed a Heatmap of the summarized expressions of each gene at each of the times using the R software and its Heatplus package (http://www.bioconductor.org/packages/2.10/bioc/html/Heatplus.html). The summarized expression of each gene was obtained by using the median value corresponding to each of the times, rescaled to have mean zero and standard deviation of one. To construct the hierarchical cluster of genes, associated with the Heatmap, we used absolute correlation as the measure of dissimilarity and the average linkage clustering strategy.

Validation of the genes differentially expressed by real-time PCR

Among the genes identified as differentially expressed in the microarray experiment, we selected eight for validation by real-time PCR because they perform important functions in the immune response (*AATK - apoptosis-associated tyrosine kinase*; *CCL2- chemokine C-C motif ligand 2*; *CCL20 - chemokine C-C motif ligand 20*; *CD40 - CD40 molecule*; *CSF2 - colony stimulating factor 2*; *IL-1β - interleukin 1β*; *INHBA - inhibin beta A*; and *NOS2A - nitric oxide synthase 2A*). Besides these eight genes, we evaluated the expression of six others by real-time PCR, even though they did not present a significant difference by the microarray technique (*IL-12 - interleukin 12*; *IL-17 - interleukin 17*; *TLR-2 - Toll-like receptor 2*; *TLR-4 - toll-like receptor 4*; *GRO-α - growth related oncogene α*; and *TGF-β1 - transforming growth*

factor β1). We analyzed these genes' expression because according to some published articles, they can have important functions in the immune response to mastitis (Riollet et al., 2006; Lahouassa et al., 2007; Oviedo-Boyso et al., 2007).

For the real-time PCR, the first strand cDNA was synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), which includes RNase H. Reverse transcription was carried out on a control sample, which contained a mix of all the RNA samples extracted and all the components of the kit, except the SuperScript III RT enzyme, to confirm the absence of any contamination by genomic DNA during the real-time PCR. The cDNA synthesized was then stored at -20 ºC until use.

The real-time PCR reactions were performed with the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). The reactions were performed twice for each sample and amplified with the 7300 Real-Time PCR System (Applied Biosystems). Each reaction contained cDNA, a pair of primers (a concentration was optimized for each target) and PCR Master Mix in a final volume of 25 µL. A mixture containing all the PCR components, without cDNA, was used as a negative control for each reaction, besides the reverse transcription control, in which the sample that did not contain the SuperScript III RT enzyme was used as a template in the real-time PCR. The amplification conditions were 2 minutes at 50 ºC, 10 minutes at 95 ºC, followed by 40 cycles of 15 seconds at 95 ºC and 1 minute at 60 ºC. At the end of each reaction, a denaturation curve was plotted to assure that each reaction produced a single fragment, that is, the curve contained only one dissociation peak.

The primers used to evaluate the expression of the genes are listed in Table 1. They were designed using the Primer Express program (Applied Biosystems) from the sequences obtained in the GenBank (http://www.ncbi.nlm.nih.gov) or data in the literature. The genes *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*), *β-actin, 18S rRNA, Ubiquitin, RPLP0* (*ribosomal protein large P0*) and *HPRT* (*hypoxanthine-guanine phosphoribosyltransferase*) were tested for endogenous control. All the endogenous controls were evaluated regarding the shape of the amplification and dissociation curves. The β-actin gene presented more than one dissociation peak and was excluded from the subsequent analyses. The other five genes were analyzed with the geNorm program (Vandesompele et al., 2002) and the

two endogenous controls considered the most stable were *Ubiquitin* and *RPLP0*, as can be seen in Figure 1.

By the real-time PCR technique, besides the contrasts in the microarray analyses for the IQ (0 h x 4 h; 0 h x 9 h; 0 h x 24 h; 4 h x 9 h; 4 h x 24 h; 9 h x 24 h), three other contrasts were considered for the NIQ (0 h NIQ x 0 h IQ; 0 h NIQ x 24 h NIQ; 24 h NIQ x 24 h IQ). The cycle threshold (Ct) data obtained during the real-time PCR were exported to an Excel spreadsheet and analyzed with the REST 2009 program, developed by M. Pfaffl (Munich Technical University) and Qiagen, to obtain data on reaction frequency and to compare the difference in expression between treatments. This program can be downloaded from http://www.qiagen.com/products/rest2009software.aspx#Tabs=t2 and permits analysis of the differential expression with efficiency correction for each gene and with normalization for more than one endogenous control, besides being adequate for data whose variance can be large and that are not normally distributed. The coefficient of variation of the duplicates of the Ct of each sample did not exceed 5% and the amplification efficiency of all the genes considered in this study were close (data not shown).

Construction and analysis of the gene networks

The data obtained from the hybridization of the microarrays were also analyzed using the Weighted Gene Co-expression Network Analysis (WGCNA) method (Langfelder and Horvath 2008). WGCNA is a method that identifies correlation patterns between genes and has been widely used to find clusters (called modules) of highly correlated (or co-expressed) genes, which can be involved in a single biological process. Therefore, the groups of genes that are differentially co-expressed or genes that are differentially connected between two groups are identified. These changes in the co-expression or connectivity pattern can correspond to a response to environmental variations or physiological disturbances.

Two sets of microarray data (0 and 24 hours after inoculation of the IQ), with five animals each, were pre-processed using the *affy* and *gcrma* packages (Bioconductor) with the R software. Then a filter was applied so that only the transcripts present in all the samples were considered. By means of the method proposed by Fuller et al. (2007), the gene co-expression networks were identified separately for each group (0 and 24 hours), by means of the WGCNA package in the

R software, and then these networks were compared to identify the modules not conserved between the two groups.

Results

The microbiological analysis of the milk samples from all the animals revealed the presence of *Strep. agalactiae* in the IQ 24 hours after intramammary inoculation, and there was a sharp increase in the SCC between 0 and 24 hours, which can be observed in Figure 2, supported by the Mann-Whitney test with $P < 0.001$. These results assure that the experimental infections were successful. Furthermore, all the cows presented clinical mastitis 24 hours after intramammary inoculation.

Of the 68 RNA samples extracted, 48 were selected according to the RNA quality for hybridization in the microarray chips. Of these, 21 were considered of poor or marginal quality regarding the percentage of present transcripts, background level, integrity of the endogenous control genes and RNA degradation, according to the statistics generated by the Bioconductor and RMAExpress packages. Hence, just 27 arrays that have passed all quality control checks were considered in the subsequent statistical analyses, remaining 12, eight, four and three chips for times 0, 4, 9 and 24 hours, respectively.

All told, 32 transcripts presented difference in the expression level ($P < 0.05$) in the IQ when analyzed by the microarray technique. However, differences in the expression level were only identified between 0 and 4 hours of the IQ samples, as can be observed in Table 2.

In the real-time PCR analysis, all the genes presented a significant difference ($P < 0.05$) in at least one of the comparisons made, as shown in Table 3. Besides this, the genes presented a similar expression profile by microarray and real-time PCR, i.e., the eight genes with significant expression difference by microarray had their expression increased at 4 hours in relation to hour 0, a condition confirmed by the real-time PCR technique in the samples collected from the IQ.

The gene network analysis identified 17 modules of co-expressed genes, three of them not conserved between the groups (0 and 24 hours after inoculation of the IQ). These modules included 35, 37 and 192 genes, respectively. In the functional enrichment analysis, carried out with the Blast2GO software (http://www.blast2go.com/b2ghome), each of these modules presented distinct

characteristics. Those in the first module (*BTG3*, *CD3E*, *MBD1*, *CHIC2*, *PLXNA3*, *MOCS3*, *NEIL1*, *VPS45* and *BCL2*) were related to apoptosis and to antigen recognition. The second module was enriched in inflammatory mediators, including genes considered to be markers of mastitis (*FGL1*, *GJA1*, *F2RL1*, *PTPRF*, *S100A2*, *TGF-β2*), while the third module included genes involved in cell division and inflammatory responses (*CD97*, *MAD2L1*, *ZFP106*, *CDKN2C*, *LOC514364*, *NOP14*, *PCBD1*, *LOC100139798*, *AP1S1*, *EDN1*, *IL-1β*, *ANXA11*).

Figure 3 contains the Heatmap of the expression profiles of the differentially expressed genes, obtained by the microarray technique. As can be observed, the greatest expression difference among the genes identified by this technique occurred in the first four hours after infection, with the majority of the genes being upregulated at hour 4. In general, the differentially expressed genes showed an increase in expression in the first four hours after inoculation, and soon afterward the level of expression declined, in some cased gradually, such as *DCN* (decorin) and *FGF2* (fibroblast growth factor 2), and in others abruptly, like *CH25H* (cholesterol 25 hydroxylase) and *GJA1* (gap junction protein alpha 1). Other genes maintained relatively constant expression level after the four-hour mark, such as *IL-1β* and *NOS2*.

In the present study, we noted that *NOS2A* gene's expression is altered following experimentally induced infection by *Strep. agalactiae*, with the expression at 4 hours being twice that at hour 0 according to the microarray technique ($P < 0.05$ - Table 2) and up to 12 times higher 24 hours after inoculation according to real-time PCR ($P < 0.01$ - Table 3). And also we observed an increase in the expression of *CD40*, both by the microarray technique and real-time PCR ($P < 0.05$ and $P < 0.001$, respectively). By the microarray technique, its expression increased almost twofold at 4 hours in relation to hour 0 (Table 2) and by real-time PCR it presented significantly higher expressions at 4, 9 and 24 hours after inoculation, but no significant differences between these three times ($P > 0.05$ - Table 3). As can be seen in Figure 4a, this gene was one of those that maintained high expression during practically the entire period analyzed in the experiment, together with *INHBA* (Figure 4b). Figure 4a also shows the increase in the expression of the *AATK* gene after inoculation ($P < 0.001$) as well in the expression of the *CSF2* gene ($P < 0.05$), in at least one of the contrasts carried out.

Among the differentially expressed genes by the real-time PCR, both the *TLR-2* and *TLR-4* genes showed a significant increase in the expression level (about six-fold) between 0 and 24 hours ($P < 0.001$ and $P < 0.01$, respectively), as can be observed in Figure 4b and Table 3. This difference also was observed between 4 and 24 hours, with the same probability values, and also between 9 and 24 hours ($P < 0.01$ and $P < 0.05$, respectively), showing there was a gradual increase in the expression of these genes up to 24 hours after inoculation of the pathogen (Figure 4b). This situation is even more evident when comparing the NIQ with the IQ in Figure 5, in both cases 24 hours after inoculation, showing greater expression of *TLR-2* and *TLR-4* in the IQ ($P < 0.001$ and $P < 0.05$, respectively), i.e., at the infection site.

In general there was an increase in the expression of *TGF-β1* at all times analyzed in relation to hour 0, with a significant difference ($P < 0.05$) in the 0 h x 4 h and 0 h x 24 h contrasts for the IQ (Table 3). At 9 hours, there was a decline in the levels of *TGF-β1*, which then increased at 24 hours, a fact shown by the twofold increase in this gene's expression ($P < 0.05$ - Figure 4b and Table 3). For this reason, there was downregulation between 4 and 9 hours ($P < 0.05$) and no significant difference between 0 and 9 hours ($P > 0.05$).

We noted a significant twofold increase in the expression of *IL-1β* gene by the microarray technique 4 hours after inoculation ($P < 0.05$ - Table 2), a finding validated by real-time PCR, in which *IL-1β* presented 25 times greater expression in the same time frame ($P < 0.001$ - Table 3). However, this difference in the level of expression diminished with time passed thereafter, being 12 times higher after 9 hours ($P < 0.001$) and 8 times higher after 24 hours ($P < 0.001$) (Table 3 and Figure 4a). Therefore, between 4 hours and 24 hours after inoculation, the expression of *IL-1β* decreased by a factor of 3 ($P < 0.01$), but it was still 8 times higher than before experimentally induced intramammary infection. We also noted a difference in the expression of this cytokine between hours 0 and 24 from the samples collected from the NIQ (approximately four times greater, $P < 0.05$ - Table 3). However, the difference was greater (about eight times) in the milk from the IQ ($P < 0.001$), showing that the main expression of *IL-1β* occurs at the infection site (Figure 4a and Table 3).

In this work, the expression of *IL-17*, a key component of the T_H17 response, also increased when analyzed by real-time PCR, being differentially expressed in

nearly all the contrasts ($P < 0.05$), except between 9 and 24 hours, despite showing a trend to continue increasing (Table 3 and Figure 4b). This cytokine's expression increased up to 23 times between 0 and 24 hours in the IQ samples ($P < 0.001$), but this gene did not present a significant difference in the microarray analyses.

We noted that the expression of *GRO-α* increased at hour 4 in relation to hour 0 and at hour 24 in relation to hours 0, 4 and 9, with the greatest difference being between 0 and 24 hours, in which the expression was nearly seven times higher ($P < 0.001$ - Table 3 and Figure 4a). Another cytokine, IL-12, presented increased expression by real-time PCR at hours 4, 9 and 24 in relation to hour 0 and at hour 24 in relation to hours 4 and 9. Only not presenting a significant difference ($P > 0.05$) between hours 4 and 9 (Table 3 and Figure 4b). Along with IL-17, IL-12 was one of the cytokines that presented the highest expression levels at hour 24, about 22 times higher than at hour 0 ($P < 0.001$).

Both the microarray and real-time PCR analyses revealed variations in the expression of the mRNA from the chemokines CCL2 and CCL20 (Tables 2 and 3). The expression profile of *CCL2* was very similar to that of *TGF-β1*, with reduced expression at hour 9 in relation to the base level of this chemokine, but with predominance of higher expression levels in relation to hour 0 during the entire period of the experiment (Figure 4a). A similar profile was also observed for the *CCL20* gene, but its peak expression occurred at hour 24, while the peak of *CCL2* happened at hour 4 (Figure 4a).

In general we observed a significant difference ($P < 0.05$) in the expression of five genes among the samples from the IQ and NIQ before inoculation (Table 3 and Figure 6) and nine genes considered in this study 24 hours after inoculation between the IQ and NIQ (Table 3 and Figure 5).

Discussion

The immune response in the first hours after infection is of great importance, because the host has to respond quickly and effectively against the invading pathogen. Generally the reaction against pathogens present in the mammary gland is governed by responses that occur within a few hours of initial infection (Bannerman 2009). For this reason, we expected a large difference in the gene expression profile in the first hours after inoculation of the strain. Furthermore the results of gene

network analysis show there are mechanisms that were altered in the Gyr cows after infection of the mammary gland by *Strep. agalactiae*.

Toll-like receptors play a major role in the innate immunity of the mammary gland against pathogenic microorganisms. The discovery these receptors and their function in the immune response opened a new field of study to better understand the primary immune response of the mammary gland (Schukken et al., 2011). These Toll-like receptors, located in the endosomal membrane, recognize the structures of lipopolysaccharides (LPS), peptidoglycan (PGN) and lipoteichoic acid (LTA), which constitute the pathogen-associated molecular patterns (PAMPs) of both gram-positive and gram-negative bacteria. TLR-4 recognizes LPS from gram-negative bacteria while TLR-2 recognizes the LTA and PGN present in gram-positive bacteria, such as *Strep. agalactiae* (Oviedo-Boyso et al., 2007). Recognition of the pathogen is the critical first step to mount an effective immune defense against the invading microorganism (Rinaldi et al., 2010). When activated, each TLR triggers a signaling route, leading to activation and development of a specific immune response to each pathogen (O'Neill 2006), increasing the expression of these types of receptors (Petzl et al., 2008). In the mammary gland, the immune system cells, together with the epithelium cells, are activated through recognition of the PAMPs of the invading microorganism via TLRs (Rainard and Riollet 2006; Griesbeck-Zilch et al., 2008). As in the present study, Goldammer et al. (2004) found an increase in the expression of both *TLR-2* and *TLR-4* in udders infected with *Staphylococcus aureus*, a gram-positive bacterium, as is *Strep. agalactiae* and suggested that the expression of these two receptors is regulated jointly during the start of the immune response in the udder. Other authors have also observed an increase in the expression of *TLR-2* and *TLR-4* in mammary tissue after experimental *in vivo* infection with *Escherichia coli* (Petzl et al., 2008), and when mammary cells are exposed *in vitro* to *E. coli* inactivated by heat (Griesbeck-Zilch et al., 2008). The activation of Toll-like receptors leads to the expression of inflammatory cytokines and other mediators involved in the immune response, cell differentiation and apoptosis (Ibeagha-Awemu et al., 2008; Yang et al., 2008; Cates et al., 2009).

The *NOS2A* gene also participates in the innate immune response and its expression is regulated by signals from Toll-like receptors (Günther et al., 2009). Nitric oxide acts as a bactericide and is important for the action of phagocytes in killing microorganisms. Lutzow et al. (2008) also found an increase in the expression

of *NOS2A* in mammary tissue infected by *Staph. aureus*, while Günther et al. (2009) noted the same effect when the infection was caused by *E. coli*. The *CD40* gene is a member of the superfamily of tumor necrosis factor (TNF) receptors and codifies a protein cell membrane receptor that has a synergetic role in the signaling triggered by Toll-like receptors. Because this gene is a co-stimulator of both T and B lymphocytes, our result can indicate that these lymphocytes were stimulated during the experimental period. Other studies have shown that the interaction between CD40 and its ligand (CD154) is an essential mechanism to activate regulatory B cells, i.e., producers of IL-10 (Mauri and Bosma 2012). In cattle, the B cells stimulated via CD40 and in the presence of TGF-β and IL-2 increase the production of IgA (Estes et al., 1998), an important antibody isotype that is responsible for immunity of the mucosa, of which the lactating mammary gland is part (Fagarasan and Honjo 2004; Brandtzaeg, 2010).

Although inflammation is an essential component of the host's response to intramammary infection, a prolonged inflammatory response can damage the mammary secretory epithelium and cause a permanent reduction in milk production (Long et al., 2001; Paape et al., 2003). In this context, the participation of anti-inflammatory cytokines such as IL-10 and members of the TGF-β family (Grutz 2005) is extremely important to minimize the effects of inflammatory cytokines. In agreement with our results, Chockalingam et al. (2005) noted an increase in the expression of the protein TGF-β1 in milk from cows with udders infected with *E. coli*, while Bannerman et al. (2005) found the same effect when the mastitis was experimentally induced by *Pseudomonas aeruginosa*. However, Lahouassa et al. (2007) did not find any variation in the expression of *TGF-β1* in mammary epithelial cells infected *in vitro* by *Staph. aureus* or *E. coli*, and suggested that perhaps these cells are not a source of the TGF-β1 found in the milk samples following experimentally induced infection. We should point out that in this work the RNA was obtained from somatic cells present in the milk, so cells from various sources can be present, explaining the apparent divergence in results. Besides this, we used zebu animals in this study, unlike others breeds used in previous studies. TGF-β has immunosuppressive activity, but also has effects on cell proliferation and differentiation. It has been suggested that during infection of the mammary gland, TGF-β is probably produced by leukocytes that invade the udder (Schukken et al.,

2011). For this reason, we expected there to be an increase in the expression of this gene with the increase in the number of somatic cells.

The *INHBA* gene is a ligand of the TGF-β superfamily (Gaddy-Kurten et al., 1995) and also has a function related to apoptosis, as does the *AATK* gene. Sheffield (1997) found that induction of apoptosis in the mammary gland after experimental infection with *Strep*. *agalactiae*, could explain the increase in the expression of these genes. It is known that damage to the mammary tissue can be induced by apoptosis or necrosis (Zhao and Lacasse 2008), and curiously, when there is an increase in the number of apoptotic cells there is also a higher proliferation of epithelial cells, which can be a mechanisms to maintain alveolar integrity (Kerr and Wellnitz 2003).

IL-1β is one of the most important inflammatory cytokines because it acts as a mediator of both the systemic and local immune response (Schukken et al., 2011). Our results shows the importance of this cytokine in the immediate response to mastitis in Gyr dairy cows, with its expression peaking four hours after infection. These results are in agreement with the findings of Strandberg et al. (2005) who showed that the expression of the mRNA of *IL-1β* increased rapidly in the first two hours after *in vitro* stimulation of mammary epithelial cells by LPS or LTA. However, this level remained relatively stable thereafter until the cells were stimulated by LPS (a component of gram-negative bacteria) and reduced gradually when they were stimulated by LTA (present in gram-positive bacteria such as *Strep*. *Agalactiae*). Lahouassa et al. (2007) also observed peak expression of *IL-1β* in the first hours after infection (3 hours) when mammary epithelial cells were stimulated with different strains of *Staph*. *aureus*. However when these cells were stimulated *in vitro* with *E. coli*, the peak expression of this cytokine occurred at 24 hours afterward. This can indicate its function in both the local and systemic immune response to mastitis. According to Schukken et al. (2011), IL-1β not only regulates the expression of a variety of genes involved in the immune response, but also genes involved in cell proliferation and apoptosis. Because it is a key component of the innate immune response (Lahouassa et al., 2007), we expected an increase in the expression of this cytokine in the first hours after infection. IL-1β, together with other cytokines, promotes an increase in vascular permeability, favoring the recruitment of leukocytes and inducing hepatic synthesis of proteins that enhance the recognition of bacteria (Bannerman et al., 2006) and stimulate the production of

prostaglandins and leukocytes, which increase the inflammatory reaction at the site (Oviedo-Boyso et al., 2007).

According to Kolls et al. (2008), pro-inflammatory cytokines like IL-1β can facilitate induction of the T_H17 response, which in turn induces the antimicrobial response by epithelial cells and recruits neutrophils and dendritic cells. The T_H17 cells, when in contact with antigens at the infection site, are stimulated to synthesize and release cytokines such as IL-17. Originally IL-17 was considered to be a cytokine able to induce inflammation mediated by neutrophils only in acquired immunity, but today it is considered very important both in innate and acquired immunity (Matsuzaki and Umemura 2007). The pro-inflammatory nature of this cytokine has been related to various inflammatory reactions, and in the mammary gland of cattle it is possibly related to the recruitment of neutrophils in the presence of chronic infection caused by *Staph*. *aureus* (Riollet et al., 2006). *In vivo* studies conducted by Tao and Mallard (2007) also show the transcription of *IL-17* induced by *Staph*. *aureus* in mammary gland cells. Other cytokine, the IL-12, a mediator between innate and acquired immunity, is produced by T lymphocytes and dendritic cells. This protein regulates the differentiation of T lymphocytes and acts as an endogenous adjuvant in the recruitment of neutrophils, which upon arriving at the infection site phagocytose the invading bacteria and release antibacterial peptides, among other compounds (Oviedo-Boyso et al., 2007). Our results corroborate the idea that IL-17 and IL-12 also have a pro-inflammatory function during bovine mastitis caused by *Strep*. *agalactiae* in zebu animals

IL-17 induces cells such as fibroblasts, epithelial cells and keratinocytes to secrete various cytokines, among them CSF, that stimulates the bone marrow to increase the production of neutrophils and macrophages (Ye et al., 2001); and GRO-α (Riollet et al., 2006) which acts to mobilize neutrophils and is produced by a variety of cells after stimulation with LPS, IL-1 and TNF. Lahouassa et al. (2007) also observed an increased expression of *GRO-α* in mammary epithelial cells stimulated with different strains of *Staph. aureus* and *E. coli*. However, the peak production of this cytokine observed by those authors happened 3 hours after infection with the different strains of *Staph. aureus* and 24 hours after infection by *E. coli*, a similar pattern to that found here with *Strep*. *agalactiae*, in which the peak expression of *GRO-α* occurred 24 hours after inoculation of the pathogen. These results suggest that different species of bacteria generate distinct patterns of immune

response to mastitis, making it necessary to study the gene expression in response to mastitis caused by different species of pathogens, both in taurine and zebuine animals.

Chemokines are a specific class of cytokines that mediate the recruitment of effector cells to the inflammation site. CCL2 is the key chemokines for recruitment of monocytes which compose the second line of cellular immune defense. It is important for innate immunity and also helps initiate the humoral immune response (Schukken et al., 2011). Only a few studies have reported inflammation induced by the increased expression of CCL2 in the udder and mammary epithelial cells (Strandberg et al., 2005; Lutzow et al., 2008; Mount et al., 2009). The present study presents the first report of an increase in the expression of this gene during mastitis caused by *Strep*. *agalactiae* in zebuine cattle. CCL20 is a chemokine that interacts with the CCR6 receptor (Schutyser et al., 2003) and the CCL20-CCR6 pair is responsible for the chemoattraction of immature dendritic cells, T effector cells and B cells. The recruitment of these cell types provides the link to the humoral immune response (Schukken et al., 2011). Other studies have also revealed an increase in the expression of *CCL20* in the udder and mammary epithelial cells infected by *E. coli* (Günther et al., 2009, 2010).

There was clear individual variation among the animals, because some presented much greater changes in the number of somatic cells than others. This variation of initial response might also have happened in the expression of the genes analyzed before inoculation of the pathogen. Base expression levels of some cytokines have also been found in *in vitro* experiments (Okada et al., 1997; Lahouassa et al., 2007), which indicates that other components of the culture medium can influence the expression of some genes. For *in vivo* experiments, in which it is even harder to control the conditions, this difference in expression even before inoculation is understandable and cannot be taken as a failure of experimental control. In addition, because the lactating mammary gland is considered part of the mucosal immune system (Brandtzaeg 2010), it has some particularities. It is estimated that at least 90% of the microorganisms that infect humans use the mucosa for entry (Brandtzaeg 2010). For this reason, this system contains a large number of effector lymphocytes, even in the absence of disease (Tanoue et al., 2010). In counterpart, there are powerful regulatory mechanisms that prevent these local responses from getting out of control (Bailey et al., 2001). This can be one more

reason for the difference in expression between the inoculated and non-inoculated quarters at hour 0. In turn, the difference in the expression of the genes considered in this study 24 hours after inoculation between the IQ and NIQ can be an indication that the genes *AATK*, *CCL2*, *CCL20*, *CD40*, *GRO-α*, *IL-17*, *INHBA*, *TLR-2* and *TLR-4* are mainly expressed at the infection site, at the place of action of their products.

During bacterial infection of the bovine mammary gland, a large number of leukocytes migrate to the udder to establish the response against the pathogen. However, the population of leukocytes that mediate this immune response is not yet well defined (Schukken et al., 2011). Studies of gene expression related to the immune response can help to better characterize this population and to understand how the host responds to a determined pathogenic microorganism. Besides this, the protective immunity of the bovine mammary gland against natural infection by bacteria is relatively brief, only lasting a few weeks (Suojala et al., 2008; Schukken et al., 2009). This incapacity of long-term defense against natural infection poses a significant challenge to the development of effective vaccines against mastitis (Schukken et al., 2011). Once again, the knowledge generated by studies of the expression of candidate genes can help in the selection of better adapted and more productive animals, enabling a reduced need to administer drugs, with consequent reduction of production costs and levels of contamination of dairy products and the environment.

In this study it was possible to verify changes in the expression of at least 14 genes related to the immune response of zebuine animals against mastitis caused by *Strep. agalactiae*. These genes can play important roles in fighting intramammary infection and maintaining the tissue during infection. However, besides carrying out proteomic studies, it is necessary to validate these results in other biological models to identify which tissues and mechanisms are involved in the response to infection. After this validation, the gene structure will be more useful in the identification of marker SNPs for the phenotypes for resistance and susceptibility to mastitis in Gyr animals.

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Table 1. Sequences of primers employed in the real-time PCR

Gene	Forward primer	Reverse primer	Reference or ID[1]
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	Wang et al., 2005
AATK	TGGTGGCCGTGTCCTTCT	ACCCGATGCCTCCCTTCTTA	XM_588863
β-actin	CGCACCACTGGCATTGTCAT	TTCTCCTTGATGTCACGCAC	Singh et al., 2008
CCL2	AGTCACCAGCAGCAAGTGTCCTAA	TTCTTGGGTTGTGGAGTGAGTGCT	NM_174006
CCL20	CAGCAAGTCAGAAGCAAGCAA	CCCACTTCTTCTTTGGATCTGC	Günther et al., 2009
CD40	AGGGCACCTTGAATACAGACACCA	ATGACACGTTGGAGAAGAAGCCGA	NM_001105611
CSF2	CTCGCCTGAAGCTGTACAAGAA	TCGTAGTGGGTGGCCATCA	NM_174027.2
GAPDH	CCTGGAGAAACCTGCCAAGT	GCCAAATTCATTGTCGTACCA	Mount et al., 2009
GRO-α	CGCCTGTGGTCAACGAACT	CTTCACGCTCTGGATGTTCTTG	NM_175700
HPRT	GCCGACCTGTTGGATTACAT	ACACTTCGAGGGGTCCTTTT	Tao et al., 2004
IL-12	TTAATTGAGGTCGTGGTAGAAGCTG	GGTCTCAGTTGCAGGTTCTTGG	Leutenegge et al., 2000
IL-17	AGATATCCCTCTGTGATCTGGGAAG	CAGGACCAGGATCTCTTGCTGGATG	Riollet et al., 2006
IL-1β	CTCTCACAGGAAATGAACCGAG	GCTGCAGGGTGGGCGTATCACC	Lahouassa et al., 2007
INHBA	GGAGGGCAGAAATGAATGAACTTAT	TCTTCCTGGCTGTGCCTGAT	NM_174363.2
NOS2A	TTGATTGCACCGCTTGGA	TCATACAAGGAAGGCCCAAGAG	NM_001076799
RPLPO	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	Mount et al., 2009
TGF-β1	CCTGAGCCAGAGGCGGACTAC	GCTCGGACGTGTTGAAGAAC	Lahouassa et al., 2007
TLR-2	CAGTTTAACCCAGTGCCTTC	CTCCAACGTCTTCAGTTGCT	Ibeagha-Awemu et al., 2008
TLR-4	ACTGCAGCTTCAACCGTATC	TAAAGGCTCTGCACACATCA	Ibeagha-Awemu et al., 2008
Ubiquitin	GGCAAGACCATCACCCTGGAA	GCCACCCCTCAGACGAAGGA	Singh et al., 2008

[1]ID: GenBank accession number (http://www.ncbi.nlm.nih.gov).

Table 2. Genes differentially expressed by the microarray technique in the inoculated quarters

Gene symbol	Gene name	Fold change	P[1]
INHBA	inhibin, beta A	3.16	0.02
Gene not annotated		3.13	0.01
CH25H	cholesterol 25-hydroxylase	3.06	0.01
CCDC80	coiled-coil domain containing 80	2.84	0.03
IL-1β	interleukin 1, beta	2.63	0.03
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	2.19	0.03
CLDN1	claudin 1	2.11	0.04
NUAK1	NUAK family, SNF1-like kinase, 1	2.08	0.03
IL-1β	interleukin 1, beta	2.05	0.03
AATK	apoptosis-associated tyrosine kinase	2.01	0.03
NOS2	nitric oxide synthase 2, inducible	2.01	0.04
CCL20	chemokine (C-C motif) ligand 20	1.94	0.04
CCL2	chemokine (C-C motif) ligand 2	1.93	0.04
FGF2	fibroblast growth factor 2 (basic)	1.88	0.04
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	1.85	0.04
CD40	CD40 molecule, TNF receptor superfamily member 5	1.82	0.03
TFRC	transferrin receptor (p90, CD71)	1.77	0.03
CISH	cytokine inducible SH2-containing protein	1.76	0.03
PROCR	protein C receptor, endothelial	1.76	0.02
CFB	complement factor B	1.73	0.04
Gene not annotated		1.73	0.03
DCN	decorin	1.7	0.03
IFI44	interferon-induced protein 44	1.63	0.04
NOS2	nitric oxide synthase 2, inducible	1.63	0.05
TAP	tracheal antimicrobial peptide	1.61	0.04
PDXK	pyridoxal (pyridoxine, vitamin B6) kinase	1.59	0.05
TM4SF1	transmembrane 4 L six family member 1	1.57	0.03
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	1.55	0.04
GJA1	gap junction protein, alpha 1, 43kDa	1.55	0.03
IL-1F6	interleukin 1 family, member 6 (epsilon)	1.53	0.04
CCNE2	cyclin E2	-1.76	0.03
Gene not annotated		-2.20	0.01

[1]P = adjusted p-values.

Table 3. Relative expression by the real-time PCR technique

		Gene													
		AATK	**CCL2**	**CCL20**	**CD40**	**CSF2**	**GRO-α**	**IL-12**	**IL-17**	**IL-1β**	**INHBA**	**NOS2A**	**TGF-β1**	**TLR-2**	**TLR-4**
0 h IQ[1] x 4 h IQ	Relative expression[3]	0.53	3.53	1.84	7.07	3.06	2.68	3.31	3.98	25.26	6.78	0.99	1.86	1.58	0.76
	Standard error	0.06 - 5.14	1.17 - 11.60	0.44 - 8.14	1.42 - 35.11	0.62 - 9.94	0.53 - 9.57	0.56 - 18.83	0.67 - 19.90	3.35 - 208.24	1.68 - 31.49	0.17 - 6.20	0.56 - 6.43	0.63 - 3.75	0.07 - 9.08
	Probability[4]	0.243	< 0.001	0.097	< 0.001	0.002	0.042	0.012	0.002	< 0.001	< 0.001	0.971	0.038	0.052	0.632
0 h IQ x 9 h IQ	Relative expression	2.8	1.43	1.4	11.5	1.88	1.77	2.36	10.03	12.36	6.56	1.59	1.16	1.55	1.3
	Standard error	0.11 - 61.75	0.41 - 4.76	0.28 - 6.67	2.98 - 48.81	0.31 - 8.93	0.37 - 6.46	0.40 - 13.52	1.75 - 65.24	1.63 - 88.45	1.51 - 29.82	0.24 - 10.06	0.40 - 3.84	0.46 - 3.96	0.11 - 14.35
	Probability	0.185	0.241	0.37	< 0.001	0.177	0.267	0.049	< 0.001	< 0.001	< 0.001	0.292	0.605	0.181	0.641
0 h IQ x 24 h IQ	Relative expression	11.17	2.07	13.21	8.47	8	6.47	21.94	23.12	7.55	8.51	12.37	2.14	6.34	5.84
	Standard error	0.80 - 97.62	0.56 - 7.56	0.65 - 178.89	1.48 - 44.72	0.98 - 67.21	1.21 - 21.59	1.07 - 387.90	1.40 - 502.61	1.12 - 49.31	1.52 - 50.22	0.45 - 259.61	0.58 - 7.10	1.71 - 28.48	0.50 - 64.57
	Probability	< 0.001	0.029	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.003	0.025	< 0.001	0.009
4 h IQ x 9 h IQ	Relative expression	5.25	0.4	0.76	1.87	0.62	0.66	0.72	2.52	0.49	0.97	1.61	0.63	0.98	1.73
	Standard error	0.22 - 173.28	0.12 - 1.51	0.24 - 2.90	0.57 - 8.81	0.08 - 4.39	0.20 - 2.21	0.15 - 2.85	0.48 - 11.91	0.08 - 3.13	0.26 - 3.14	0.39 - 6.24	0.26 - 1.58	0.30 - 2.57	0.15 - 21.49
	Probability	0.057	0.006	0.383	0.067	0.369	0.188	0.357	0.033	0.12	0.912	0.167	0.041	0.94	0.346
4 h IQ x 24 h IQ	Relative expression	20.93	0.59	7.18	1.2	2.62	2.41	6.63	5.81	0.3	1.26	12.56	1.15	4	7.72
	Standard error	1.27 - 413.76	0.14 - 2.32	0.44 - 96.11	0.30 - 6.54	0.21 - 27.52	0.71 - 8.32	0.35 - 121.26	0.37 - 99.82	0.05 - 1.77	0.26 - 5.57	0.37 - 214.70	0.38 - 3.40	1.00 - 16.77	0.55 - 97.27
	Probability	< 0.001	0.125	0.004	0.642	0.099	0.009	0.008	0.011	0.009	0.581	0.001	0.598	< 0.001	0.003
9 h IQ x 24 h IQ	Relative expression	3.99	1.45	9.47	0.72	4.26	3.66	9.28	2.31	0.61	1.3	7.81	1.85	4.1	4.48
	Standard error	0.10 - 161.91	0.33 - 6.21	0.55 - 124.54	0.23 - 2.72	0.35 - 59.45	1.05 - 12.38	0.42 - 154.78	0.17 - 57.96	0.14 - 2.79	0.27 - 5.71	0.27 - 139.51	0.68 - 5.92	0.90 - 20.81	0.34 - 56.21
	Probability	0.135	0.295	< 0.001	0.343	0.03	< 0.001	0.002	0.223	0.216	0.5	0.006	0.023	0.004	0.021
0 h NIQ[2] x 0 h IQ	Relative expression	0.6	2.09	3.18	0.72	0.62	1.17	-	1.71	0.61	0.76	0.03	0.37	2.68	0.78
	Standard error	0.04 - 18.62	0.83 - 5.13	0.50 - 18.14	0.14 - 3.36	0.10 - 4.71	0.31 - 6.47	-	0.14 - 21.33	0.06 - 5.70	0.14 - 3.84	0.01 - 0.18	0.09 - 1.53	1.01 - 7.60	0.07 - 11.62
	Probability	0.455	0.006	0.02	0.44	0.344	0.789	-	0.364	0.421	0.511	< 0.001	0.003	< 0.001	0.689
0 h NIQ x 24 h NIQ	Relative expression	0.54	0.73	1.25	0.96	2.85	1.27	-	0.78	4.22	2.07	1.13	0.89	1.43	0.83
	Standard error	0.02 - 16.42	0.27 - 1.96	0.21 - 7.58	0.23 - 3.45	0.36 - 26.31	0.29 - 5.89	-	0.05 - 10.55	0.42 - 38.00	0.47 - 10.58	0.29 - 4.12	0.24 - 3.31	0.44 - 4.80	0.06 - 11.60
	Probability	0.458	0.328	0.639	0.921	0.067	0.536	-	0.722	0.02	0.059	0.712	0.726	0.237	0.751
24 h NIQ x 24 h IQ	Relative expression	12.374	5.94	33.585	6.336	1.727	5.94	-	50.544	1.089	3.133	0.296	0.892	11.936	5.54
	Standard error	0.56 - 271.20	1.21 - 27.98	1.68 - 465.92	1.48 - 32.78	0.15 - 20.14	1.41 - 24.95	-	3.06 - 1.047.17	0.17 - 6.63	0.67 - 14.08	0.01 - 4.40	0.24 - 3.09	2.50 - 63.03	0.40 - 64.50
	Probability	0.003	< 0.001	< 0.001	< 0.001	0.338	< 0.001	-	< 0.001	0.858	0.004	0.09	0.73	< 0.001	0.013

[1]IQ = inoculated quarter.

[2]NIQ = non-inoculated quarter.

[3]Numbers greater than 1 = higher expression at the second time; numbers less than 1 = higher expression at the first time.

[4]Results with $P < 0.05$ were considered significant.

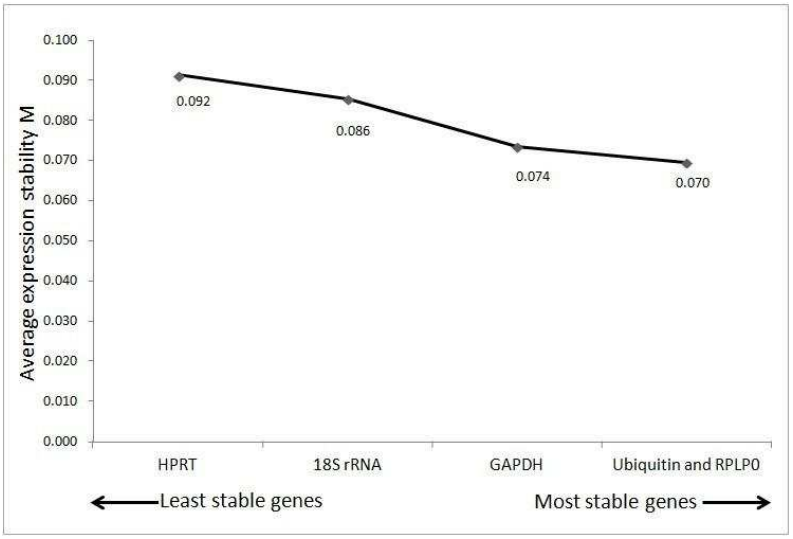

Fig. 1 Estimate of the M stability of the five genes tested as endogenous controls carried out in geNorm. In this analysis, the variation of expression and stability of each gene were calculated, given by an M stability value, which is inversely proportional to the variation in expression of the particular gene.

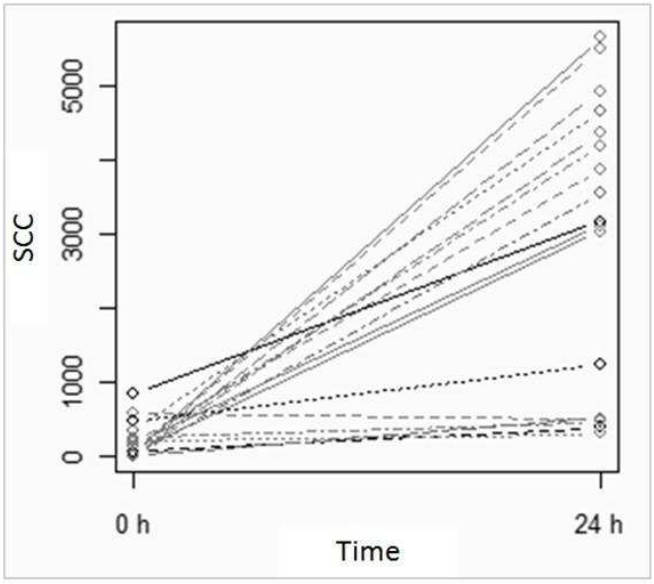

Fig. 2 Result of the somatic cell count (SCC) of the 17 cows at the time of inoculation of the strain of *Streptococcus agalactiae* in the inoculated quarter and 24 hours later.

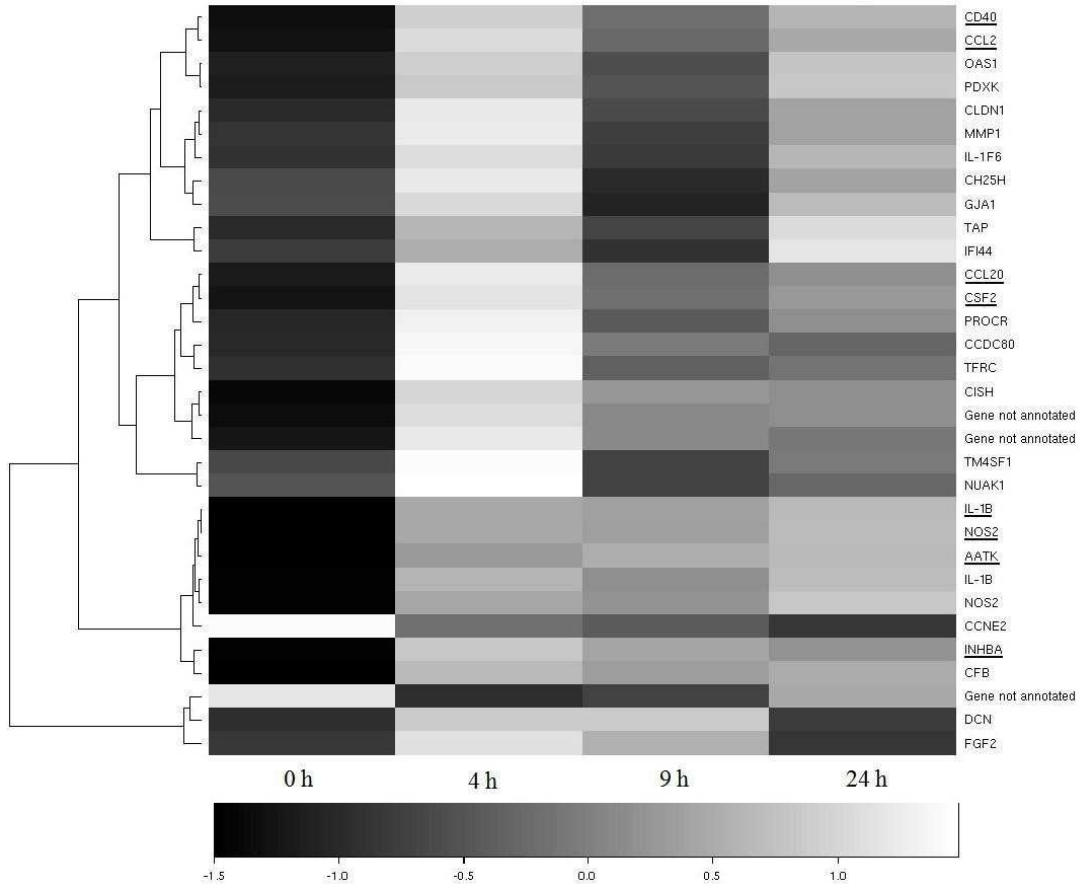

Fig. 3 Heatmap of the expression profiles of the differentially expressed genes by the microarray technique. The darker color represents the downregulated genes and the lighter color the upregulated genes. The underlined genes are those whose expression was validated by real-time PCR.

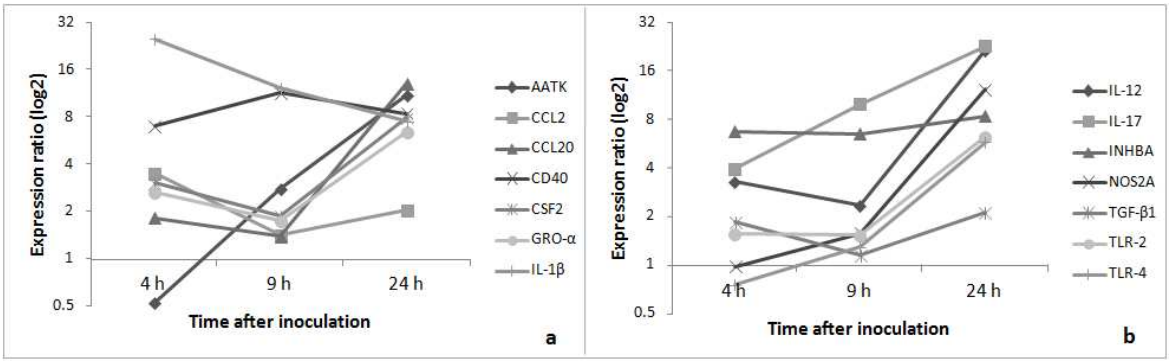

Fig. 4 Ratio of the gene expression at 4, 9 and 24 hours after inoculation of the strain of *Streptococcus agalactiae* in relation to hour 0 by the real-time PCR technique.

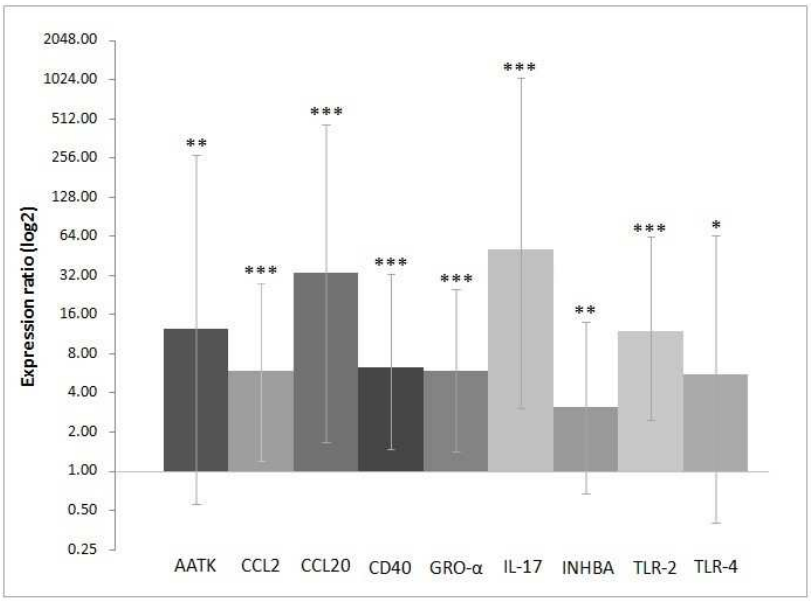

Fig. 5 Expression level of the genes with statistical difference 24 hours after inoculation with the *Streptococcus agalactiae* strain from the inoculated quarter (IQ) in relation to the non-inoculated quarter (NIQ) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

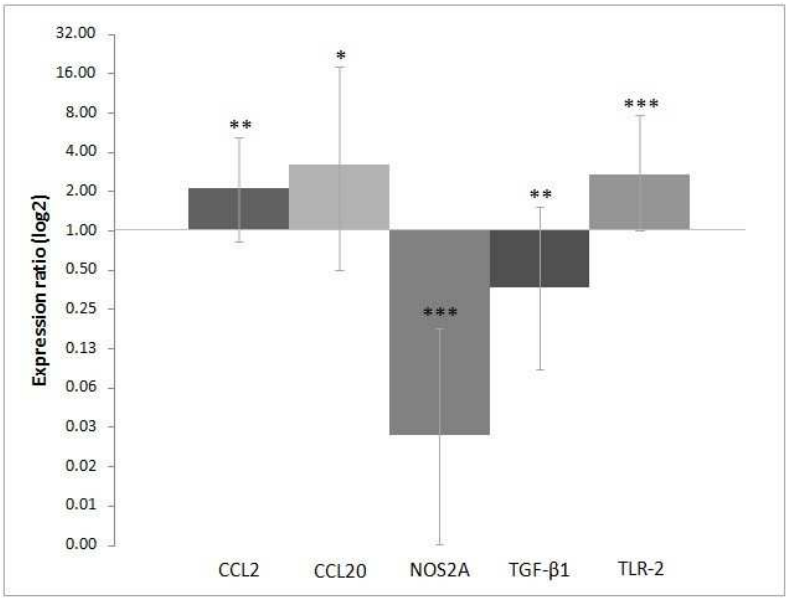

Fig. 6 Level of expression of the genes with statistically significant differences before inoculation of the *Streptococcus agalactiae* strain (0 h) from the inoculated quarter (IQ) in relation to the non-inoculated quarter (NIQ) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

References

Alluwaimi, A.M., Leutenegger, C.M., Farver, T.B., Rossitto, P.V., Smith, W.L., Cullor, J.S., 2003. The cytokine markers in *Staphylococcus aureus* mastitis of bovine mammary gland. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 50, 105-111.

Bailey, M., Plunkett, F.J., Rothkötter, H.-J., Vega-Lopez, M.A., Haverson, K., Stokes, C.R., 2001. Regulation of mucosal immune responses in effector sites. Proc. Nutr. Soc. 60, 427-435.

Bannerman, D.D., Paape, M.J., Lee, J.W., Zhao, X., Hope, J.C., Rainard, P., 2004a. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11, 463-472.

Bannerman, D.D., Paape, M.J., Goff, P.P., Kimura, K., Lippolis, J.D., Hope, J.C., 2004b. Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*. Vet. Res. 35, 681-700.

Bannerman, D.D., Chockalingam, A., Paape, M.J., Hope, J.C., 2005. The bovine innate immune response during experimentally-induced *Pseudomonas aeruginosa* mastitis. Vet. Immunol. Immunopathol. 107, 201-15.

Bannerman, D.D., Paape, M.J., Chockalingam, A., 2006. *Staphylococcus aureus* intramammary infection elicits increased production of transforming growth factor-alpha, beta1, and beta2. Vet. Immunol. Immunopathol. 112, 309-315.

Bannerman, D.D., 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87, 10-25.

Brandtzaeg, P., 2010. The mucosal immune system and its integration with the mammary glands. J. Pediatr. 156, S8-15.

Cates, E.A., Connor, E.E., Mosser, D.M., Bannerman, D.D., 2009. Functional characterization of bovine TIRAP and MyD88 in mediating bacterial lipopolysaccharide-induced endothelial NF- kappaB activation and apoptosis. Comp. Immunol. Microbiol. Infect. Dis. 32, 477-90.

Chockalingam, A., Paape, M.J., Bannerman, D.D., 2005. Increased milk levels of transforming growth factor-α, β1, and β2 during *Escherichia coli*-induced mastitis. J. Dairy Sci. 88, 1986-1993.

Contreras, G.A., Rodríguez, J.M., 2011. Mastitis: Comparative Etiology and Epidemiology. J. Mammary Gland Biol. Neoplasia. 16, 339-56.

Cui, X.G., Hwang, J.T.G., Qiu, J., Blades, N.J., Churchill, G.A., 2005. Improved statistical tests for differential gene expression by shrinking variance components estimates. Biostatistics. 6, 59-75.

Denis, M., Parlane, N.A., LacyHulbert, S.J., Summers, E.L., Buddle, B.M., Wedlock, D.N., 2006. Bactericidal activity of macrophages against *Streptococcus uberis* is different in mammary gland secretions of lactating and drying off cows. Vet. Immunol. Immunopathol. 114, 111-120.

Embrapa Gado de Leite. 2005. Sistema de Produção. Accessed Apr. 29, 2012. http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Leite/LeiteRecriadeNovilhas/racas.htm

Estes, D.M., Tuo, W., Brown, W.C., Goin, J., 1998. Effects of type I/type II interferons and transforming growth factor-β on B-cell differentiation and proliferation: definition of costimulation and cytokine requirements for immunoglobulin synthesis and expression. Immunology. 95, 604-611.

Fagarasan, S., Honjo, T., 2004. Regulation of IgA synthesis at mucosal surfaces. Curr. Opin. Immunol. 16, 277-283.

Fonseca, I., Silva, P.V., Lange, C.C., Guimarães, M.F.M., Weller, M.M.C.A., Sousa, K.R.S., Lopes, P.S., Guimarães, J.D., Guimarães, S.E.F., 2009. Expression profile of genes associated with mastitis in dairy cattle. Genet. Mol. Biol. 32, 776-781.

Fonseca, I., Antunes, G.R., Paiva, D.S., Lange, C.C., Guimarães, S.E.F., Martins, M.F., 2011. Differential expression of genes during mastitis in Holstein-Zebu crossbreed dairy cows. Genet. Mol. Res. 10, 1295-1303.

Fuller, T.F., Ghazalpour, A., Athen, J.E., Drake, T.A., Lusis, A.J., Horvath, S., 2007. Weighted gene coexpression network analysis strategies applied to mouse weight. Mamm. Genome. 18, 463-472.

Gaddy-Kurten, D., Tsuchida, K., Vale, W., 1995. Activins and the receptor serine kinase superfamily. Recent Prog. Horm. Res. 50, 109-129.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J., 2004.

Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.J., Brunner, R.M., Kata, S.R., Seyfert, H.M., 2004. Mastitis increases mammary mRNA abundance of β-defensin 5, Toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab. Immunol. 11, 174-185.

Griesbeck-Zilch, B., Meyer, H.H., Kuhn, C.H., Schwerin, M., Wellnitz, O., 2008. *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. J. Dairy Sci. 91, 2215-2224.

Grönlund, U., Johannisson, A., Waller, K.P., 2006. Changes in blood and milk lymphocyte sub-populations during acute and chronic phases of *Staphylococcus aureus* induced bovine mastitis. Res.Vet. Sci. 80, 147-154.

Grutz, G., 2005. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. J. Leukoc. Biol. 77, 3-15.

Günther, J., Koczan, D., Yang, W., Nürnberg, G., Repsilber, D., Schuberth, H.J., Park, Z., Maqbool, N., Molenaar, A., Seyfert, H.M., 2009. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with *Escherichia coli*. Vet. Res. 40, 31-39.

Günther, J., Liu, S., Esch, K., Schuberth, H.J., Seyfert, H.M., 2010. Stimulated expression of TNF-α and IL-8, but not of lingual antimicrobial peptide reflects the concentration of pathogens contacting bovine mammary epithelial cells. Vet. Immunol. Immunopathol. 135, 152-157.

Ibeagha-Awemu, E.M., Lee, J.W., Ibeagha, A.E., Bannerman, D.D., Paape, M.J., Zhao, X., 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Vet. Res. 39, 11.

Keefe, G.P., 1997. *Streptococcus agalactiae* mastitis: A review. Can. Vet. J. 38, 429-37.

Kerr, D.E., Wellnitz, O., 2003. Mammary expression of new genes to combat mastitis. J. Anim. Sci. 81, 38-47.

Kolls, J.K., McCray, P.B., Chan, Y.R., 2008. Cytokine-mediated regulation of antimicrobial proteins. Nat. Rev. Immunol. 8, 829-835.

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

Langfelder, P., Horvath, S., 2008. WGCNA: An R package for weighted correlation network analysis. BMC Bioinformatics. 9, 559.

Lee, J.W., Bannerman, D.D., Paape, M.J., Huang, M.K., Zhao, X., 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR. Vet. Res. 37, 219-229.

Leutenegger, C.M., Alluwaimi, A.M., Smith, W.L., Perani, L., Cullor, J.S., 2000. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan polymerase chain reaction. Vet. Immunol. Immunopathol. 77, 275-287.

Long, E., Capuco, A.V., Wood, D.L., Sonstegard, T., Tomita, G., Paape, M.J., Zhao, X., 2001. *Escherichia coli* induces apoptosis and proliferation of mammary cells. Cell Death Differ. 8, 808-816.

Lutzow, Y., Donaldson, L., Gray, C., Vuocolo, T., Pearson, R., Reverter, A., Byrne, K., Sheehy, P., Windon, R., Tellam, R., 2008. Identification of immune genes and proteins involved in the response of bovine mammary tissue to *Staphylococcus aureus* infection. BMC Vet. Res. 4, 18-25.

Mauri, C., Bosma, A., 2012. Immune regulatory function of B cells. Annu. Rev. Immunol. 30, 221-241.

Matsuzaki, G., Umemura, M., 2007. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. Microbiol. Immunol. 51, 1139-1147.

Mount, J., Karrow, N., Caswell, J., Boermans, H., Leslie, K., 2009. Assessment of bovine mammary chemokine gene expression in response to lipopolysaccharide, lipotechoic acid + peptidoglycan, and CpG oligodeoxynucleotide 2135. Can. J. Vet. Res. 73, 49-57.

Neave, F.K., Dodd, F.H., Kingwill, R.G., Westgarth, D.R., 1969. Control of mastitis in the dairy herd by hygiene and management. J. Dairy Sci. 52, 696-707.

Okada, H., Ito, T., Ohtsuka, H., Kirisawa, R., Iwai, H., Yamashita, K., Yoshino, T., Rosol, T.J., 1997. Detection of interleukin-1 and interleukin-6 on cryopreserved bovine mammary epithelial cells in vitro. J. Vet. Med. Sci. 59, 503-507.

O'Neill, L.A.J., 2006. How Toll-like receptors signal: what we know and what we don't know. Curr. Opin. Immunol. 18, 3-9.

Oviedo-Boyso, J., Valdez-Alarcón, J.J., Cajero-Juárez, M., Ochoa-Zarzosa, A., López-Meza, J.E., Bravo-Patiño, A., Baizabal-Aguirre, V.M., 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. J. Infect. 54, 399-409.

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

Petzl, W., Zerbe, H., Gunther, J., Yang, W., Seyfert, H., Nurnberg, G., Schuberth, H., 2008. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Vet. Res. 39, 18.

R Development Core Team., 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rainard, P., Riollet, C., 2003. Mobilization of neutrophils and defense of the bovine mammary gland. Reprod. Nutr. Dev. 43, 439-457.

Rainard, P., Riollet, C., 2006. Innate immunity of the bovine mammary gland. Vet. Res. 37, 369-400.

Richards, V.P., Lang, P., Bitar, P.D.P., Lefébure, T., Schukken, Y.H., Zadoks, R.N., Stanhope, M.J., 2011. Comparative genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae*. Infect. Genet. Evol. 11, 1263-1275.

Rinaldi, M., Li, R.W., Bannerman, D.D., Daniels, K.M., Evock-Clover, C., Silva, M.V.B., Paape, M.J., Ryssen, B.V., Burvenich, C., Capuco, A.V., 2010. A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to *E. coli* mastitis. Funct. Integr. Genomics. 10, 21-38.

Riollet, C., Mutuel, D., Duonor-Cérutti, M., Rainard, P., 2006. Determination and characterization of bovine interleukin-17 cDNA. J. Interferon Cytokine Res. 26, 141-149.

Schukken, Y.H., Hertl, J., Bar, D., Bennett, G.J., González, R.N., Rauch, B.J., Santisteban, C., Schulte, H.F., Tauer, L., Welcome, F.L., Gröhn, Y.T., 2009. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. J. Dairy Sci. 92, 3091-3105.

Schukken, Y.H., Gunther, J., Fitzpatrick, J., Fontaine, M.C., Goetze, L., Holst, O., Leigh, J., Petzl, W., Schuberth, H.J., Sipka, A., Smith, D.G.E., Quesnell, R., Watts, J., Yancey, R., Zerbe, H., Gurjar, A., Zadoks, R.N., Seyfert, H.M., 2011. Host-response patterns of intramammary infections in dairy cows. Vet. Immunol. Immunopathol. 144, 270-289.

Schutyser, E., Struyf, S., Van Damme, J., 2003. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev. 14, 409-426.

Sheffield, L.G., 1997. Mastitis increases growth factor messenger ribonucleic acid in bovine mammary glands. J. Dairy Sci. 80, 2020-2024.

Singh, K., Davis, S.R., Dobson, J.M., Molenaar, A.J., Wheeler, T.T., Prosser, C.G., Farr, V.C., Oden, K., Swanson, K.M., Phyn, C.V., Hyndman, D.L., Wilson, T., Henderson, H.V., Stelwagen, K., 2008. cDNA microarray analysis reveals that antioxidant and immune genes are upregulated during involution of the bovine mammary gland. J. Dairy Sci. 91, 2236-2246.

Storey, J.D., 2002. A direct approach to false discovery rates. J. R. Stat. Soc. Series B Stat. Methodol. 64, 479-498.

Strandberg, Y., Gray, C., Vuocolo, T., Donaldson, L., Broadway, M., Tellam, R., 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine. 31, 72-86.

Suojala, L., Orro, T., Jarvinen, H., Saatsi, J., Pyorala, S., 2008. Acute phase response in two consecutive experimentally induced *E. coli* intramammary infections in dairy cows. Acta Vet. Scand. 50, 18-24.

Tao, W., Mallard, B., Karrow, N., Bridle, B., 2004. Construction and application of a bovine immune-endocrine cDNA microarray. Vet. Immunol. Immunopathol. 101, 1-17.

Tao, W., Mallard, B., 2007. Differentially expressed genes associated with *Staphylococcus aureus* mastitis of Canadian Holstein cows. Vet. Immunol. Immunopathol. 120, 201-211.

Tanoue, T., Umesaki, Y., Honda, K., 2010. Immune responses to gut microbiota-commensals and pathogens. Gut Microbes. 1, 224-233.

The Bovine HapMap Consortium, 2009. Genome-wide survey of SNP variation uncovers the genetic structure of cattle breeds. Science. 324, 528-532.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR

data by geometric averaging of multiple internal control genes. Genome Biol. 3(7), research0034.I-0034.II.

Wang, Y.H., Byrne, K.A., Reverter, A., Harper, G.S., Taniguchi, M., McWilliam, S.M., Mannen, H., Oyama, K., Lehnert, S.A., 2005. Transcriptional profiling of skeletal muscle tissue from two breeds of cattle. Mamm. Genome. 16, 201-210.

Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C., R. Paules, S., 2001. Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. 8, 625-637.

Wu, H., Kerr, M.K., Cui, X., Churchill, G.A., 2003. MAANOVA: a software package for the analysis of spotted cDNA microarray experiments. Pages 313-341 in The Analysis of Gene Expression Data: Methods and Software. Edited by: Parmigiani G., E. Garrett, R. Irizarry, and S. Zeger. New York: Springer-Verlag.

Yang, W., Zerbe, H., Petzl, W., Brunner, R.M., Gunther, J., Draing, C., von Aulock, S., Schuberth, H.J., Seyfert, H.M., 2008. Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin- 8 (CXCL8) expression in the udder. Mol. Immunol. 45, 1385-1397.

Ye, P., Rodriguez, F.H., Kanaly, S., Stocking, K.L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., Shellito, J.E., Bagby, G.J., Nelson, S., Charrier, K., Peschon, J.J., Kolls, J.K., 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J. Exp. Med. 194, 519-527.

Zadoks, R.N., Middleton, J.R., McDougall, S., Katholm, J., Schukken, Y.H., 2011. Molecular Epidemiology of Mastitis Pathogens of Dairy Cattle and Comparative Relevance to Humans. J. Mammary Gland Biol. Neoplasia. 16, 357-372.

Zhao, X., Lacasse, P., 2008. Mammary tissue damage during bovine mastitis: causes and control. J. Anim. Sci. 86, 57-65.

CHAPTER 2

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Expression of genes related to mastitis in cells of Holstein-Zebu crossbreed dairy cows

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Running title: Gene analysis in mastitis

Abstract

Among the potential public health problems of animal production, infectious-contagious diseases stand out. Mastitis is among the main diseases affecting dairy cattle. One of the most promising options to reduce the problems caused by this disease, besides proper sanitary and management practices, is the selective breeding of resistant animals. To shed light on the immune response mechanisms involved in the resistance/susceptibility phenotype to this disease, we quantified the relative expression of the genes *IL-2*, *IL-6*, *IL-8*, *IL-12*, *IFN-γ*, *TNF-α*, *TLR-2*, *SEMA5A* and *FEZL* in cells of crossbreed dairy cows, divided into two groups, one healthy and the other suffering from clinical mastitis. Total RNA was extracted from the cells in the milk from the animals in each group (with and without clinical mastitis). Gene expression was determined using the real-time PCR method. The levels of gene expression were compared, and the cows with mastitis were found to express 2.5

times more *TLR-2* than those free of mastitis (P<0.05). There was no significant difference (P>0.05) in the expression of the other genes.

Key words: Immune response; Real-time PCR; Resistance to mastitis

Introduction

Dairy cattle breeds of European origin are recognized as being more productive and also more demanding in terms of management and nutrition than are Zebu breeds. Therefore, the expected higher production is not always borne out in tropical regions on dairy farms that are less technically advanced, since the animals do not receive sufficient management and nutrition to reach their full genetic potential. The use of crossbreed cattle to produce milk, in a pasture-based system, is a common option among dairy farmers in Brazil and other tropical regions. Various crosses of European and Zebu breeds are capable of profitably and sustainably, producing milk under tropical conditions. These animals thus make up an important contingent of Brazil's dairy herds: an estimated 70% of the country's milk comes from Holstein-Zebu crossbreeds.

In recent decades, the dairy production chain in Brazil has undergone intense transformations, with restructuring of all links, making the sector more competitive. This is reflected in the 70% increase in productivity (liters/cow/year) between 1980 and 2007. Although the statistics show continuous development, there are still various obstacles that need to be overcome, particularly by smaller, less technically advanced dairy operators, to be able to maintain the sector's sustainability and competitiveness. Several factors affect dairy production, such as climate, installations, herd health, labor availability and zootechnical and genetic potential. Among animal health problems, infectious-contagious diseases stand out the most, and mastitis is the main such disease afflicting dairy cattle from an economic standpoint (Oviedo-Boyso et al., 2006).

One of the most promising ways to reduce the problems caused by mastitis, besides adequate sanitary conditions, is the selective breeding of resistant animals. According to Detilleux et al. (1994), it is hard to produce effective vaccines against this disease due to the large variety of microorganisms causing it and its multifactor character. Therefore, studies to better understand the biological processes involved in

determining resistance to diseases are essential to resolve problems and develop innovative solutions.

Mastitis is one of the most prevalent and costly diseases affecting the dairy industry worldwide (Bradley, 2002; Petrovski et al., 2006). It is characterized by inflammation of the mammary gland and is generally caused by bacteria. The speed and efficacy of the host's immune response to the invading microorganisms is a crucial factor for the establishment, persistence and severity of the infection (Bannerman et al., 2009). Epithelial and endothelial cells perform important functions in the first-line defense against local infections, by producing cytokines and other inflammatory mediators (Strandberg et al., 2005; Corl et al., 2008; Griesbeck-Zilch et al., 2008). In the mammary gland, cells from the immune system together with epithelial cells are responsible for recognizing the invading microorganism via toll-like receptors, or TLRs (Rainard and Riollet, 2006; Griesbeck-Zilch et al., 2008). Activation of TLRs triggers the expression of inflammatory cytokines and other mediators related to immune response, cell differentiation and apoptosis (Ibeagha-Awemu et al., 2008; Yang et al., 2008; Cates et al., 2009).

The importance of inflammatory cytokines to the development of an effective immune response against mastitis has been documented in several works, which have evaluated changes in their concentrations in milk during tests on animals with experimentally infected udders (Burvenich et al., 2003; Bannerman et al., 2009). The resistance response to mastitis is a complex characteristic and the genes involved in the immune response have been indicated as strong candidates in determining animal resistance (Shuster et al., 1993; Ferens et al., 1998; Alluwaimi et al., 2003; Rambeaud et al., 2003; Oviedo-Boyso et al., 2006; Fonseca et al., 2009). In light of this premise, the aim of this study was to characterize the expression of genes related to the phenotype determining resistance/susceptibility to mastitis, to better understand the immune response mechanisms. For this purpose, we carried out field tests to investigate the expression of the following genes: *IL-2* (interleukin 2), *IL-6* (interleukin 6), *IL-8* (interleukin 8), *IL-12* (interleukin 12), *IFN-γ* (interferon-gamma), *TNF-α* (tumor necrosis factor-alpha), *TLR-2* (toll-like receptor 2), *SEMA5A* (semaphorin 5A) and *FEZL* (forebrain embryonic zinc-finger-like) in cells present in the milk of crossbreed cows (Holstein x Gyr) with and without clinical mastitis, using naturally infected animals.

Material and Methods

We used 20 crossbreed cows from the Santa Mônica Experimental Field of the Embrapa Dairy Cattle Research Unit, located in the municipality of Valença in the state of Rio de Janeiro. We selected two groups, one free of infection and the other with clinical mastitis, each composed of 10 animals with different birth orders, blood degree and age.

All animals underwent clinical udder examination and strip-cup testing before collection of the milk samples. From each cow, 200 mL of milk were taken in sterile tubes. The milk samples from the cows with mastitis were collected immediately after the appearance of clinical signs and before treatment with drugs. Hence, there was no artificial infection or treatment effect. Additionally, all the samples from cows with mastitis were submitted to microbiological tests to identify the pathogen, performed according to NMC (1987).

Total RNA of the milk samples was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendations. To remove any contamination by genomic DNA, the samples were submitted to digestion with DNase (RNase-free DNase Set – Qiagen). The quality of the RNA samples was evaluated by the Agilent Bioanalyzer 2100 (Agilente, Palo Alto, CA) and the concentrations were determined by spectrophotometry using a NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA). The mean RNA integrity index (RIN) of the samples was 6.3 (data not shown).

The first cDNA strand was synthesized using the SuperScript™ III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA), and the average DNA concentrations were estimated by spectrophotometry, after which the single cDNA strand was stored at -20 °C until use in the real-time PCR reaction (qPCR).

The qPCRs were carried out with the SYBR Green® PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. The primers used to evaluate the gene expressions were designed according to data from the literature, as shown in Table 1.

Six endogenous controls were used: *β-actin*, *18S rRNA*, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *Ubiquitin*, *RPLP0* (ribosomal protein, large P0) and *HPRT* (hypoxanthine-guanine phosphoribosyltransferase). We chose the two best endogenous controls according to the profile of the amplification and

dissociation curves, and also analyzed them with the geNorm software (Vandesompele et al., 2002). After 40 amplification cycles, all the samples were submitted to dissociation curve analysis to verify the absence of non-specific products and primer dimers.

After real-time quantification, the reactions were optimized for all the genes, by testing three quantities of cDNA (100, 200 and 400 ng/reaction) and six primer dilutions (50, 100, 200, 400, 600 and 900 nM). After determining the best conditions, we plotted the standard curve for each gene, in which each series cDNA dilution was graphed against the respective Ct (cycle threshold).

Each test was performed in duplicate in optical 96-well reaction plates, sealed with optical adhesive film and amplified in an ABI Prism 7300 Sequence Detection Systems device (Applied Biosystems), with each target amplified separately. The data obtained from the qPCRs, generated by the device, were analyzed by the REST© 2009 program, developed by M. Pfaffl (Technical University of Munich) and by Qiagen, available at http://www.gene-quantification.de/rest-2009.html, to compare the expression difference between the treatments.

Table 2 shows the amplification sequence for each gene. In the dissociation curves, two peaks could be observed for *GADPH* in the samples from two animals, so this primer was excluded from the analyses by the geNorm software to choose the best endogenous control. For the other genes, no peaks were observed referring to primer dimers or non-specific products (data not shown). Table 2 also shows the quantity of cDNA and primer optimized for each gene, besides the dissociation temperature of the amplified fragment. The coefficient of variation of the duplicate Ct readings for each sample did not exceed 5% (data not shown).

The analyses with the geNorm software indicated that the two most efficient endogenous controls were *18S rRNA* and *Ubiquitin*, as can be seen in Figure 1, because these showed the lowest expression variation among the animals.

Results

Table 3 shows the results of the microbiological examination of cows with mastitis, according to which three cows were infected by *Corynebacterium* spp., three by *Streptococcus agalactiae*, one by *Streptococcus* spp and one by coagulase-negative *Staphylococcus* spp. No bacterial growth was detected in the milk of the other two cows, even though the animals showed clinical signs of mastitis. Reports

indicate that 25-40% of all clinical samples are negative on routine culture. The number of the organism less than the minimum detection limit of the assay, absence of the microorganism in the sample or phagocytosis of the microorganism by somatic cells may be some of the reasons for these cases (NMC, 1987).

We also compared the gene expression level of the crossbreed animals with and without clinical mastitis (Table 4). The expression in the animals with mastitis was 2.5 times greater for *TLR*-2 than in the animals without mastitis ($P<0.05$). There was no significant difference in expression for the other genes ($P>0.05$) according to the analyses carried out with the REST© 2009 program.

Discussion

Innate immunity predominates in the initial phase of infection and is mediated by macrophages, neutrophils, natural killer cells (NK) and cytokines. It recognizes and responds to different pathogens, even if the organism has never been infected by the particular species. Bacteria are especially likely to have different cell wall structures, which are recognized by membrane receptors. These structures are lipopolysaccharides (LPS), peptidoglycans (PGN) and lipoteichoic acid (LTA), which constitute the pathogen-associated molecular patterns (PAMPs). These PAMPs are recognized by toll-like receptors (TLRs), which are located in the cell and endosome membranes. Interaction between the PAMPs and TLRs of immune system cells induces the production of cytokines and other endogenous mediators, which are essential for protecting the organism against pathogenic microorganisms. The *TLR-2* gene is responsible for recognizing the LTA and PGN of Gram-positive bacteria (Hirschfeld et al., 2000; Takeuchi et al., 2000). As can be observed in Table 2, all the microorganisms identified as causing mastitis in this study are Gram-positive bacteria. This corroborates other findings reported in the literature, according to which there was greater expression of the *TLR-2* gene during mastitis caused by Gram-positive microorganisms (Bannerman et al., 2004a; Goldammer et al., 2004; Swanson et al., 2009).

We did not observe significant differences ($P>0.05$) between the groups studied in relation to the expression level of the other genes. The *IL-2* gene, produced mainly by Th_1 lymphocytes, induces the proliferation of mononuclear cells and activates some epithelial cells. In contrast, *IFN-*γ is associated with conversion of Th_0 into Th_1 lymphocytes and activation of macrophages, as well as potentiating the

action of TNF-α (Janeway et al., 2002). Therefore, this cytokine is related to the Th_1 immune response profile, i.e., the immune response of this cell type. The *IL-8* gene, together with *IL-1* and *TNF-α*, has been indicated as an important mediator of neutrophil recruitment to the inflamed site. Some studies have shown that TNF-α and IL-8 are present in the milk from udders infected by Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*, but they are present in lower concentrations or not detected in the milk from cows with mammary glands infected by *Staphylococcus aureus* (Shuster et al., 1997; Riollet et al., 2000; Bannerman et al., 2004a; Bannerman et al., 2004b; Bannerman et al., 2005). Another study by our group with commercial cattle with and without mastitis caused by natural infection showed a difference in the expression profile of the *IFN-γ*, *IL-2* and *IL-10* genes among animals of the Holstein BW and Gyr breeds (Fonseca et al., 2009).

Despite the non-significant expression for the other genes, these results improve the understanding of how immunological reactions occur in response to mastitis, greatly affecting milk quality and quantity, especially in crossbreed animals, which are the basis of the dairy industries in Brazil. Mastitis is a multifactor disease and resistance is influenced by many genes. For this reason, gene-expression studies are particularly important to clarify how immune responses to this disease occur. The present study is groundbreaking in this respect for studying crossbreed animals. The majority of studies have examined European breeds with artificially induced infection. Like this study, that by Fonseca et al. (2009) also evaluated the expression of some genes in naturally infected cows, but of separate Holstein BW and Gyr breeds rather than crossbreed animals. In that study, the *IL-6*, *IL-8* and *TNF-α* genes also failed to show significantly different expression between cows with and without clinical mastitis.

The immune response can differ according to the bacterial strain and the host, because there is great individual variation. Lahouassa et al. (2007) demonstrated, by means of *in vitro* studies, that different strains of *S. aureus* provoke different responses in epithelial cells and the udder. Besides this, the intensity and level of expression of the genes considered in that study (*IL-8*, *GRO-α*, *GRO-β*, *TNF-α*, *IL-1β*, *TGF-β1* and *IL-10*) varied according to the infection phase (3, 10 and 24 h after addition of the bacteria to the cell culture). According to the authors, these different responses imply alternate activation pathways or different levels of signal

transduction, reflecting what is observed *in vivo*. Swanson et al. (2009) also showed that after infection of the udder by a pathogen, complex cellular and physiological processes occur, and the changes in the expression of genes related to the immune response by epithelial cells in the mammary gland appear to be specific for each pathogen. These works highlight the complexity of the immune response to an infective pathogen. In the present study, the milk samples were collected just after the detection of clinical signs of natural infection, so there was no control of the time of infection. Besides this, there was no control of the type of strain that caused the infection, and in two cows it was not even possible to isolate the pathogen. It should be pointed out that this study was an attempt to demonstrate what happens in a commercial herd under natural conditions, and thus, it is not possible to state that these animals were free of other infections or diseases that could have influenced the results.

From the results of this study, we cannot affirm that the genes studied are only expressing the messenger RNA or are also expressing a protein, since there are post-transcriptional regulation factors that were not analyzed. For this reason, it would be interesting to perform proteomic studies to better understand these results. Besides this, the analysis of the structure of the *TLR-2* gene, which showed a significant difference in expression, could be useful to identify SNP markers for mastitis resistance and susceptibility phenotypes in crossbreed animals.

It was possible to verify the difference in the expression profile of the *TLR-2* gene in relation to the mastitis resistance/susceptibility phenotype among the animals examined. We intend to perform further studies including a larger number of samples and specific experimental conditions, to evaluate the potential of the *TLR-2* gene as a marker, and also to include new genes, so as to obtain a better understanding of the physiopathological mechanisms of mastitis.

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Table 1. Primer sequences used in the qPCRs.

Gene	Primer F	Primer R	Reference
IL-2	GGATTTACAGTTGCTTTTGGAGAAA	GCACTTCCTCTAGAAGTTTGAGTTCTT	Leutenegge et al., 2000
IL-6	TCAGCTTATTTTCTGCCAGTCTCT	TCATTAAGCACATCGTCGACAAA	Leutenegge et al., 2000
IL-8	CACTGTGAAAAATTCAGAAATCATTGTTA	CTTCACCAAATACCTGCACAACCTTC	Leutenegge et al., 2000
IL-12	TTAATTGAGGTCGTGGTAGAAGCTG	GGTCTCAGTTGCAGGTTCTTGG	Leutenegge et al., 2000
IFN-γ	TGGATATCATCAAGCAAGACATGTT	ACGTCATTCATCACTTTCATGAGTTC	Leutenegge et al., 2000
TNF-α	TCTTCTCAAGCCTCAAGTAACAAGT	CCATGAGGGCATTGGCATAC	Leutenegge et al., 2000
TLR-2	CAGTTTAACCCAGTGCCTTC	CTCCAACGTCTTCAGTTGCT	Ibeagha-Awemu et al., 2008
*SEMA*5A	TGTGGGACCAACGCTTTCA	TCATGGATCTCCGTCAGGTTACT	Sugimoto et al., 2006
FEZL	CTACAAGCCCTTCGTCTGTGAAT	GCTGTGGGTCAGCTTGTGATT	Sugimoto et al., 2006
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	Wang et al., 2005
GAPDH	CCTGGAGAAACCTGCCAAGT	GCCAAATTCATTGTCGTACCA	Mount et al., 2009
Ubiquitin	GGCAAGACCATCACCCTGGAA	GCCACCCCTCAGACGAAGGA	Singh et al., 2008
RPLPO	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	Mount et al., 2009
HPRT	GCCGACCTGTTGGATTACAT	ACACTTCGAGGGGTCCTTTT	Tao et al., 2004

Table 2. Concentration of primer and cDNA, reaction efficiency and dissociation temperature (DT) of the amplified fragment for each gene.

Gene	Primer (nM)	cDNA (ng/reaction)	Efficiency	DT (°C)
IL-2	400	400	0.7	75.9
IL-6	400	400	0.8	76.1
IL-8	400	100	0.8	76.6
IL-12	200	200	0.9	76.7
TNF-α	400	200	0.8	82.5
IFN-γ	400	100	0.9	77.8
TLR-2	50	200	0.9	75.3
*SEMA*5A	900	400	0.7	74.3
FEZL	900	400	0.7	80.6
18S rRNA	200	100	0.9	80.7
GAPDH	200	200	1.0	83.3
Ubiquitin	200	100	0.9	83.4
RPLPO	400	200	1.0	83.2
HPRT	400	200	0.9	78.6

Table 3. Results of the microbiological examination of animals with clinical mastitis.

Animal	Microbiological test	Gram classification
0642-0	Negative result	-
0797	*Corynebacterium* spp.	Gram-positive
0801-0	*Corynebacterium* spp.	Gram-positive
0860-0	*Corynebacterium* spp.	Gram-positive
1759	Negative result	-
3604	*Streptococcus agalactiae*	Gram-positive
3754	*Streptococcus* spp.	Gram-positive
4523	Coagulase negative *Staphylococcus* spp.	Gram-positive
8726-9	*Streptococcus agalactiae*	Gram-positive
9838	*Streptococcus agalactiae*	Gram-positive

Table 4. Relative expression of the genes in crossbreed cows with mastitis in relation to the animals without mastitis.

Gene	Relative expression	Standard error	Probability
IL-2	0.687	0.062 - 6.007	0.618
IL-6	0.763	0.043 - 6.571	0.707
IL-8	1.409	0.168 - 8.271	0.620
IL-12	0.514	0.031 - 6.668	0.449
TNF-α	0.551	0.159 - 2.153	0.226
IFN-γ	0.459	0.050 - 2.674	0.213
*TLR-2**	2.504	0.645 - 8.691	0.047
SEMA5A	0.476	0.036 - 5.020	0.350
FEZL	0.476	0.034 - 4.677	0.325

Numbers greater than 1: greater expression in animals with mastitis; Numbers less than 1: lower expression in animals with mastitis (* $P<0.05$).

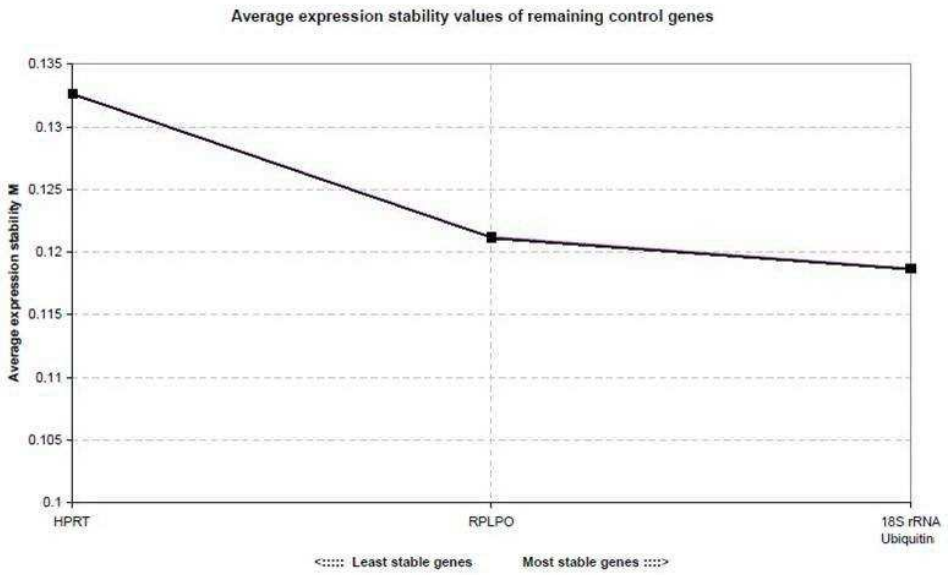

Figure 1. Result of the best endogenous control analysis, performed with the geNorm software.

References

Alluwaimi AM, Leutenegger CM, Farver TB, Rossitto PV, et al. (2003). The Cytokine Markers in *Staphylococcus aureus* Mastitis of Bovine Mammary Gland. *J. Vet. Med*., 50(3):105-111.

Bannerman DD, Paape MJ, Lee JW, Zhao X, et al. (2004a). *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin. Diagn. Lab. Immunol*., 11:463-472.

Bannerman DD, Paape MJ, Hare WR, Hope JC (2004b). Characterization of the bovine innate immune response to intramammary infection with *Klebsiella pneumoniae*. *J. Dairy Sci*., 87:2420-2432.

Bannerman DD, Chockalingam A, Paape MJ, Hope JC (2005). The bovine innate immune response during experimentally-induced *Pseudomonas aeruginosa* mastitis. *Vet. Immunol. Immunopathol*., 107:201-215.

Bannerman DD, Rinaldi M, Vinyard BT, Laihia J, et al. (2009). Effects of intramammary infusion of cis-urocanic acid on mastitis-associated inflammation and tissue injury in dairy cows. *Am. J. Vet. Res*., 70:373–382.

Bradley A (2002). Bovine mastitis: an evolving disease. *Vet. Journal*, 164:116–128.

Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A, et al. (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet. Res*., 34:521–564.

Cates EA, Connor EE, Mosser DM, Bannerman DD (2009). Functional characterization of bovine TIRAP and MyD88 in mediating bacterial

lipopolysaccharide-induced endothelial NF-kB activation and apoptosis. *Comp. Immunol. Microbiol. Infect. Dis*., 32:477–490.

Corl CM, Gandy JC, Sordillo LM (2008). Platelet activating factor production and proinflammatory gene expression in endotoxin-challenged bovine mammary endothelial cells. *J. Dairy Sci.*, 91:3067–3078.

Detilleux JC, Koehler KJ, Freeman AE, Kehrli Jr M.E, et al. (1994). Immunological parameters of periparturient Holstein cattle: genetic variation. *J. Dairy Sci.*, 77:2640-2650.

EMBRAPA GADO DE LEITE (2008). Estatística do Leite. Disponível em: <http://www.cnpgl.embrapa.br/nova/informacoes/estatisticas/producao/tabela0230.php>. Acesso em: 05 out. 2009.

EMBRAPA GADO DE LEITE (2003). Sistema de Produção de Leite (Zona da Mata Atlântica). Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Leite/LeiteZonadaMataAtlantica/racas1.html>. Acesso em: 05 out. 2009.

Ferens WA, Goff WL, Davis WC, Fox LK, et al. (1998). Induction of type-2 cytokines by a Staphylococcal enterotoxins superantigen. *J. Nat. Toxins*, 7:193–213.

Fonseca I, Silva PV, Lange CC, Guimarães MFM, et al. (2009). Expression profile of genes associated with mastitis in dairy cattle. *Genet. Mol. Biol*., 32(4):776-781.

Goldammer T, Zerbe H, Molenaar A, Schuberth RM, et al. (2004). Mastitis increases mammary mRNA abundance of β-Defensin 5, Toll-Like-Receptor 2 (TLR2) and TLR4 but not TLR9 in cattle. *Clin. Diag. Lab. Immunol*., 11:174-185.

Griesbeck-Zilch B, Meyer HH, Kuhn CH, Schwerin M, et al. (2008). *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J. Dairy Sci*., 91:2215–2224.

Hirschfeld M, Ma Y, Weis JH, Vogel SN, et al. (2000). Cuttingedge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol*., 165:618–622.

Ibeagha-Awemu EM, Lee JW, Ibeagha AE, Bannerman DD, et al. (2008). Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and down-stream TLR signaling molecules in bovine mammary epithelial cells. *Vet. Res*., 39:11.

Janeway CA, Tavers P, Walport M, Sholmchik MJ (2002). Imunologia: O Sistema Imune na saúde e na doença. 5ª Ed, Artmed, Porto Alegre.

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

Leutenegger CM, Alluwaimi AM, Smith WL, Perani L, et al. (2000). Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan® polymerase chain reaction. *Vet. Immunol. Immunopathol*., 77:275-287.

Mount JA, Karrow NA, Caswell JL, Boermans HJ, et al. (2009). Assessment of bovine mammary chemokine gene expression in response to lipopolysaccharide, lipotechoic acid 1 peptidoglycan, and CpG oligodeoxynucleotide 2135. *Can. J. Vet. Res*., 73:49-57.

NMC (National Mastitis Council) (1987). Laboratory and Field Handbook on Bovine Mastitis. NMC, Arlington.

Oviedo-Boyso J, Valdez-Alarcón JJ, Cajero-Juárez M, Ochoa-Zarzosa A, et al. (2006). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *J. Infect*., 54:399-409.

Petrovski KR, Trajcev M, Buneski G (2006). A review of the factors affecting the costs of bovine mastitis. *J. S. Afr. Vet. Assoc*., 77:52–60.

Rainard P, Riollet C (2006). Innate immunity of the bovine mammary gland. *Vet. Res*., 37:369–400.

Rambeaud M, Almeida RA, Pighetti GM, Oliver SP (2003). Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. *Vet. Immunol. Immunopathol*., 96:193-205.

REST (Relative Expression Software Tool) 2009 Software. Michael W. Pfaffl and Qiagen. Disponível em: < http://www.gene-quantification.de/rest-2009.html >. Acesso em 19 jul. 2010.

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

Shuster DE, Kehrli Jr ME, Stevens MG (1993). Cytokine production during endotoxin-induced mastitis in lactating dairy cows. *Am. J. Vet. Res*., 54(1):80-85.

Shuster DE, Kehrli Jr ME, Rainard P, Paape M (1997). Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. *Infect. Immun*., 65:3286-3292.

Singh K, Davis SR, Dobson JM, Molenaar AJ, et al. (2008). cDNA microarray analysis reveals that antioxidant and immune genes are upregulated during involution of the bovine mammary gland. *J. Dairy Sci*., 91:2236–2246.

Sugimoto M, Fujikawa A, Womack JE, Sugimoto Y (2006). Evidence that bovine forebrain embryonic zinc finger-like gene influences immune response associated with mastitis resistance. *Proc. Natl. Acad. Sci*. USA, 103:6454-6459.

Strandberg Y, Gray C, Vuocolo T, Donaldson L, et al. (2005). Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine*, 31:72–86.

Swanson, KM, Stelwagen K, Dobson J, Henderson HV, et al. (2009). Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. *J. Dairy Sci*. 92:117-129.

Takeuchi O, Hoshino K, Akira S (2000). Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol*., 165:5392–5396.

Tao W, Mallard B, Karrow N, Bridle B (2004). Construction and application of a bovine immune-endocrine cDNA microarray. *Vet. Immunol. Immunopathol*., 101:1–17.

Vandesompele J, De Preter K, Pattyn F, Poppe B, et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.*, 3(7): research0034.1–0034.11.

Wang YH, Byrne KA, Reverter A, Harper GS, et al. (2005). Transcriptional profiling of skeletal muscle tissue from two breeds of cattle. *Mamm. Genome*, 16:201–210.

Yang W, Zerbe H, Petzl W, Brunner RM, et al. (2008). Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNF alpha and interleukin- 8 (CXCL8) expression in the udder. *Mol. Immunol*., 45:1385–1397.

CHAPTER 3
Gene networks in animal improvement

Abstract

The generation of data using sequencing and other technologies is only the first step to understand the molecular bases of the functioning of an organism. The next step requires understanding how the genes and their products interact with each other and the environment. One of the principal objectives of studying the behavior of gene networks is to discover genes that determine specific phenotypes and to model the activities of genes to enable identifying their behavior. In this context, gene networks can be useful to better understand how the response to a determined stimulus occurs, for example in response to bovine mastitis. Therefore, in this review we present the methods currently used in the study of gene networks and their possible applications.

Key words: gene expression; signalling pathways; systems biology

Introduction

The development of ever-more advanced computers and programs, the sequencing of genomes and the parallel development of technologies to monitor gene expression have generated a wealth of data in the field of molecular and quantitative genetics. However, the generation of these data is only the first step to understand the molecular bases of the functioning of an organism. The next step requires understanding how the genes and their products interact with each other and the environment. These interactions are highly complex, and their study has opened a vast new field of molecular biology, also called the "systems biology", whose objective is to identify gene networks.

The focus of systems biology is to visualize the biological system as a whole and to study the effects of diseases and the interactions with the environment, so as to facilitate comprehension of these biological processes (Hood et al., 2004; Burrage et al., 2006). According to Hood et al. (2008), the five key components of the systems biology are overall biomolecular measures, integration of biological information, identification of molecular responses to perturbations, construction of models that can be tested, and refinement of these models by hypothesis testing.

Once specific biological responses are precisely represented, the models can be used to understand the progression of a disease, identify points for intervention and predict the responses to molecular perturbations (Hood et al., 2008).

In this context, gene networks can be useful to better understand how the response to a determined disease occurs. In this review, we present the methods currently used in the study of gene networks and their possible applications, such as in the study of bovine mastitis.

Gene expression and networks

Cells can be understood as complex systems where changes in the medium in which they are found provide input signals that can cause various internal reactions. These reactions encompass, for example, interference in gene expression and propagation of biochemical signals. The various signals propagated can interact with each other, forming a complex network of interactions, and in this process of signal propagation, genes can be expressed and translated into proteins, which in turn can act as transcription factors, triggering the transcription of other genes, and so on.

In eukaryotes, gene expression is a complex process that involves methylation of DNA, modification of chromatin, imprinting and RNA interference (Mattick and Makunin, 2006). The immediate regulators are the transcription factors, which bind to specific sequences in the promoter region or enhancer regions of individual genes. The activity of the transcription factors can be regulated by the presence of other transcription factors and cofactors, methylation of the promoter region or enhancer region and accessibility to the DNA due to chromatin compaction (Loose et al., 2013). And even after the DNA is transcribed to mRNA, the latter can be degraded before being translated into proteins, such as by the action of microRNAs (Li and Cho, 2012).

To understand the exact mechanisms of a biological process, it is first necessary to identify the relevant components that are involved, such as the DNA, transcripts, proteins and metabolites. The second challenge is to determine how these interact in the networks, which can be done by means of biological systems that aim to elucidate the architecture of the functional relations among genes, proteins and metabolites (Tegnér and Björkegren, 2007). In this network, the expression level of each gene depends both on the value of its own expression and the expression values of other genes, besides external stimuli. A gene can activate or deactivate another

gene, and this gene can then increase (upregulate) or decrease (downregulate) the expression of a third gene. For this reason, a change in the expression of a single gene can cause changes in the expression of various other genes. In the case of miRNAs, for example, each miRNA is capable of regulating a large number of genes at the post-transcriptional level by degrading the mRNA or blocking the translation of the transcript (Jackson et al., 2003).

Methods used to identify gene networks

The current study of gene networks traces its origins to the work of various mathematicians in the 1950s, among which the paper by Erdös and Rényi (1959) stands out. They developed a mathematical theory of networks where the vertices (or nodes) are connected by edges. Since then, physicists and mathematicians have been working to understand the interaction between the topology and dynamics of networks (Strogatz, 2001), so that today there are a variety of methods to construct gene networks (Gill et al., 2010).

By means of statistical and computational methods, it is possible to identify the main dependence relations in a complex gene network, in which the genes influence each other by activation or inhibition. One of the principal objectives of studying the behavior of gene networks is to discover genes that determine specific phenotypes (to identify diseases, for example) and to model the activities of genes to enable identifying their behavior.

Various gene network models have been proposed (De Jong, 2002), among them continuous, discrete, deterministic and stochastic models. Boolean networks are the simplest type of discrete networks, consisting of *n* nodes, each one representing a gene that can be characterized by 1 (turned on) or 0 (turned off). By this approach it is also assumed that the regulatory control can be approximated by rules of Boolean logic and that all the elements of the network have their states updated simultaneously. The continuous models use differential equations to monitor the temporal evolution of the concentrations of the molecules of interest. These more faithfully represent physical-chemical phenomena, but they are very demanding in terms of modeling and computation, because it is necessary to use numerical methods to solve equations that generally to not have known analytic solutions (Vêncio et al., 2006).

Identifying gene networks is experimentally difficult, because there are various possible diagrams that can arise, even before considering the detailed kinetics of the reaction of interest. Just for a network of three genes, the basic challenge to identifying an algorithm for the network is the large number traits (called nodes), which can generate many different diagrams in relation to the small number of experimental samples available to differentiate these networks. Among the strategies to overcome this problem, it is possible to increase the number of experiments; incorporate the *a priori* knowledge, which can partly offset the small number of samples in relation to the number of nodes by reducing the number of possible networks that are consistent with the experimental observations evaluated; or reduce the number of nodes based on correlations in gene expression activity (Tegnér and Björkegren, 2007).

Integration of the existing knowledge of genes is essential to interpret the results of experiments. The study of the dynamic properties of these networks is becoming increasingly important, because it includes the kinetics of the interactions between and beyond the genes and proteins and allows determining whether an interaction is activating or suppressing a particular pathway. Computational tools are necessary to identify these properties of the experimental data (Tegnér and Björkegren, 2007) and many programs have been developed to visualize the interactions of genes, helping users to understand how genes are interconnected with each other.

Various computational methods have been proposed to produce a network model, describing how the expression level of each gene in the network depends on an external stimulus and the expression levels of other genes. Additional information on gene networks can be obtained experimentally by applying a direct perturbation to the network and then observing the genes' expression levels in the presence of this perturbation (Ideker et al., 2000). These perturbations can be genetic, in which the expression levels of one or more genes are fixed by deletion or overexpression; or biological, in which one or more non-genetic factors are altered, such as a change in the growth medium or a temperature increase. Once the expression data are obtained from a series of experiments with perturbations, analytic methods are necessary to make inferences about these networks (Ideker et al, 2000).

The integration of computational techniques with experimental perturbations is increasingly utilized to assess the response to drugs. The reason is that compounds

with many targets interact by various pathways and can be investigated through inferences from gene networks based on experimental perturbations. Knowledge of the architecture of these gene networks can also be useful to detect possible side effects of drugs and compounds.

It is currently possible to construct networks from expression data provided by microarrays or RNAseq, or also from data from genome-wide association studies (GWAS). Various programs have been used to construct these networks, such as Pathway Studio (http://www.ariadnegenomics.com/products/pathway-studio/expression-analysis/algorithms), Ingenuity Pathways Analysis - IPA (http://www.ingenuity.com/) and MetaCore™ (http://thomsonreuters.com/metacore/), all of which are commercial, along with Cytoscape (http://www.cytoscape.org/) and Exploratory Gene Association Networks - EGAN (http://akt.ucsf.edu/EGAN/), which are free. We can also mention GenMAPP (Dahlquist et al., 2002), PubGene (Jenssen et al., 2001) and VisANT (Hu et al., 2007). In recent years other tools have been created to study gene networks, such as iBIG (integrative BIoloGy), developed by Sun et al. (2013), which includes information such as protein-protein interaction, pathways, prediction of transcription factors and microRNA targets to generate a regulatory network based on microarray data, for instance. This tool is free and can be accessed at http://lei.big.ac.cn/ibig.

Application of gene networks to identify new candidate genes

Experiments with RNAseq and microarray have been used to evaluate the gene expression patterns in individuals with genotypic information by means of molecular markers. Expression values of the different genes are treated as multiple phenotypic traits so that QTL (Quantitative Trait Loci) mapping procedures can be used to detect polymorphisms associated with the variability of the expression patterns. These are generally called eQTL (expression Quantitative Trait Loci). The effect of allelic variations on the gene expression in a single chromosome region is called the *cis* effect, while the effect of polymorphisms on the expression of genes in other regions of the genome is called the *trans* effect (Jansen and Nap, 2001). This type of methodology has been utilized to identify candidate genes (Bystrykh et al., 2005; Hubner et al., 2005) and chromosome regions that control the expression of multiple genes, denominated *hot spots* (Bystrykh et al., 2005; Chesler et al., 2005; Hubner et al., 2005; Morley et al., 2004).

Schadt et al. (2005) experimentally identified and validated the involvement of three genes in susceptibility to obesity in a segregating population of mice, based on gene network studies. According to the authors, the identification of a common site for cis-eQTL and QTL chromosome regions for diseases is used to name genes in a disease susceptibility locus, so it is not necessary to perform complete fine mapping of the region. However, in cases when a causal gene is almost completely correlated with a complex trait of interest, or strongly regulates the expression of other genes related to the complex trait, the power to resolve the true relations will be reduced. Besides this, the procedure introduces a very simplistic view of the gene networks associated with diseases, since the true situation is more complicated due to the interaction of genes in a larger network and the possibility of negative and positive feedback. Despite this, the capacity to identify target genes associated with complex traits of interest in a gene network offers a promising approach for comprehension of these networks associated with complex traits, such as common human diseases, as well as identification of new ways to fight these diseases.

Chen et al. (2008) developed an alternative to the classic "forward" genetics, where instead of identifying susceptibility genes for a determined disease directly affected by variations in the DNA sequence, gene networks are identified that are perturbed by susceptibility loci, which in turn leads to development of the disease. This research team aimed to discover co-expression components of the gene network that respond to variations in the DNA associated with obesity, diabetes and atherosclerosis. After constructing co-expression networks of the liver and adipose tissue in segregating mouse populations, they identified sub-networks significantly associated with a complex of connected loci related to obesity, diabetes and atherosclerosis.

Characterizing the molecular networks that constitute the base of complex traits like disease susceptibility can provide a more comprehensive vision, leading to the direct identification of genes related to diseases and their roles. Other studies to characterize gene networks showed how loci associated with expression traits can be combined with clinical data to infer causal associations between expression of traits and disease (Chesler et al., 2005; Monks et al., 2004; Schadt et al., 2005).

Studies of networks applied to dairy cattle breeding

Just as knowledge of gene networks can help identify genes responsible for disease susceptibility in humans and mice, it also can be applied to identify genes or pathways responsible for traits of interest in stock breeding. This was the objective of Reverter and Fortes (2013), who evaluated the promoter region of differentially expressed genes to identify transcription factors responsible for their regulation. This information was then compiled to make inferences about a regulatory gene network. In this network, the nodes are the differentially expressed genes, the transcription factors or both, and the edges represent sequences related to the regulation of transcription. They also performed pairwise testing of the associated SNPs for epistatic interactions, and in this way they constructed an epistatic network. In other words, the regulatory network was constructed from the microarray data while the epistatic network was constructed from GWAS data. Finally, the two networks were merged and their intersection revealed the genes with functional relevance, i.e., differentially expressed genes that contain SNPs involved in epistatic interactions. The authors also explained that other functional categories, like kinases, interleukins, secreted proteins, membrane receptors, post-translational modifiers and micro-RNAs, can be considered as key regulatory molecules in these studies, in addition to transcription factors.

Bickhart and Liu (2013) have taken advantage of the huge quantity of sequencing data that has become available for some species in recent years to infer networks in species in which these data are not so abundant. They identified thousands of transcription factor binding sites in cattle by comparison with human and dog genomes, and opened the way for comparison of gene regulation networks between bovine and other mammal models (Loose et al., 2013).

During mastitis caused by different pathogens, it is observed that the pathways related to the immune response, cell proliferation and apoptosis generally are activated, and differences only occur in some genes within these pathways (Günther et al., 2009; Moyes et al., 2009; Rinaldi et al., 2010). However, although the response is initiated in the same form, the profile of the disease is different depending on the agent causing mastitis. In 2011 Breems carried out a dynamic analysis of the gene regulation in mammary epithelial cells in response to two pathogens: *Escherichia coli* and *Staphylococcus aureus*. In this work, by means of studies of the signaling pathways, the author verified differences in gene expression

in the pathway of the toll receptors depending on the invading bacteria and identified the main genes that make this response different in the two cases.

Another factor that has been studied in recent years is the influence of nutrition on gene expression. With respect to gene networks, feed can act as a stimulus to activate, deactivate or modify the expression level of different genes. Palin et al. (2013) studied the effects of fatty acids on the expression of genes related to milk cattle reproduction and verified that the abundance of mRNA of various genes was modulated by different fatty acid ingestion rates, supplied by linseed oil, which is rich in α-linolenic acid. The authors also verified differences in the secretion of prostaglandin in the uterus, which can affect the animal's reproductive efficiency. With the aim of characterizing the profiles of embrionary and endometrial expression in dairy cows fed with linseed oil supplements, the same research team customized a microarray chip in 2009 with sequences of genes found to be differentially expressed in previous nutrigenomic studies (Palin et al., 2009).

As can be observed, there are various ways to apply the study of gene networks, be it to identify genes for yield, disease resistance and even changes in the gene expression profile according to nutrition. The method to identify these key genes in the diverse networks can vary, but more than identifying these targets, it is important to understand their complex relations, which can result in different phenotypes and make a difference in a dairy farm.

References

Breems, N.V.L. A systems biology approach to signalling pathway and gene network regulation modelling in Mastitis. 2011. Thesis - Lincoln University, 2011.

Buitenhuis, B.; Røntved, C.M.; Edwards, S.M.; Ingvartsen, K.L.; Sørensen, P. In depth analysis of genes and pathways of the mammary gland involved in the pathogenesis of bovine *Escherichia coli*-mastitis. BMC Genomics, v.12, p.130, 2011.

Burrage, K.; Hood, L.; Ragan, M.A. Advanced computing for systems biology. Briefings in Bioinformatics, v.7, p. 390-398, 2006.

Bystrykh, L.; Weersing, E.; Dontje, B.; Sutton, S.; Pletcher, M.T.; Wiltshire, T.; Su, A.I.; Vellenga, E.; Wang, J.; Manly, K.F.; Lu, L.; Chesler, E.J.; Alberts, R.; Jansen, R.C.; Williams, R.W.; Cooke, M.P.; de Haan, G. Uncovering regulatory

pathways that affect hematopoietic stem cell function using “genetical genomics”. Nature Genetics, v.37, p. 225-232, 2005.

Chen, Y.; Jun Zhu, J.; Lum, P.Y.; Yang, X.; Pinto, S.; MacNeil, D.J.; Zhang, C.; Lamb, J.; Edwards, S.; Sieberts, S.K.; Leonardson, A.; Castellini, L.W.; Wang, S.; Champy, M.F.; Zhang, B.; Emilsson, V.; Doss, S.; Ghazalpour, A.; Horvath, S.; Drake, T.A.; Lusis, A.J.; Schadt, E.E. Variations in DNA elucidate molecular networks that cause disease. Nature, v. 452, n. 7186, p. 42-435, 2008.

Chesler, E.J.; Lu, L.; Shou, S.; Qu, Y.; Gu, J.; Wang, J.; Hsu, H.C.; Mountz, J.D.; Baldwin, N.E.; Langston, M.A.; Threadgill, D.W.; Manly, K.F.; Williams, R.W. Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. Nature Genetics, v. 37, p. 233-242, 2005.

Dahlquist, K.D.; Salomonis, N.; Vranizan, K.; Lawlor, S.C.; Conklin, B.R. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nature Genetics, v. 31, p. 19-20, 2002.

De Jong, H. Modeling and simulation of genetic regulatory systems: a literature review. Journal of Computational Biology, v. 9, n. 1, p. 67-103, 2002.

Erdos, P.; Renyi, A. On random graphs. Publicationes Mathematicae, v. 6, p. 290-297, 1959.

Gill, R.; Datta, S.; Datta, S. A statistical framework for differential network analysis from microarray data. BMC Bioinformatics, v. 11:95, 2010.

Günther, J.; Koczan, D.; Yang, W.; Nürnberg, G.; Repsilber, D.; Schuberth, H.J.; Park, Z.; Maqbool, N.; Molenaar, A.; Seyfert, H.M. Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with *Escherichia coli*. Veterinary Research, v. 40, p. 31-39, 2009.

Hood, L.; Heath, J.R.; Phelps, M.E.; Lin, B. Systems biology and new technologies enable predictive and preventative medicine. Science, v. 306, p. 640-643, 2004.

Hood, L.; Rowen, L.; Galas, D.J.; Aitchison, J.D. Systems biology at the Institute for Systems Biology. Briefings in Functional Genomics and Proteomics, v. 7, p. 239-248, 2008.

Hu, Z.; Ng, D.M.; Yamada, T.; Chen, C.; Kawashima, S.; Mellor, J.; Linghu, B.; Kanehisa, M.; Stuart, J.M.; DeLisi, C. VisANT 3.0: new modules for pathway visualization, editing, prediction and construction. Nucleic Acids Research, v. 35, p. W625–W632, 2007.

Hubner, N.; Wallace, C.A.; Zimdahl, H.; Petretto, E.; Schulz, H.; Maciver, F.; Mueller, M.; Hummel, O.; Monti, J.; Zidek, V.; Musilova, A.; Kren, V.; Causton, H.; Game, L.; Born, G.; Schmidt, S.; Müller, A.; Cook, S.A.; Kurtz, T.W.; Whittaker, J.; Pravenec, M.; Aitman, T.J. Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease. Nature Genetics, v. 37, p. 243-253, 2005.

Ideker, T.E.; Thorsson, V.; Karp, R.M. Discovery of regulatory interactions through perturbation: inference and experimental design. Pacific Symposium on Biocomputing, v. 5, p. 302-313, 2000.

Jackson, A.L.; Bartz, S.R.; Schelter, J.; Kobayashi, S.V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P.S. Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnology, v. 21, p. 635-637, 2003.

Jansen, R.C.; Nap, J.P. Genetical genomics: the added value from segregation. Trends in Genetics, v. 17, p. 388-391, 2001.

Jenssen, T.K.; Laegreid, A.; Komorowski, J.; Hovig, E. A literature network of human genes for high-throughput analysis of gene expression. Nature Genetics, v. 28, p. 21-28, 2001.

Li, T.; Cho, W.C.S. MicroRNAs: mechanisms, functions and progress. Genomics Proteomics Bioinformatics, v.10, p. 237-8, 2012.

Loose, M.; Patient, R.; Fang, X.; Lei, H. Gene Regulatory Networks in the Genomics Era. Genomics Proteomics Bioinformatics, v. 11, p. 133-134, 2013.

Mattick, J.S.; Makunin, I.V. Non-coding RNA. Human Molecular Genetics, v. 15, p. R17-R29, 2006.

Monks, S.A.; Leonardson, A.; Zhu, H.; Cundiff, P.; Pietrusiak, P.; Edwards, S.; Phillips, J.W.; Sachs, A.; Schadt, E.E. Genetic inheritance of gene expression in human cell lines. The American Journal of Human Genetics, v. 75, p. 1094-1105, 2004.

Morley, M.; Molony, C.M.; Weber, T.M.; Devlin, J.L.; Ewens, K.G.; Spielman, R.S.; Cheung, V.G. Genetic analysis of genome-wide variation in human gene expression. Nature, v. 430, p. 743-747, 2004.

Moyes, K.M.; Drackley, J.K.; Morin, D.E.; Bionaz, M.; Rodriguez-Zas, S.L.; Everts, R.E.; Lewin, H.A.; Loor, J.J.Gene network and pathway analysis of bovine mammary tissue challenged with Streptococcus uberis reveals induction of cell

proliferation and inhibition of PPARgamma signaling as potential mechanism for the negative relationships between immune response and lipid metabolism. BMC Genomics, v.10, p.542, 2009.

Palin, M.F.; Hallé, C.; Goff, A.K.; Petit, H.V.; Blouin, R. Effects of Different N-6/N-3 Fatty Acid Ratios and Of the Mammalian Lignan Enterolactone on Dairy Cow Endometrial Cells. WCDS Advances in Dairy Technology, v. 25, p. 382, 2013.

Palin, M.F.; Beaudry, D.; Vallée, M.; Bissonnette, N.; Murphy, B.D.; Petit, H.V. Development of a two-species cDNA microarray for transcriptional profiling of sow and dairy cow reproductive traits. Journal of Animal Science, v. 87 (E-Suppl. 2), 2009.

Reverter, A.; Fortes, M.R.S. Building single nucleotide polymorphism-derived gene regulatory networks: Towards functional genome-wide association studies. Journal of Animal Science, v. 91, p. 530-6, 2013.

Rinaldi, M.; Li, R.W.; Bannerman, D.D.; Daniels, K.M.; Evock-Clover, C.; Silva, M.V.B.; Paape, M.J.; Ryssen, B.V.; Burvenich, C.; Capuco, A.V. A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to *E. coli* mastitis. Functional and Integrative Genomics, v. 10, p. 21-38, 2010.

Schadt, E.E.; Lamb, J.; Yang, X.; Zhu, J.; Edwards, S.; Guhathakurta, D.; Sieberts, S.K.; Monks, S.; Reitman, M.; Zhang, C.; Lum, P.Y.; Leonardson, A.; Thieringer, R.; Metzger, J.M.; Yang, L.; Castle, J.; Zhu, H.; Kash, S.F.; Drake, T.A.; Sachs, A.; Lusis, A.J. An integrative genomics approach to infer causal associations between gene expression and disease. Nature Genetics, v. 37, p.710-717, 2005.

Strogatz, S.H. Exploring complex networks. Nature, v. 410, 268-276, 2001.

Sun, J.; Pan, Y.; Feng, X.; Zhang, H.; Duan, Y.; Lei, H. IBIG: an integrative network tool for supporting human disease mechanism studies. Genomics Proteomics Bioinformatics, v. 11, p. 166-71, 2013.

Tegnér, J.; Björkegren, J. Perturbations to uncover gene networks. TRENDS in Genetics, v. 23, p. 34-41, 2007.

Vêncio, R.Z.N.; Oliveira, G.B. Integração entre Biologia e Física: construindo redes de regulação gênica usando equações diferenciais. Revista Brasileira de Ensino de Bioquímica e Biologia Molecular, n. 1, 2006.

GENERAL CONCLUSIONS

Considering the crossbred animals with natural infection, higher expression was observed of the *TLR-2* gene (2.5 times greater) in the sick animals in relation to the healthy ones ($P < 0.05$). In turn, in the Gyr Dairy cows artificially infected with *S. agalactiae*, changes were observed in the expression of at least 14 genes related to the immune response ($P < 0.05$).

It is important to stress that these results cannot be directly compared because the mode of infection, the microorganisms causing mastitis and the breeds were different in the two studies. Nevertheless, these results suggest that genes that present significant differences in expression can perform important functions in fighting intramammary infection, with highlight on the *TLR-2* gene, which presented difference of expression in the two studies.

However, for these genes to be used as mastitis markers, it is necessary to validate these results in another biological model or by another method, such as by analysis of gene networks. This type of analysis has proved efficient in the identification of the main genes responsible for a determined trait.

The results presented in this thesis can be useful to test new hypotheses in future studies and to create new strategies to combat mastitis, especially in crossbred and Gyr Dairy animals, which are so important for the Brazilian dairy industry.

It is important to stress that Brazil is the only country that has an official genetic breeding program for Gyr Dairy breed and this study is the only one that analyzed the expression of genes related to the immune response to mastitis for the Gyr and crossbread dairy cows, therefore this is a first approach. From these results, proteomic studies and analysis of gene structure can be performed to identify SNPs present in genes differentially expressed in this work. These studies will not only to evaluate the potential of the genes as a marker, so as to obtain a better understanding of the physiopathological mechanisms of mastitis. Thus, the information here presented can be applied in other breeding programs and complement the selection work that has been carried out in Brazil for more than 60 years.