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EXPRESSION PROFILE OF IMMUNE RESPONSE GENES IN GOATS WITH EXPERIMENTALLY INDUCED *STAPHYLOCOCCUS AUREUS* MASTITIS.

Doctoral Thesis

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INTRODUCTION

1. MASTITIS IN SMALL RUMINANTS

Mastitis is defined as an inflammatory reaction of udder tissue to microbial organisms (mainly bacteria, but exceptionally virus and fungi), chemical, thermal or mechanical injury. The inflammatory response consists of an increase in blood proteins and white blood cells in the mammary tissue and the milk. The purpose of the response is to destroy the irritant, repair the damaged-tissue and return the udder to normal function.

Intramammary infections (IMI) in dairy small ruminants are mainly of bacterial origin (Bergonier et al. 2003b). Ovine and caprine IMI epizootiology and control share numerous common points, but are different from some physiopathological points of view: in goats, there are shorter (or even no) dry period, more varied variation factors of somatic cell counts (SCC), lentiviral mammary infection, higher 'stress' susceptibility, etc. These also differ due to certain husbandry methods: in goats, generally lower kidding synchronisation, a short or absent suckling period, diversity of alimentation systems from zero-grazing to traditional extensive husbandry, etc. Thus control strategies are based upon the same principles, but vary to a certain extent. On the contrary, important differences exist regarding cow IMI, which first led to improving basic knowledge and more recently to validating specific control programmes (Bergonier et al. 2003b).

Mastitis can be divided into three groups:

1- Clinical mastitis

2- Sub-clinical mastitis

3- Chronic mastitis

1.1. Bacterial Pathogens

A number of different organisms have been implicated in small ruminant clinical and subclinical mastitis. Sporadic cases of clinical mastitis most

frequently are caused by *Staphylococcus aureus*, coagulase-negative *Staphylococci* (CNS), *Arcanobacterium pyogenes*, *Corynebacterium*, *Pasteurella* spp., and *Pseudomonas* spp. Outbreaks of clinical mastitis most frequently involve *S. aureus*, *Streptococcus* spp. (*S. uberis, S. agalactiae,* and *S. suis*), and opportunists such as *Aspergillus, Pseudomonas, Burkholderia,* and *Serratia* (Croft et al. 2000; Plummer and Plummer 2011).

Numerous studies have identified CNS as by far the most important cause of subclinical mastitis in both the ewe (78%) and doe (71%). *S. epidermidis* and *S. caprae* are isolated most frequently, although other species are commonly identified (Poutrel et al. 1997; Croft et al. 2000). Shedding of coagulase-negative staphylococci often is cyclic, in inverse proportion to SCC elevation, and may be missed on single culture. From 60% to 80% of cultured strains of coagulase-negative staphylococci are hemolytic; hemolytic strains, and *S. epidermidis* as a species, tend to cause very high elevations in SCC, whereas other coagulase-negative staphylococcal species may not be obviously associated with an elevated SCC (Croft et al. 2000). *S. aureus* is the second most frequently isolated subclinical mastitis agent in the ewe (4%) and doe (8%), whereas *Streptococcus* spp. and *Corynebacterium* are less frequently identified (Croft et al. 2000). Unlike in dairy cattle, gram-negative bacteria are infrequent causes of mastitis in the ewes (3%) and does (8%) (Croft et al. 2000). Although rarely involved in mastitis, Listeria and *Salmonella* spp. are worth mentioning owing to their zoonotic potential; Listeria can be shed from clinically normal udders(Contreras et al. 2007; Plummer and Plummer 2011).

1.2. Clinical Mastitis

Although clinical mastitis constitutes a small percentage of mastitis cases in small ruminants, usually less than 5%, it frequently is the form of mastitis that the producer is most aware of (Bergonier et al. 2003a). Clinical signs of mastitis

include hard and swollen glands, enlarged supramammary lymph nodes, and possibly fever. Milk from affected glands may have an "off" color, contain flakes or clots, or be thinner or thicker than normal. Lameness or abnormal gait may be observed in some animals as a consequence of pain in the affected gland. Clinical mastitis usually is limited to sporadic cases, but occasional herd outbreaks have been observed (Kinde et al. 1994; Yeruham et al. 2005; Sela et al. 2007; Plummer and Plummer 2011). Even with treatment, clinical mastitis can become subclinical mastitis in many cases.

The most common cause of clinical mastitis is *S. aureus*. Other organisms that have been implicated include coagulase-negative staphylococci, *Enterobacteria* spp., *Mannheimia haemolytica*, *Pseudomonas* spp., *Arcanobacterium pyogenes*, *Streptococcus* spp., *Bacillus* spp., mycoplasmas, and fungal organisms (Bergonier et al. 2003b).

1.2.1. *Staphylococcus aureus* mastitis

S. aureus is the most common cause of clinical mastitis in small ruminants, accounting for 11% to 65.3% of the cases (Suarez et al. 2002; Lafi et al. 1998; Mork et al. 2007). This organism is a gram-positive coccus that occurs in clumps or pairs. It forms large colonies that are surrounded by a zone of incomplete hemolysis and up to 2 mm of complete hemolysis. Most but not all isolates exhibit such double-zone hemolysis. The clinical presentation in *S. aureus* mastitis ranges from severe gangrenous mastitis to subclinical mastitis. Acute infections manifest with a swollen, hot, and painful udder half accompanied by systemic illness. Chronic infections are associated with decreased production accompanied by induration and abscess formation within the udder (Smith et al. 2009). Subclinical infections are extremely difficult to treat and should be considered contagious. *S. aureus* is thought to be transmitted primarily through milking. The organism resides in microabcesses in chronically

infected animals, which then serve as a source of infection for other members of the herd or flock. *S. aureus* mastitis can be very difficult to cure, and all culture-positive animals should either be culled or milked last to prevent spread to flock- or herdmates. *S. aureus* is shed intermittently, so a single negative culture does not mean that an animal is truly clear of the organism. Before an animal can be returned to the main milking string, negative results on serial cultures and persistently low SCCs must be documented. *S. aureus* milk should be pasteurized before it is fed to kids or lambs, because diarrhea, pneumonia, and even death have been reported in kids and lambs consuming infected milk. (Plummer and Plummer 2011).

1.2.1.1. Bluebag (Gangrenous mastitis)

Bluebag is a form of acute mastitis characterized by ischemic necrosis of the udder causing discoloration of the udder. The most common bacterium isolated in gangrenous mastitis is *S. aureus* (Mork et al. 2007; Abu-Samra et al. 1998). *M. haemolytica, Clostridium* spp., and the coliforms also have been isolated in cases of gangrenous mastitis (Mork et al. 2007). In one study, *S. aureus* was isolated in 60% of cases (Hogan et al. 2003). Gangrenous mastitis typically is seen during lactation but occasionally appears during the last week of gestation as well. In some cases, death may occur within 24 hours of onset of clinical signs. If the animal survives the initial stage of infection, a demarcation line will form on the udder, and the affected portion of the udder will slough. Supramammary lymph nodes also will become enlarged, edematous, and hemorrhagic.

1.2.2. Coliform mastitis

Coliforms, mainly *Escherichia coli* and *Klebsiella,* have been isolated in cases of small ruminant clinical mastitis. Both organisms are gram-negative rods and

form large gray or yellow, moist colonies. Coliforms account for between 1.4% and 14.2% of reported cases (Suarez et al. 2002; Lafi et al. 1998; Mork et al. 2007). Although coliforms are very common in clinical mastitis in dairy cattle, these organisms are not a common cause of clinical mastitis in small ruminants. The relatively lower incidence of coliform mastitis in small ruminants probably is due to the difference in fecal consistency between small ruminants and cattle. The drier feces of small ruminants contribute to less fecal contamination of the udder. Coliform mastitis is most common in periparturient does. Coliforms can cause an endotoxin release that leads to severe systemic illness in the affected animal. Many of the clinical signs of coliform mastitis are associated with release of lipopolysaccharides and the systemic response to these endotoxins (Plummer and Plummer 2011).

1.2.3. *Mannheimia* mastitis

M. haemolytica is a common cause of mastitis in sheep and occasionally has been isolated from goat's milk. This organism is a gram-negative bipolar rod that forms medium, gray-tinged, transparent colonies on blood agar. Hemolysis also can be seen on blood agar. *M. haemolytica* probably is transmitted by suckling kids or lambs, where it often is found as part of the normal flora of the upper respiratory tract (Scoth et al. 1998; Gougoulis et al. 2008). Clinical signs can mimic those of *S. aureus* mastitis, so this infection should be a consideration in the differential diagnosis for bluebag (Plummer and Plummer 2011).

1.2.4. *Pseudomonas* mastitis

Pseudomonas is a gram-negative rod that forms granular and dry-appearing colonies of a variety of colors. The source of *Pseudomonas* may be contaminated water or teat dips, old pitted inflations on the milking machine, and wet bedding. Case presentations range from subclinical to gangrenous

mastitis (Sela et al. 2007; Leitner et al. 2007). Affected animals show clinical signs of systemic disease such as inappetence, fever, and depression, in addition to a firm, swollen, painful udder. Culling of infected and carrier animals is recommended; however, aggressive therapy may be successful (Hall et al. 2007). When this organism is cultured from a mastitis specimen, careful attention should be paid to the water in the parlor and the teat dip as a possible source (Plummer and Plummer 2011).

1.2.5. *Arcanobacterium pyogenes* infection

Arcanobacterium pyogenes is a small gram-negative rod that grows slowly on blood agar and forms very small "peach fuzz" colonies. *A. pyogenes* infections are associated with multiple abscesses in the udder. It is believed that such wounds predispose the affected animal to entry of the organism. *A. pyogenes* infections are more severe in nonlactating animals than in lactating animals. With chronic infection, culling is advised. If no evidence of spread to any other organs is found, amputation of the affected teat or gland can be performed (Smith et al. 2009).

1.2.6. Other species associated with clinical mastitis

Additional species that have been isolated in clinical mastitis cases include *Streptococcus* spp., *Micrococcus* spp., *Corynebacterium* spp., and *Bacillus* spp. (Lafi et al. 1998; Hall et al. 2007; Ndegwa et al. 2001).

1.2.7. *Mycoplasma* mastitis

Mycoplasma mastitis frequently is suspected when signs of clinical mastitis appear but repeated bacterial cultures are negative. *Mycoplasma* also should be considered as the infecting organism in mastitis associated with arthritis,

pneumonia, or conjunctivitis in the herd (Rodriguez et al. 1995). Several different species of *Mycoplasma* cause mastitis in sheep and goats. These species vary in their geographic distribution and clinical signs of disease.

1.2.8. *Mycoplasma agalactiae*

Mycoplasma agalactiae is the etiologic agent associated with the specific disease entity contagious mastitis. At present, *M. agalactiae* infection is rare in the United States but commonly is found in Mediteranean countries, Europe, Middle East, and South Africa. Clinical signs of contagious mastitis include septicemia with localization in the udder, joints, or eyes. The organism is shed in the milk, urine, feces, and ocular and nasal discharge for months, which can be a source of infection for other animals in the flock or herd (Bergonier et al. 2003b). Transmission of *M. agalactiae* is through ingestion or inhalation. Environmental contamination can occur and can be a source of infection as well.

1.2.9. *Mycoplasma mycoides* subsp. *mycoides* (Large Colony)

Mycoplasma mycoides subsp. *mycoides* (Large Colony) has been identified in cases of mastitis in the United States, Israel, and Europe (Kinde et al. 1994; Mork et al. 2007; Rodriguez et al. 1995; De massa et al. 1983; Bar-Moshe et al. 1981; East et al. 1983; Blikslager et al. 1992). This species is associated with respiratory disease as well (Rodriguez et al. 1995). It also has been classified by some workers as a cause of contagious agalactia. The disease associated with the organism occurs frequently in Europe (Plummer and Plummer 2011).

1.2.10. *Mycoplasma putrifaciens*

Mycoplasma putrifaciens has been associated with outbreaks of mastitis, abortion, and arthritis in California, Europe, and the Middle East (Smith et al. 2009; Dhondt et al. 1977). *M. putrifaciens* also has been identified in cases of subclinical mastitis characterized by fibrosis or palpable inflammation within the udder with no visible changes in the milk. This organism does not always cause fever in affected animals (Plummer and Plummer 2011).

1.2.11. Other Mycoplasmas

Several other *Mycoplasma* species have been described in association with mastitis. *Mycoplasma mycoides* subsp. *capri* and *M. mycoides* subsp. *capricolum* have both been implicated in cases of mastitis in goats in France. Experimental infections with *M. mycoides* subsp. *capricolum* resulted in severe clinical mastitis in does, manifesting with thick yellowish secretions, increased somatic cells, agalactia, and enlarged lymph nodes. Pneumonia, polyarthritis, and keratoconjunctivitis also were observed in the nursing kids. *M. arginini* has been associated with purulent mastitis in does in India but usually is considered nonpathogenic (Plummer and Plummer 2011).

1.2.12. Fungal mastitis

Although uncommon, fungal mastitis does occur and usually is the result of prolonged antibiotic use. A variety of organisms have been implicated, including *Candida albicans, Aspergillus fumigatus, Aspergillus terreus, Cryptococcus albidus, Cryptococcus neoformans, Yersinia pseudotuberculosis, Nocardia* spp., *Rhodotorula glutinis,* and *Geotrichum candidum*. Clinical signs of fungal mastitis include purulent mammary secretions, induration of the affected gland, fever, and weight loss (Jensen et al. 1996). Generally treatment is not

recommended owing to the lack of approved drugs for use in food-producing species (Plummer and Plummer 2011).

1.3. Subclinical Mastitis

Subclinical mastitis is a significant cause of elevated SCC and decreased production levels in small ruminants. Subclinical disease accounts for a majority of mastitis cases in a flock or herd and is a common cause of high bacterial counts or SCCs. Identification of animals affected by subclinical mastitis is much more difficult than recognition of those with clinical mastitis. In subclinical mastitis, few outward signs emerge to indicate presence of a problem. Occasionally the affected milk may have a slightly "off" color and may contain clots or blood, but frequently the affected milk may be completely normal in appearance. Some producers will note a decrease in production levels for an animal subsequently found to have subclinical mastitis. Detection of subclinical mastitis may require some additional testing such as with CMT or by SCC (Plummer and Plummer 2011).

1.3.1. Bacterial Subclinical Mastitis

The most common cause of subclinical mastitis in most herds or flocks will be bacterial in origin. Coagulase-negative staphylococci have been implicated as the leading cause of subclinical mastitis, with prevalence rates of 71% and 78%, respectively, in goats and sheep (Bergonier et al. 2003b). The second most common reported cause of subclinical mastitis is *S. aureus*, with reported prevalence rates of 8% in goats and 4% in sheep (Bergonier et al. 2003b). Subclinical *S. aureus* infections may start as clinical mastitis, which subsequently progresses to chronic, subclinical mastitis.

1.3.1.1. Coagulase-Negative *Staphylococci*

A variety of species have been implicated in causing subclinical mastitis, including *S. epidermidis, S. caprae, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus lugdunensis, Staphylococcus chromogenes,* and *Staphylococcus warneri* (Valle et al. 1991; Deinhofer et al. 1995; Ariznabarreta et al. 2002; Moroni et al. 2005; Leitner et al. 2004). *S. epidermidis* and *S. caprae* are the most common isolates. These subclinical infections tend to persist through the lactation cycle and also are more common in older does and with later lactation. Coagulase-negative staphylococci commonly are found on the skin or in the environment. An ongoing debate concerns the clinical significance of infections due to coagulase-negative staphylococci (Deinhofer et al. 1995; Ariznabarreta et al. 2002; Moroni et al. 2005; Leitner et al. 2004). Overall, the economic importance is unclear, because these infections do not cause severe illness or major production losses. A high prevalence of these infections is seen in many dairy goat herds. (Plummer and Plummer 2011).

1.3.1.2. Coagulase-Positive Staphylococci

S. aureus is the most common coagulase-positive staphylococcal isolate in subclinical mastitis. Many of these subclinical cases started as clinical mastitis, which did not resolve completely because the organism was not fully eradicated from the udder. Chronic *S. aureus* mastitis can be very difficult to clear, and any culture-positive animals either should be culled from the milking herd or should be milked last to decrease the potential to spread the organism to other animals in the herd. Only after multiple negative cultures and a low SCC have been obtained should an animal be returned to the main milking string (Plummer and Plummer 2011).

1.3.1.2. *Streptococcus* spp.

Streptococci also have been isolated in cases of subclinical mastitis. Prevalence rates range between 1.1% and 6.8% of subclinical mastitis cases (Lafi et al. 1998; Mork et al. 2007; Hall et al. 2007; Ndegwa et al. 2001). With the exception of *Streptococcus agalactiae*, these organisms are environmental contaminants and should be treated as such.

1.3.2. Retroviral Mastitis

The caprine and ovine retroviruses that are the agents of caprine arthritis-encephalitis (CAE) and ovine progressive pneumonia (OPP), respectively, both can be the cause of subclinical mastitis. Although mastitis may not be the primary clinical sign observed with each of these infections, the mastitis caused by these viruses can significantly affect the productivity of the doe or ewe.

Retroviral mastitis commonly is referred to as “hard udder” or “hard bag.” It is an interstitial mastitis that frequently is recognized at the time of parturition. The primary clinical manifestation in interstitial mastitis is a firm udder with loose overlying skin. No edema in the skin, heat, or erythema is noted. At the start of lactation, the affected animal may produce little to no milk, but milk production may gradually increase over the first couple of weeks after parturition. Any milk that is produced will be normal in appearance but will have significantly elevated cell counts. Evidence of systemic illness is lacking in affected animals. Supramammary lymph nodes also may be enlarged. Firmness also may be noted in the udder of does or ewes that are milking normally. In addition, affected animals may show signs of arthritis or respiratory problems.

Unfortunately, no treatment is available for CAE or OPP. Therefore culling of affected animals is recommended. Control of CAE and OPP is aimed at

eradicating the viral infection within the herd or flock (Plummer and Plummer 2011).

1.4. Prevention and Control

Mastitis is an “economic, hygienic, and legal” problem for producers (Bergonier et al. 2003b). Although the incidence of small ruminant clinical mastitis typically is less than 5% per year (Bergonier et al. 2003b; Contreras et al. 2007) problem herds may have clinical mastitis rates of 30% to 50%. The prevalence of subclinical mastitis in the average herd is very high, especially during late lactation, when chronic infections are at their highest prevalence (Bergonier et al. 2003b). Mastitis in small ruminants, especially the goat, often persists through the lactation and dry periods, and re-infection is common. Self-cure rates for subclinical mastitis during the dry period are 35% to 67% in the ewe and 20% to 60% in the doe (Bergonier et al. 2003b). New infections are associated with the first third of lactation, the start of machine milking, and the suckling-to-milking transition (Bergonier et al. 2003b). Mastitis control programs should focus on hygiene, the milking system and process, dry-off protocols, and culling. Culling often is the best recommendation for animals with clinical mastitis and for those with subclinical disease that do not respond to dry therapy (Bergonier et al. 2003b; Smith et al. 2009).

1.4.1. Hygiene

Skin flora and IMIs are the main reservoir for staphylococcal and streptococcal pathogens. Infections are spread and established during milking or nursing (Bergonier et al. 2003b). Although supporting evidence for their use is minimal, udder hygiene practices common to cattle dairies are encouraged in sheep and goat dairy operations (Smith et al. 2009). The dry, pelleted form of small

ruminant feces facilitates good udder hygiene scores; this is especially important at milking, when the teats must be clean and dry. Teat-dipping is recommended, especially "post-dipping," which can reduce the incidence of new IMI by 30% to 40% and also improve bulk tank SCCs (Paape et al. 2001); in goat herds, use of individual or single use towels also can reduce IMI rates (East et al. 1997).

Farm hygiene can have a direct effect on udder health. Bedding areas should be kept clean and dry to prevent coliform invasion. Moldy feed and bedding may introduce fungal pathogens (Bergonier et al. 2003b). Strict attention should be paid to stocking density; poor air ventilation and increased humidity associated with overcrowding will result in high airborne bacterial counts and a more favorable cutaneous environment for pathogens.

1.4.2. Milking Processes

Milking practices and the milking system may have a critical impact on udder health by causing mechanical insult or by providing bacterial reservoirs in dirty equipment. Producers are advised to implement a milking order whereby primiparous and nonmastitic animals are milked first. This strategy will decrease major (clinical) mastitis rates among first-lactation animals and decrease minor (subclinical) mastitis rates among multiparous animals (Bergonier et al. 2003b).

Milking practices that should be avoided include overmilking and undermilking, claw removal under vacuum, and vigorous udder massage or stripping.

Machine milking systems should optimize equipment to production levels, teat conformation, and operation size. Most milking system mechanical recommendations are based on data from dairy cattle, ideal small ruminant milking systems have not been fully characterized, and it is likely that current recommendations will continue to evolve. Institution of a program of annual system inspection and maintenance is a reasonable and often-overlooked step (Plummer and Plummer 2011).

1.4.3. Dry-Off

The dry period permits udder involution and colostrum development before the next lactation cycle. If well managed, it is an excellent opportunity to improve udder health and cure existing IMIs. Institution of dry-off should be prompted by decreased milk production or increased bacterial or somatic cell concentration, or should coincide with the next kidding date. Generally, it is better for udder health to abruptly decrease milking than to gradually decrease milking frequency. Dry therapy is used to cure existing infections or to prevent new infections in the close post-dry period; the former is more important in sheep and goats (Abu-Samra et al. 1988).

Animals with IMIs that persist through the dry period despite appropriate treatment should be culled (Bergonier et al. 2003b). Intramammary dry treatments significantly improve mastitis cure rates in the ewe (65% to 95.8%) and doe (50% to 92.5%) in comparison with untreated control animals (Bergonier et al. 2003b; Fox et al. 1992); coagulase-negative staphylococcal infections are more responsive to dry therapy than are *S. aureus* infections (Poutrel et al. 1997).

1.5. Inflammatory Response

Although much work has been carried out in dairy ruminants to understand the complex physiological and cellular events that occur in the mammary gland in response to pathogens (Sordillo et al. 2005; Paape et al. 2002; Kehrli et al. 1994), the protective mechanisms are still obscure. Schematically, when pathogens enter the udder lumen via the teat canal, they are detected by both immune and non-immune cells, and this is followed by the release of chemoattractants. As a consequence, neutrophils migrate from the blood flow to the infection site (Paape et al. 2002). These cells can phagocyte bacteria and

exert bactericidal activities by releasing potent oxidative products (Paape et al. 2003). This massive recruitment of neutrophils in the udder incurs a dramatic increase in the milk SCC (Kehrli et al. 1994).

Accordingly, SCC has been widely advocated as an easy-to-measure tool for predicting mastitis and discriminating between chronically infected and non-infected animals (Kehrli et al. 1994; Bergonier et al. 2003b; Bonnefont et al. 2011).

1.6. Somatic Cells

Somatic cells are normal constituent of milk and only when they become excessive do they indicate a problem. Somatic cells are composed of leucocytes and epithelial cells. Leucocytes (white blood cells) increase in milk in response to infection or injury while increase in epithelial cells is the result of infection or injury. The number of cells reflects the severity of mastitis. Somatic cells are expressed either as cells/ml of milk (SCC) or as its logarithmic transformation, the Somatic Cell Score (SCS) (Ali and Shook 1980).

Neutrophils are the most common leukocyte in both the infected and uninfected caprine mammary gland, making up 74% to 80% of the cell population in late lactation (Croft et al. 2000). By comparison, the noninfected ovine mammary gland cell population is comparable to that in cattle, being largely composed of macrophages (45% to 85%), with fewer neutrophils (10% to 35%), lymphocytes (10% to 17%), and epithelial cells (2% to 3%); neutrophil numbers increase during infection and are highly correlated with SCC (Fox et al. 1992; Croft et al. 2000).Normal somatic cell populations differ dramatically between the species. The SCC average concentration in milk is $150x10^3$/mL for goats and $15x10^3$/mL for ewes (Bergonier et al. 2003b). Higher counts are considered abnormal and indicate possible infections. In small ruminants, increased somatic cell counts (SCCs) are associated with increased parity, days in milk, stressors, and onset of

estrus, as well as with infection. The contribution of these factors is compounded in a seasonally producing herd. Apocrine milk production in small ruminants complicates SCC determination because some testing methods will miscount normal DNA-free cytoplasmic droplets; goats produce 10 times more cytoplasmic droplets than sheep (Fox et al. 1992). The Levowitz-Weber stain used for cattle SCC determinations does not adequately differentiate between leukocytes and cytoplasmic droplets (Fox et al. 1992).

Although nonpathologic increases in SCC are unavoidable, especially in goats, it is possible to influence SCC by controlling subclinical mastitis (Plummer and Plummer 2011).The degree to which infection directly correlates with SCC is controversial. Some workers suggest that IMI status is the major variable factoring into SCC (Croft et al. 2000; Godden et al. 2009). In one goat dairy, however, although SCC increased with IMI prevalence, 90% of SCC variability relates to factors other than mastitis (Murphy et al. 2004). Higher SCCs in early and midlactation are more likely to indicate infection than equivalent counts in late lactation (Contreras et al. 2007; Croft et al. 2000) and repeated tests, or comparative samples between udder halves, are more informative than single test points (Croft et al. 2000). Focusing on the annual average bulk tank SCC will help control for lactation-stage confounding factors and identify herd-level IMI. A strong correlation (r^2 = 0.845) between the annual average bulk tank SCC and persistent subclinical mastitis has been documented in ewes. Each 100,000 cell/mL–step increase in average bulk tank SCC equals a 2.5% increase in flock IMI prevalence (e.g., 250,000 cells/mL = 16% prevalence; 1 million cells/mL = 35% prevalence) (Bergonier et al. 2003a). Although interpretation of SCC in goats is more complex, a survey of 155 French goat dairies demonstrated a similar association: bulk tank SCC of 750,000 cells/mL = 30% (±12%) prevalence; 1 million cells/mL = 39% (±8%) prevalence; and 1.5 million cells/mL = 51% (±8%) prevalence (Bergonier et al. 2003a). For these reasons elevations in bulk tank SCC should be treated as an udder health

problem until proven otherwise (Plummer and Plummer 2011). Measured on a monthly basis, it can therefore be interpreted as an effect of infection and a good indirect indicator of chronic mastitis (Rupp et al. 2009). Estimates of heritability of the lactation SCS for sheep ranged between 0.10 and 0.20 (El Saied et al. 1999; Mavrogenis et al. 1999; Barillet et al. 2001; Othmane et al. 2002; Rupp et al. 2003; Serrano et al. 2003; Legarra and Ugarte 2005; Riggio et al. 2007; Sechi et al. 2007). No data are reported for goats.

1.7. Selection for Mastitis Resistance

Clinical mastitis is the primary health reason for involuntary culling in dairy small ruminants and causes additional economic losses from costs of veterinary treatments. Furthermore, some of the classical prophylactic measures sometimes appear too demanding to breeders in terms of time and care, and efficient vaccination against the main pathogens is still lacking; thus, complementary strategies are needed (Rupp et al. 2009). Over the past few decades, accumulating research has given strong evidence that the host's udder health is under genetic control in dairy ruminants (Shook 1989; Mrode and Swanson 1996; Heringstad et al. 2000; Detilleux 2002; Rupp and Boichard 2003). Moreover, there is accumulating evidence that the highly successful selection for milk production achieved over recent decades has led to a deterioration of mastitis resistance (Emanuelson et al. 1988; Heringstad et al. 2003a; Carlen et al. 2004). Accordingly, many countries (Heringstad et al. 2000; Miglior et al. 2005) have recently updated their breeding objective to include or increase the weight for nonproductive traits, with special attention given to mastitis resistance. Most research is concerned with quantitative studies on milk SCC using the SCS. However, given the low frequency of clinical mastitis and probably the very low heritability, further investigations are needed to confirm this trend. Despite the lack of well focused experiments in dairy sheep, SCC is

the criterion most used for evaluating dairy ewes' resistance to clinical and subclinical mastitis (Bonnefont et al. 2011) and breeding programs for mastitis resistance have been implemented throughout the world in dairy cattle (Heringstad et al. 2000; Rupp et al. 2003) and sheep (Rupp et al 2009) using indirect predictor traits such as clinical mastitis and SCS. In goat instead the significance of SCC values for parameters of udder health and the susceptibility against mastitis is still unknown.

Results of an experiment based on 2 lines of Lacaune ewes divergently selected for SCC suggested that selection for reducing milk SCC leads to improved resistance to clinical and subclinical intramammary infections (Rupp et al. 2009). Currently, selection strategies are based on a linear decrease of milk SCS, ideally as a tool to decrease both subclinical and clinical IMI. Scandinavian countries, where a large-scale recording scheme for clinical mastitis (CM) is available, also directly include the reduction of CM as a selection criterion (Heringstad et al. 2003b).

Genetic correlation between SCS and bacterial infection was estimated to be near unity in the only available large-scale study (9,784 recorded cows; Weller et al. 1992), indicating that SCS and subclinical infections are essentially the same trait.

Additionally, there is evidence that SCS-based selection should efficiently reduce CM incidence. The genetic correlation between SCS and CM is high, ranging from 0.60 to 0.70 (Heringstad et al. 2000, 2006; Carlen et al. 2004; Koivula et al. 2005). Furthermore, the correlation between SCS and CM does not show any sign of nonlinearity (McDaniel and Adkinson 1993; Philipsson et al. 1995; Rupp et al. 2000; Cranford and Pearson 2001), and 2 studies reported that the cows with the lowest observed SCC were cows with the lowest risk of CM (Rupp et al. 2000). The advantage of using SCS rather than CM directly in selection relies on greater heritability (e.g., 0.15 vs. 0.02, on average) on a linear scale (Heringstad et al. 2003b; Rupp and Boichard 2003). Furthermore, only

Scandinavian countries have recording schemes for CM for many years. In those countries, where CM is also included in the selection index, the low heritability of CM is counterbalanced by the large size of the progeny groups to obtain high accuracy of the breeding values of a sire (Rupp et al. 2009).

However, data are lacking on the genetic relationships between SCC or SCS, CM, and IMI across the environment and over time, and on the field evolution of IMI frequencies as a result of SCS- or CM-based breeding schemes. Both SCS and CM are used as a phenotypic black box selection tool that might not fully consider the variety of pathogens responsible for mastitis and the complexity of the resistance traits (Rupp et al. 2009). There is little knowledge of the genes and mechanisms mobilized by phenotype-based selection. Recently Bonnefont et al. (2011) highlighted some of the possible mechanisms leading to improved immune responses and consequently lower susceptibility to infection in two divergent –SCS-lines of dairy sheep. The study suggested a list of the differentially expressed genes between the resistant and susceptible animals and provided relevant information for the identification of candidates for the genetic basis underlying resistance to staphylococcal IMI in sheep.

The long-term effects and efficacy of sole-SCS selection, however, are still unknown. A better understanding of the defense mechanisms affected and modified by SCS-based selection would be helpful to predict the indirect response for CM, pathogen-specific infections, and resistance to other diseases in the long term (Rupp et al. 2009). A limited number of studies have examined one or a few candidate markers associated with cow, sheep and goat response to *S. aureus* infection (Riollet et al. 2001; Alluwaimi et al. 2003; Wesson et al. 2003; Yanke et al. 2000; Lee et al. 2006) however, the genetic interactions and their regulated expression in response to infection and disease are still poorly understood. It is a shared idea that the trait is not governed by a single major gene, but rather represents a polygenic trait, such a condition contributes to make the parameters for selection problematic (Cremonesi et al. 2011).

2. MARKER ASSISTED SELECTION

The classical selection has been very successful in improving performance related to quantitative traits, above all medium-high hereditable traits. The knowledge of genomic region responsible of the genetic variability of quantitative traits (the so called quantitative traits loci, QTL) can be useful to develop more efficient selection systems. Marker-assisted selection (MAS) is indirect selection process where a trait of interest is selected, not based on the trait itself, but on a marker linked to it. The assumption is that linked allele associates with the gene and/or QTL of interest. MAS can be useful for traits that are difficult to measure, exhibit low heritability, and/or are expressed late in development and targeting to particular classes of loci rather than globally may be more effective than simple index selection. In such a contest the finding of polymorphic markers like microsatellites and single nucleotide polymorphism (SNP) has made the MAS more realistic.

3. GENOME-WIDE SCANNING AND THE CANDIDATE GENE APPROACH

There are two approaches for genetic dissections of complex and quantitative traits, genome-wide scanning and candidate gene (CG) approach, which each has specific advantages and disadvantages. A CG is a gene, located in a chromosome region suspected of being involved in the expression of a trait such as a disease, whose protein product suggests that it could be the gene in question.

Genome-wide scanning usually proceeds without any presuppositions regarding the importance of specific functional features of the investigated traits, but of which the principal disadvantage is expensive and resource intensive. In general,

genome-wide scanning only locates the glancing chromosomal regions of quantitative trait loci (QTLs) at cM-level with the aid of DNA markers under family-based or population-based experimental designs, which usually embed a large number of candidate genes (Mengjin Zhu and Shuhong Zhao 2007). Using those families, investigators can identify genetic regions associated or "in linkage" with the disease by observing that affected family members share certain marker variants located in those regions more frequently than would be expected by chance. These regions can then be isolated, or cloned, for further analysis and characterization of the responsible genes (Kwon and Goate 2000). The primary advantage of linkage mapping is that investigators need no prior knowledge of the physiology or biology underlying the disorder being studied, which is important for complex disorders. In comparison, the alternative CG approach has been proven to be extremely powerful for studying the genetic architecture of complex traits, which is a far more effective and economical method for direct gene discovery (Mengjin Zhu and Shuhong Zhao 2007). The CG approach involves assessing the association between a particular allele (or set of alleles) of a gene that may be involved in the disease (i.e. , a candidate gene) and the disease itself. In other words, this type of association study tries to answer the question, " Is one allele of a candidate gene more frequently seen in subjects with the disease than in subjects without the disease?". Studies of CG do not require large families with both affected and unaffected members, but can be performed with unrelated cases and control subjects or with small families (Kwon and Goate 2000). Nevertheless, the practicability of traditional CG approach is largely limited by its reliance on existing knowledge about the known or presumed biology of the phenotype under investigation, and unfortunately the detailed molecular anatomy of most biological traits remains unknown (Mengjin Zhu and Shuhong Zhao 2007).

A useful way to identify CG is by microarray, this allows to analyze the expression levels of specific genes in a case control study, therefore if a gene is

dysregulated in a disease it may be identified by microarray. After a CG has been identified, its sequence and the correlated sequences (i.e. the promoter region) are analyzed to characterize the primary structure, to find polymorphism and to identify variants and alleles. The final goal is to identify functional SNP in CG and to find haplotypes to search for in the population.

A DNA microarray (also commonly known as gene chip, DNA chip, or biochip) is a collection of microscopic DNA spots attached to a solid surface. DNA microarrays are useful to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles of a specific DNA sequence, known as probes. These can be a short section of a gene or other DNA element that are used to hybridize a target cDNA or cRNA sample under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetics tests in parallel dramatically accelerating many types of investigation. DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or resequence mutant genomes.

The microarray technology, that nowadays enable to simultaneously study the expression of multiple genes in tissues as a response to a given treatment or physiological condition, enables the examination of complex interactions between the host and bacterial pathogens (Zheng et al. 2006).

The identification of a large number of bovine sequences (Womack 2005) has allowed the development of extensive oligonucleotide resources and microarrays tools in this species. By contrast, in caprine species, the absence of large sequencing programs has hampered the development and commercialization of caprine microarrays. However, structural genomic studies of domestic animals

have shown that goats are relatively closely related to bovine species (Schibler et al. 1998) and the cross-species hybridization approach for transcriptome analyses has already been reported to be successful in other species (Adjaye et al. 2004; Ollier et al. 2007). These facts, as well as microarray results so far published suggest that bovine tools may be used in transcriptome analyses in goats (Pisoni et al. 2008, 2010).

In the last decade, gene expression profiling microarrays have been widely used in animal genomics and this technique has enabled researchers to monitor, on a broad scale, the effects of pathogens on host cells and tissues, aiming to gain insight into the molecular mechanisms that are involved in the host-pathogen interactions. In dairy ruminants transcriptome analysis during experimental infection by various pathogens has enabled the identification of genes, pathways and regulatory networks activated in mammary tissues, (Gunther et al. 2009; Pareek et al. 2005; Lutzow et al. 2008; Moyes et al 2009; Rinaldi et al. 2010; Swanson et al. 2009; Brand et al. 2011), milk somatic cells (MSC) (Pisoni et al. 2010; Bonnefont et al. 2011) and peripheral blood mononuclear cells (Tao et al. 2007). More recently, also a meta-analysis of transcription-profiling data from six independent studies of infections with mammary gland pathogens, including samples from cattle challenged in vivo with *S. aureus*, *E. coli*, and *S. uberis* as well as samples from goats challenged in vivo with *S. aureus* were performed in order to test different responses to mastitis infection: overall (common signature), early stage, late stage, and cattle-specific (Genini et al. 2011).

AIMS

Counteracting infectious diseases of farm animals are an everlasting challenge in food production from livestock and preserving the health of farm animals is highly relevant to maintaining high standards of food quality. Although the incidence of small ruminant clinical mastitis typically is less than 5% per year problem herds may have clinical mastitis rates of 30% to 50%. Complementary strategies are needed to counteract the IMI in dairy small ruminants, since some of the classical prophylactic measures sometimes appear too demanding to breeders in terms of time and care and efficient vaccination against the main pathogens is still lacking,. The identification of specific genes and gene pathways associated with complex infectious diseases such as mastitis can contribute new knowledge for marker assisted selection studies aimed at increasing disease resistance in ruminants.

Consequently, there is an urgent need to understand the basic mechanisms regulating the host's immune defense against the most relevant pathogens and its modulation by the conditions of modern husbandry. Knowledge of these basic mechanisms will help to the design new and optimized strategies to prevent infections and, at the same time, significantly aid the improvement of food safety for the consumer.

The objectives of the present study were: (i) to identify the network of genes that becomes activated in caprine blood and milk somatic cells in early response towards a *S. aureus* challenge in order to better understand the local and sistemic response and (ii) to search any difference in this immune response by using two animal groups belonging to a caprine reference family established based on founders with adverse SCC breeding values, (iii) to develop a set of internal reference genes useful to normalize RT-qPCR data in studies of gene expression in caprine milk somatic cells (MSCs), these reference genes could be valuable during the investigation of the transcriptional status of the mammary gland of goats in relation to its genotype, nutritional and pathologic status, and under influence of hormonal factors.

The overall goal was to improve the understanding of host response to *S. aureus* mastitis and eventually aid in genetic selection of disease resistance.

MATERIALS AND METHODS

4. ANIMALS

Ten primiparous dairy alpine goats from Institut National de la Recherche Agronomique (France) were used. To provide enhanced insight into the genetic mechanisms involved in SCC-based selection, two groups of five primiparous French Alpine goats were issued from divergent selection based on extreme breeding values for the somatic cell counts (Low Somatic Cell Count (LSCC) and High Somatic Cell Count (HSCC) goats). The goats at the peak of lactation (48 ± 2 days in milking) were chosen according to the following criteria: similar milk production (3.2 ± 0.5 kg/d), first parity and absence of intramammary infection. Clinical examination of the mammary gland, bacteriological analyses and SCC confirmed that all animals were free from udder infections before challenges. Goats were monitored for intramammary infections (particularly for *S. aureus*) throughout the lactation period (from parturition to the day of challenge) with bacteriological analysis of weekly milk samples. Bacteriological analysis and somatic cell counts were performed as previously described (Moroni et al. 2005). At the moment of challenge, no inflammation in the udders was present, as was indicated by the absence of mastitis pathogens in foremilk samples tested for 11 consecutive days just before experimental challenge (Cremonesi et al. 2011).

Experiments were performed according to the Italian legislation, successfully notified and hence approved by the Italian ethics committee.

5. *STAPHYLOCOCCUS AUREUS* STRAIN

The organism used for the inoculum was *S. aureus* strain DV137, which was originally isolated from a chronic case of caprine mastitis (Vimercati et al. 2006). *S. aureus* DV137 was positive for clumping factor, free coagulase, enterotoxins C and L, toxic shock syndrome toxin TSST-1 and leukocidin LukDE.

Before challenge exposure, 10 mL of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Milan, Italy) were inoculated with the strain and incubated for 6 h at 37°C. Thereafter, 1 mL of the inoculum was transferred to aerating flask containing 99 mL of tryptic soy broth (TSB, Difco, Milan, Italy) and incubated overnight at 37°C. After incubation, the flask was placed in an ice water bath and mixed by swirling. One mL from the flask was serially diluted in PBS and 1 mL of the resulting dilution was mixed with 9 mL of pre-melted trypticase soy agar in petri dishes. The plates were allowed to solidify at room temperature and then transferred to a 37°C incubator overnight. The aerating flasks containing the stock inoculum were maintained at 4°C overnight. Once the concentration of the stock had been determined based on the prepared pour plates, the stock was diluted in sterile pyrogen-free Phosphate Buffered Saline (PBS, Invitrogen, Milan, Italy) to a final concentration of 10^3 Colony Forming Unit/mL (CFU/mL) (Cremonesi et al. 2011).

6. INTRAMAMMARY CHALLENGE

Prior to intramammary challenge, both udder halves were milked by hand and emptied, then the teat ends were carefully disinfected with chlorhexidine. The left udder half of each goat was infused with 1 mL (10^3 CFU/mL) inoculum of *S. aureus*. The right udder half was infused with 1 mL of sterile pyrogen-free PBS. Inoculation was administered intracisternally through the teat canal by the use of a sterile blunt needle. Milk samples were collected from all goats from both udder just prior to the challenge and 6, 12, 18, 24 and 30 hours after inoculation.

During experimental challenge the goats were also monitored by performing a general health check and udder examination. Before the milk sampling, each goat was examined using the following protocol: general impression of the animal which included behaviour, stance, position, food/water intake and

vocalization, general health check with temperature measurement and udder examination with temperature of the udder, swelling, color, pain, lumps, injuries to the teats/udder, milk extraction, milk colour and milk clots (Cremonesi et al. 2011).

7. BACTERIOLOGY AND MILK SOMATIC CELLS ANALYSIS

Milk sampling was performed under strict hygienic conditions. Disposable latex gloves disinfected with chlorhexidine prior to the sampling, were worn during the milking. Teats were wiped with chlorhexidine, and after the first streams of milk were discarded, 20 mL of sample milk was collected from each udder half into two sterile 10 ml tubes. One of the tubes was used for the bacterial counts, while the other tube of milk was used for SCC analysis. Subsequently, foremilk was collected from the left infected udders for milk cells isolation and further analysis. For the determination of milk SCC, a 2-mL aliquot of milk was heated for 15 min at 60°C and maintained at 40°C until counted in duplicate on automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Milan, Italy). For differential cell counts (DCCs), 10 ml of milk were centrifuged for 15 min at 400g, the cream layer and supernatant were discarded and the cells were washed twice in PBS. DCCs of isolated cell suspensions were estimated by means of esterase stain (Cremonesi et al. 2011).
Aseptically collected milk samples were serially diluted and plated onto blood agar plates and the number of CFU enumerated after 16 h of incubation at 37°C. Colonies displaying hemolysis were initially counted as *S. aureus*, and subsequently confirmed microscopically and biochemically by the presence of Gram-positive cocci that were both catalase- and coagulase-positive. *S. aureus* isolates were tested with RAPD-PCR (Van Leeuwen et al. 1996) to confirm that they were identical to the strain of challenge.

8. BLOOD SAMPLES ANALYSIS

Blood samples were collected from the jugular vein using a vacutainer system (sleeve, 18G 1" vacutainer needle and 10 ml EDTA vacutainer blood collection tube). About 10 ml of blood in EDTA have been collected from all animals just prior to the challenge, and 6, 12, 18, 24 and 30 hours after inoculation.

Blood samples were analyzed using the ADVIA 120 (Siemens Healthcare) haematology system, using the multispecies software provided by the manufacturer. The following parameters were calculated: erythrocyte (RBC) count, haemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte (WBC) count, platelet (Plt) count, mean platelet volume (MPV). Leukocyte differentials (percentage and number of neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells or LUC), were also performed (Cremonesi et al. 2011).

9. BLOOD LEUKOCYTE RNA EXTRACTION

Fresh blood was centrifuged at 250g for 20 minutes, then the white cell layer was transferred to fresh tubes and red blood cells were lysed with distilled sterile water. RNA was extracted immediately using the TRI Reagent following the instructions of the supplier (Sigma Aldrich, St. Louis, MO). High-quality RNA was obtained through purification with RNeasy MinElute spin column (Qiagen, Carlsbad, CA) and eluted in RNase-free water. RNA was then quantified using a NanoDrop spectrophotometer (Agilent, Santa Clara, CA) and quality-checked using a Bioanalyser 2100 (Agilent, Santa Clara, CA) (Cremonesi et al. 2011).

10. MILK SOMATIC CELLS ISOLATION AND RNA EXTRACTION

Foremilk collected aseptically from each mammary gland at 0 (T0), 6 (T1), 12 (T2), 18 (T3), 24 (T4), and 30 (T5) hours post-infection was transferred into 50 mL falcon and centrifuged at 2000 rpm at 4°C for 10 min. After the fat layer and the supernatant were discarded the cell pellet was washed and suspended in 10 mL of PBS pH 7.2 (added EDTA). All the solution (cells +PBS) was recovered and transferred in a new falcon; PBS was added to 50 mL final volume. After a centrifugation at 1500 rpm for 10 min, the supernatant was discarded and the pellet was resuspended in 3-5 mL of Trizol for total RNA extraction. Total RNA was extracted from at least 10^6 milk somatic cells with Trizol reagent following the instructions of the supplier (Invitrogen, Foster City, CA). High-quality RNA was obtained through purification with RNeasy MinElute spin column (Qiagen, Carlsbad, CA) and eluted in RNase-free water. RNA was then quantified using a NanoDrop spectrophotometer (Agilent, Santa Clara, CA) and quality-checked using a Bioanalyser 2100 (Agilent, Santa Clara, CA).

11. MICRORRAY DESIGN

All available bovine transcript sequence information was downloaded from Ensembl release 5.0, and Unigene and dbEST databases (Sept. 2008). A bioinformatic pipeline was created to align the sequences and select a unique set of minimally redundant bovine transcripts. This dataset was used to design 43,768 unique probes with a length of 35 nucleotides, each representing a single bovine transcript. These probes were synthesized in duplicate, along with negative and quality controls, on a 90K feature array from CombiMatrix (Seattle, WA)(Figure 1).

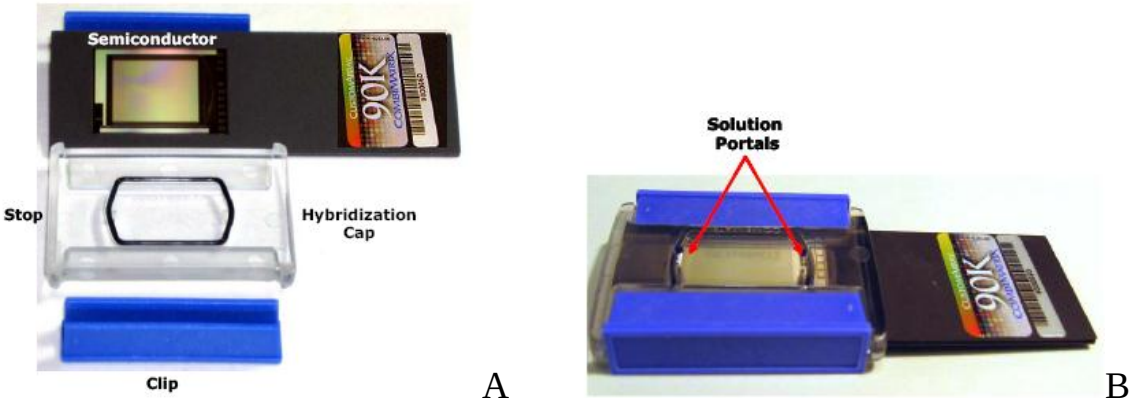


Figure 1. CustomArray™ 90K microarray components (A) and its assembling with Hybridization Cap and Clips (B)

12. ARRAY HYBRIDIZATION AND DATA ANALYSIS

About 1 µg RNA was amplified and labelled with Cy5-ULS using the RNA Amplification and Labelling Kit from CombiMatrix (ampULSe Cat. no. GEA-022; Kreatech Biotechnology, Amsterdam, The Netherlands).

All procedures were carried out according to the manufacturer's protocols (Figure 2). The purified labelled aRNA was quantified using a NanoDrop spectrophotometer (Agilent, Santa Clara, CA). Four µg of labelled RNA were fragmented to a uniform size and hybridized to the custom array following the CustomArray 90K Microarray Hybridization and Imaging Protocol.

Arrays were stripped and re-hybridized using the CustomArray Stripping Kit for 90K (Cat. No. 610049) following the protocols of the manufacturer. Each array was used up to 4 times with no deterioration in signal or increase in background.

The hybridized arrays were scanned with a GenePix 4000B microarray scanner (Axon, Toronto, CA) and the images (TIF format) were exported to the CombiMatrix Microarray Imager Software, to perform a quality check of the hybridizations and the spots on the slide. Data were extracted and loaded into R software using the Limma analysis package from Bioconductor and the signal

intensities were processed and normalized using standard procedures. Limma performs a linear regression analysis on the hybridizations, using a group-means parameterization approach to compare the different conditions and performs a false discovery rate adjustment with Benjamini-Hochberg correction for multiple testing (Smyth 2004). Differentially expressed (DE) genes were selected using an adjusted P-value cut off equal to 0.01 (Cremonesi 2011).

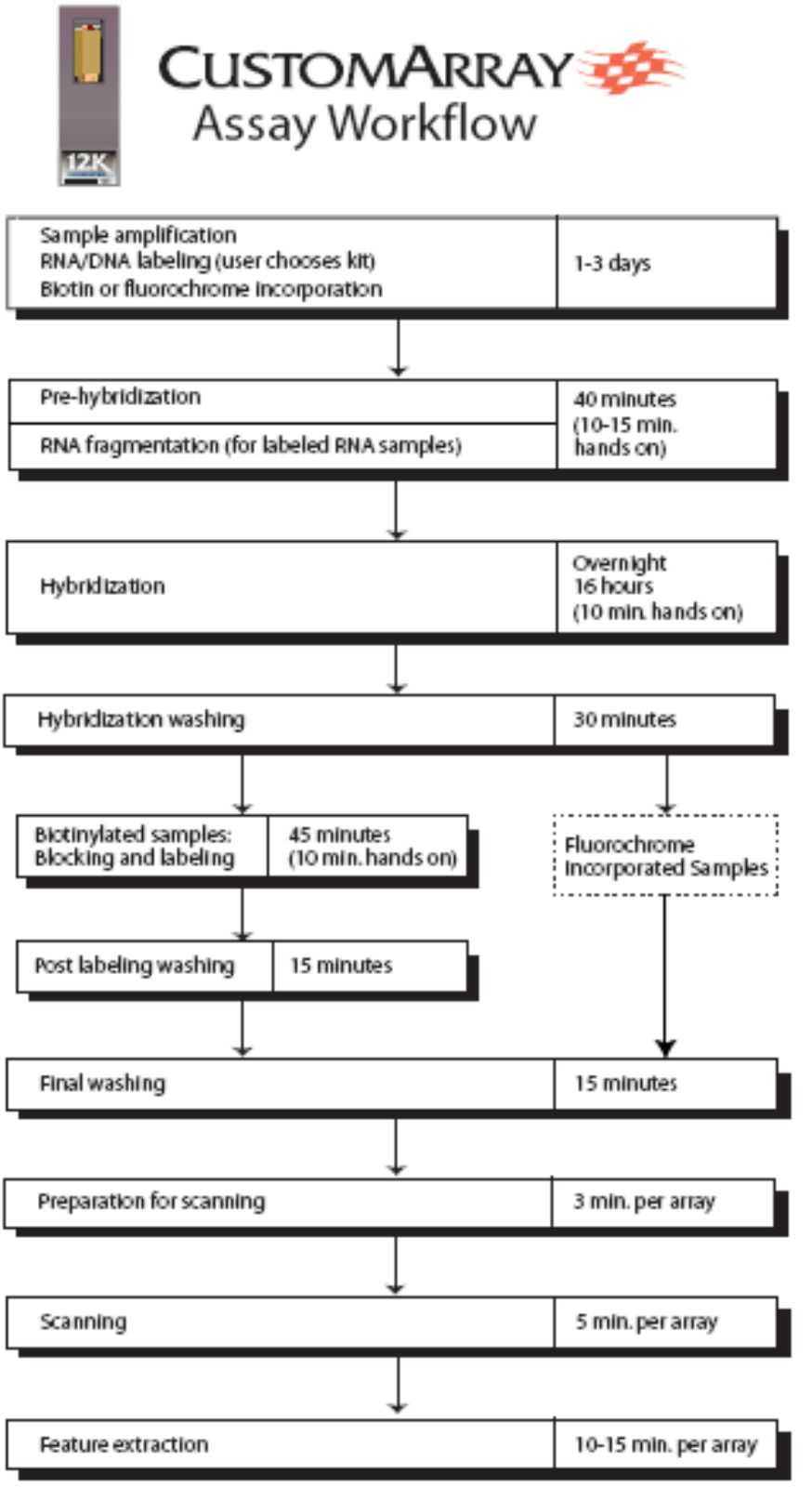


Figure 2. CustomArray™ assay workflow according to the manufacturer's protocols

13. EVALUATION OF INTERNAL REFERENCE GENES FOR QUANTITATIVE EXPRESSION ANALYSIS BY REAL-TIME PCR IN SOMATIC CELLS FROM GOAT MILK

Sample processing and experiments were carried out according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). Total RNA extracted (as described above) from MSCs isolated before infection (T0) was used for the evaluation of internal reference genes for quantitative expression analysis by real-time PCR (RT-qPCR). To avoid any genomic DNA contamination during RT-qPCR 500 ng of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Carlsbad, CA), according to the manufacturer's instructions. This kit ensures complete digestion of genomic DNA by a brief incubation of the sample at 42°C with a specific Wipeout buffer before retrotranscription.

Fourteen genes involved in basic cell metabolism and frequently used as references in RT-qPCR gene expression experiments were selected (Table 1).

Symbol	Gene name
ACTB	B-actin
B2M	β-2-microglobulin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxantine phosphoribosyltransferase 1
PGK1	phosphoglycerate kinase 1
RPL19	ribosomal protein L19
SDHA	succinate dehydrogenase complex

TFRC	transferrin receptor
YWHAZ	tyrosine 3-monooxygenase
G6PD	glucose-6-phosphate dehydrogenase
GYPC	glycophorin C
18S rRNA	18S rRNA
UBQ	ubiquitin
TUBB	β-tubulin

Table 1: Gene selected for the **e**valuation of internal reference genes for quantitative expression analysis by real-time PCR in somatic cells from goat milk.

Primers for SDHA, G6PD, TUBB and 18S rRNA were based on previous publications (Garcia-Crespo et al. 2005; Frota et al. 2010). The Primer3 software, freely available online, was used to design the other primers on conserved regions after the alignment of the caprine sequences available in GenBank with bovine and ovine homologous genes (Figure 3).Primers were selected to produce amplicons spanning two or more exons, moreover the possibility to recognize non-specific amplification of the genomic DNA on the basis of the melting curves analysis was taken into account during the selection of primers. Preliminary PCR assays using caprine cDNA and genomic DNA were performed to test primer specificity. One µl of cDNA was used in a 25 µl PCR reaction using 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Carlsbad, CA) and 900 nM of each primer. PCR amplification was run on a Mx 3005P QPCR System (Stratagene-Agilent Technologies, Santa Clara, CA). Amplification products were loaded and checked on a 2 % agarose gel, purified with a PCR Clean-Up System (Nucleospin Extract II- Macherey-Nagel, Düren, Germany) and sequenced using BigDye® Terminator v1.1Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). After purification with (Illustra^{TM} AutoSeq^{TM} G50 Dye Terminator Removal Kit- GE Healthcare) they were run on

a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Finally, sequences were checked for their specificity using Blast software to compare them with sequences available on GenBank.

Primer3 Output

```
PRIMER PICKING RESULTS FOR ywhaz e4f e5r

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER        600   20   59.01   55.00  3.00  2.00 CTGAACTCCCCTGAGAAAGC
RIGHT PRIMER       776   20   59.89   55.00  4.00  0.00 CTGCTTCAGCTTCGTCTCCT
SEQUENCE SIZE: 846
INCLUDED REGION SIZE: 846

PRODUCT SIZE: 177, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00
TARGETS (start, len)*: 639,99

    1 TGCTGAGCCCCGTCCGTCCGCCGCCACCTACTCCGGACACAGnAACATCCAGTCATGGAT

   61 AAAAACGAGCTGGTACAGAAGGCCAAACTGGCCGAGCAGGCTGAGCGATATGATGACATG

  121 GCAGCCTGCATGAAGTCTGTAACTGAGCAAGGAGCTGAATTATCCAATGAGGAGAGGAAT

  181 CTTCTCTCAGTTGCTTATAAAAATGTTGTAGGAGCCCGTAGGTCATCTTGGAGGGTCGTC

  241 TCCAGTATTGAGCAAAAGACGGAAGGTGCTGAGAAAAAACAGCAGATGGCTCGAGAATAC

  301 AGAGAGAAAATAGAGACCGAGCTAAGAGATATCTGCAATGATGTACTGnTCTCTTTTGGA

  361 AAAGTTCTTGATCCCCAACGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGAAAATGAA

  421 AGGAGACTACTACCGCTACTTGGCTGAGGTTGCAGCTGGTGATGACAAGAAAGnGGATTG

  481 TGGACCAGTCACAGCAAGCATACCAAGAAGCTTTTGAAATCAGCAAAAAGGAAATGCAAC

  541 CAACACATCCTATCAGACTGGGTCTGGCCCTTAACTTCTCTGTGTTCTATTATGAGATTC
                                                                  >

  601 TGAACTCCCCTGAGAAAGCCTGCTCTCTTGCAAAGACAnGCATTTGATGAAGCCATTGCT
      >>>>>>>>>>>>>>>>>>>>                  **********************

  661 GAACTTGATACATTAAGTGAAGAGTCATACAAAGACAGCACGCTAATAATGCAGTTACTG
      ************************************************************

  721 AGAGATAACTTGACAnTTGTGGACATCGGATACCCAAGGAGACGAAGCTGAAGCAGGAGA
      *****************                  <<<<<<<<<<<<<<<<<<<<<

  781 AGGAGGGGAAAATTAACCTGCCTTCCAACTTTTGTCTGCCTCATTCTAAAATTTACACAG

  841 TAGACC

KEYS (in order of precedence):
****** target
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                   start  len      tm      gc%   any   3'  seq

 1 LEFT PRIMER        600   20   59.01   55.00  3.00  2.00 CTGAACTCCCCTGAGAAAGC
   RIGHT PRIMER       779   20   59.89   55.00  4.00  0.00 CTCCTGCTTCAGCTTCGTCT
   PRODUCT SIZE: 180, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 3.00

 2 LEFT PRIMER        600   20   59.01   55.00  3.00  2.00 CTGAACTCCCCTGAGAAAGC
   RIGHT PRIMER       773   20   60.13   55.00  4.00  2.00 CTTCAGCTTCGTCTCCTTGG
   PRODUCT SIZE: 174, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00
```

Figure 3. Primer3 output. This software has a web interface and was used to study primers for the amplification of the candidate reference genes.

The protocol of amplification of four out of the fourteen genes did not satisfy the requirements and they were excluded from further analysis. For the remaining ten pair of primers, after preliminary real-time assays, the optimal primer

concentration appeared to be 600 mM, which generates the lowest Ct value and a sharp peak, with no amplification of non-specific products. For each pair of primers, efficiency of RT-qPCR (E), slope values, and correlation coefficients (R^2) were determined, using serial 1:10 dilutions of template cDNA, on a Mx 3005P QPCR System (Stratagene- Agilent Technologies, Santa Clara, CA) (Table 2).

Gene	Forward Primer Sequence [5'→3']	Primer conc. (nM)	Amplicon Length (bp)	Tm (°C)	Slope	E%	R^2
ACTB	CTTCCAGCCGTCCTTCCT TGTTGGCATACAGGTCCTTTC	600	214	56	-3.25	100	0.997
GAPGH	GGGTCATCATCTCTGCACCT ACAGTCTTCTGGGTGGCAGT	600	211	56	-3.392	95.6	0.998
G6PD	TGACCTATGGCAACCGATACAA CCGCAAAAGACATCCAGGAT	600	76	56	-3.61	94,6	0.997
PGK1	GGAAGGGAAGGGAAAAGATGC TCCCCTAGCTTGGAAAGTGA	600	92	56	-3.15	100	0.994
18SrRNA	TTTGGTGACTCTAGATAACCTCGGGC TCCTTGGATGTGGTAGCCGTTTCT	600	184	56	-3.344	99.1	0.999
RPL13A	CCCTGGAGGAGAAGAGAAAGG AATTTTCTTCTCGATGTTCTTTTCG	600	104	56	-3.065	100	0.999
SDHA	CATCCACTACATGACGGAGCA ATCTTGCCATCTTCAGTTCTGCTA	600	90	56	-3.44	99.9	0.985
YWHAZ	CTGAACTCCCCTGAGAAAGC CTGCTTCAGCTTCGTCTCCT	600	177	56	-3.197	100	0.995
TUBB	TTCATTGGCAACAGCACAGCCA TCGTTCATGTTGCTCTCAGCCT	600	150	58	-3.39	97.2	0.995
TFRC	TGGAAAAATCAGTTTTGCTGAA GTCCAAAAACTGGAAGATTTGC	600	124	56	-3.21	100	0.994

Table 2 Details of primers and amplicons for each of the 10 evaluated genes with efficiency of RT-PCR (E), slope values, and correlation coefficients (R^2).

Retrotrascripted total RNA from MSCs isolated in the time point T0, T4 and T5 from each goat (n=10) was amplified by Real-Time qPCR, using SYBR Green

detection chemistry, run in triplicate on 96-wells reaction plates with the Mx 3005P QPCR System (Stratagene- Agilent Technologies, Santa Clara, CA).

Reactions were carried out in a total volume of 25 µl containing: 2 µl cDNA, 1.5 µl of each 10 µM primer (600 mM each; Invitrogen, Foster City, CA), 12.5 µl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Carlsbad, CA) and 7.5 µl RNase,/DNase-free sterile water (Qiagen, Carlsbad, CA). Blank controls and genomic DNA template were run for each master mix. The cycle conditions were set as follows: initial template denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 45 s and elongation at 72°C for 30 s. For the TUBB gene annealing/elongation temperature was 58°C for 45s. This cycle was followed by a melting curve analysis of 95°C for 10 s, 45°C for 60 s, and a slow ramp (0.05°C/sec) to 95°C with continuous fluorescent acquisition.

Baseline and threshold values were automatically determined for all plates using the MxPro^{TM} QPCR software (Stratagene - Agilent Technologies, Santa Clara, CA). To ensure comparability between data obtained from different experimental plates, the threshold value has been subsequently manually set to the value automatically determined thresholds annotated previously; then all data have been reanalyzed. Raw Cq values were transformed to quantities using the comparative Ct method ($Q=E^{(\text{Min Cq- Sample Cq})}$). The data obtained have been converted into correct input files, according to the requirements of the software, and analyzed using *geNorm* (version 3.5) (Vandesompele et al. 2002) and *NormFinder* (version 0.953) (Andersen C.L. et al. 2004) applets.

The geNorm VBA applet for Microsoft Excel determines the most stable reference genes from a set of tested genes in a given cDNA sample panel, and calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of reference genes. geNorm calculates the gene expression stability measure M for a reference gene as the average pairwise variation V for that gene with all other tested reference genes.

Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability from the most stable (lowest M values) to the least stable (highest M values).

NormFinder is an algorithm for identifying the optimal normalization gene among a set of candidates. It ranks the set of candidate normalization genes according to their expression stability in a given sample set and given experimental design. The algorithm is rooted in a mathematical model of gene expression and uses a solid statistical framework to estimate not only the overall expression variation of the candidate normalization genes, but also the variation between sample subgroups of the sample set (e.g. normal and cancer samples). Notably, NormFinder provides a stability value for each gene, which is a direct measure for the estimated expression variation enabling the user to evaluate the systematic error introduced when using the gene for normalization.

14. NORMALIZATION OF RELATIVE QUANTITIES OF TRANSCRIPTS OF GENES OF INTEREST (GOI)

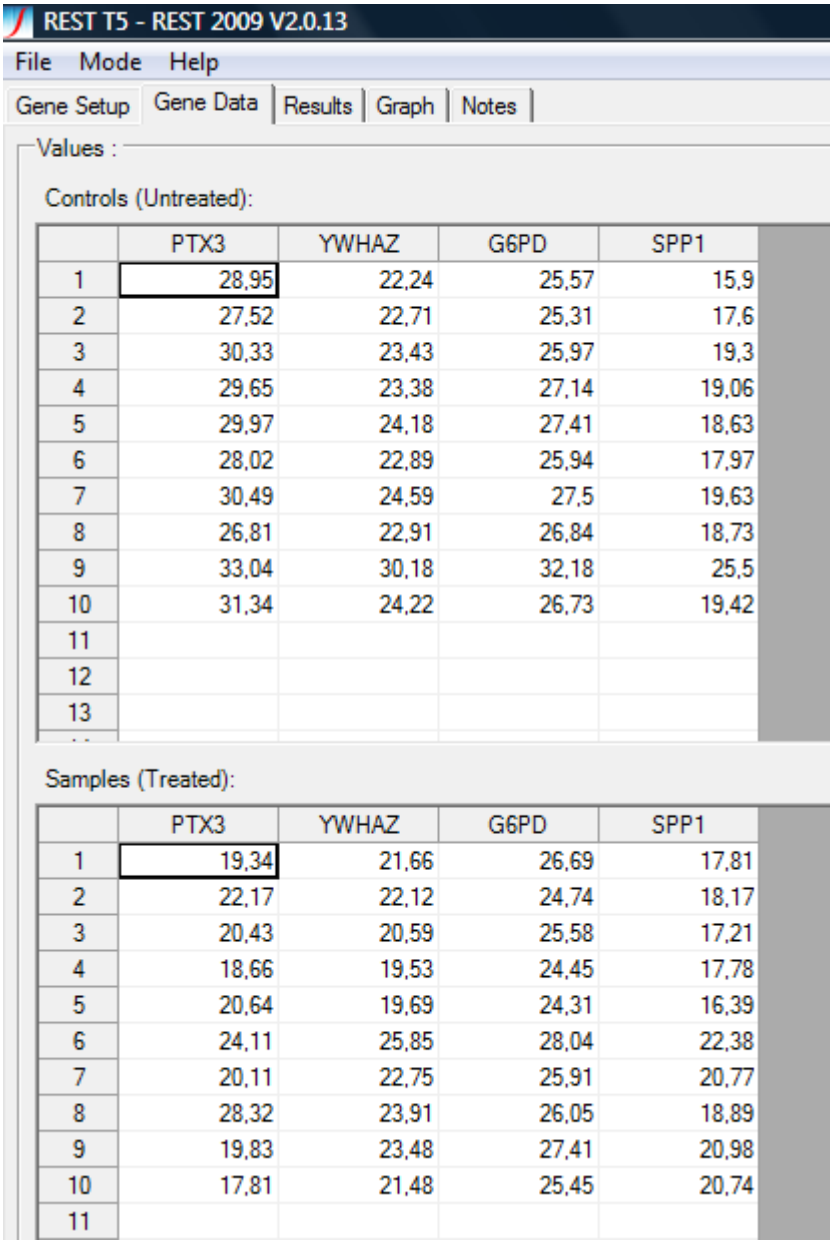
The expression of the genes with the highest difference of expression (pentraxin-related protein, PTX3 and secreted phosphoprotein 1, SPPI), as shown by microarray analysis, was validated by RT-qPCR.

Total RNA extracted from MSCs isolated at time point T0, T4 and T5 was used to analyze the expression level of PTX3 and SPPI genes. The same protocol used for the evaluation of the internal reference genes was followed. To avoid any genomic DNA contamination during qRT-PCR 500 ng of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Carlsbad, CA), according to the manufacturer's instructions. As no goat sequences for PTX3 and SPPI genes were available in GenBank, at the moment of writing, primers were designed using Primer3 software on conserved regions

of the bovine and ovine homologous genes. They were selected to produce amplicons spanning two or more exons, and to allow identification of non-specific genomic DNA amplification on the basis of the melting curves analysis. Preliminary PCR assays using caprine cDNA and genomic DNA were performed to test primers specificity. Amplification products were loaded and checked on a 2% agarose gel, purified with a PCR Clean-Up System (Nucleospin Extract II- Macherey-Nagel, Düren, Germany) and sequenced using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). After purification (Illustra™ AutoSeq™ G50 Dye Terminator Removal Kit- GE Healthcare) they were run on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Finally, sequences were checked for gene specificity using Blast software and comparing them with sequences available on GenBank.

Genes expression were quantified on the same batch of cDNA, to minimize experimental variation (in large part due to cDNA synthesis) and the same gene was tested in the 10 different samples collected at T0, T4 and T5 in the same PCR run to exclude further variation. The Cq values were transformed to quantities using the comparative Ct method.

The REST 2009 v2.0.13 software was used to analyze gene expression data from quantitative RT-q PCR experiments. This software applies a mathematic model that takes into account the different PCR efficiencies of the gene of interest and reference genes. It provides statistical information that is suitable for comparing expression in groups of treated and untreated samples in a robust manner. The integrated randomization and bootstrapping methods used in REST 2009 Software test the statistical significance of calculated expression ratios and can be used even when the data includes outliers. The analysis of experimental data were carried out setting default value for randomization and bootstrapping.



	PTX3	YWHAZ	G6PD	SPP1
1	28,95	22,24	25,57	15,9
2	27,52	22,71	25,31	17,6
3	30,33	23,43	25,97	19,3
4	29,65	23,38	27,14	19,06
5	29,97	24,18	27,41	18,63
6	28,02	22,89	25,94	17,97
7	30,49	24,59	27,5	19,63
8	26,81	22,91	26,84	18,73
9	33,04	30,18	32,18	25,5
10	31,34	24,22	26,73	19,42
11				
12				
13				

	PTX3	YWHAZ	G6PD	SPP1
1	19,34	21,66	26,69	17,81
2	22,17	22,12	24,74	18,17
3	20,43	20,59	25,58	17,21
4	18,66	19,53	24,45	17,78
5	20,64	19,69	24,31	16,39
6	24,11	25,85	28,04	22,38
7	20,11	22,75	25,91	20,77
8	28,32	23,91	26,05	18,89
9	19,83	23,48	27,41	20,98
10	17,81	21,48	25,45	20,74
11				

Figure 4. REST 2009 software interface.

RESULTS

15. EXPERIMENTAL CHALLENGES

15.1. Milk Bacteriological Analysis and Somatic Cell Counts Before Challenge

Bacteriological analyses and SCC confirmed that all animals were free from udder infections before challenges. Milk samples were taken from both udders every days from 11 days prior to intramammary *S. aureus* (strain DV137) infusions and tested for bacterial growth and somatic cell counts. No significant difference was observed in mean composite milk SCC between Low Somatic Cell Count (LSCC) and High Somatic Cell Count (HSCC) goats (Figure 5).

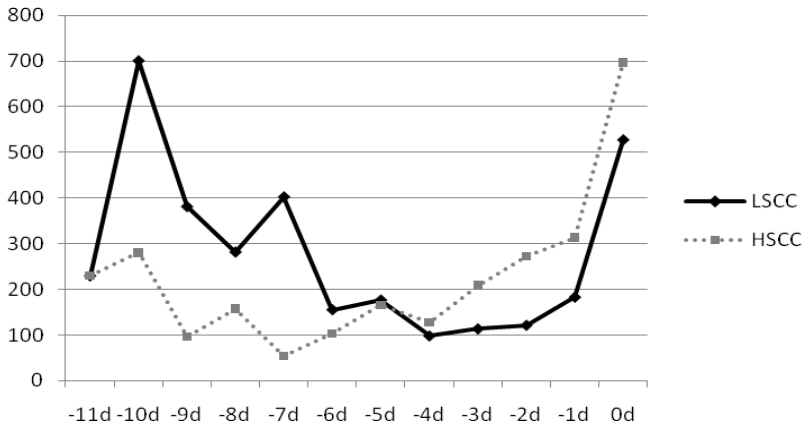


Figure 5. Mean composite milk SCC of LSCC and HSCC goats from 11 to 0 days pre-infection (x10^3/ml).

15.2. Intramammary *S. Aureus* Growth Following Experimental Infection

S. aureus was recovered from the milk of all 10 experimentally infected left udders within 6 h (T1) of infusion (Table 3). At the final sampling 30h post-infection (T5), S.aureus was found in 9 of the 10 left udders, showing they were still infected. *S. aureus* counts in milk peaked at 18h (T3) post-infection (mean 5.8 and 6.1 log10 CFU/ml in LSCC and HSCC selected goats respectively) and

remained relatively constant for the duration of the experiment (Figure 6). RAPD analysis confirmed that *S. aureus* isolated from infected udders were identical to the strain of challenge. PBS-infused udders remained free of infection throughout the study. No significant difference was observed in *S. aureus* counts at different time points in milk from LSCC and HSCC goats.

	T0	T1	T2	T3	T4	T5
1	0	1135	58500	18750000	7300000	87500
2	0	60	3550	24500	140000	1,9E+08
3	0	100	10200	955000	46000	63000
4	0	530	3550	24500	140000	65000
5	0	335	21100	1040000	31500	6500
6	0	245	28650	1100000	68750	10500
7	0	150	18600	106300	314000	63000
8	0	10	10	5	140	650
9	0	100	18050	2275000	5475000	1,6E+08
10	0	198	62000	1,9E+07	5650000	2,4E+07

Table 3 *S. aureus* recovered from the milk of all 10 left udders during experimental challenge (CFU/ml).

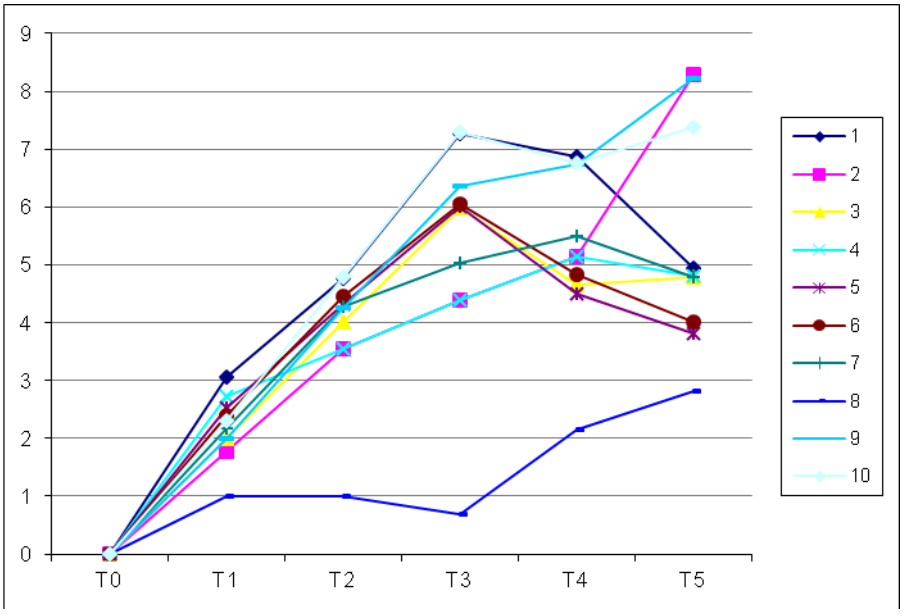


Figure 6. S*taphylococcus aureus* log10 CFU/ml in milk from left udders at T0, T1, T2, T3, T4 and T5 (0, 6,12, 18, 24 and 30 hours post infection respectively).

15.3. Systemic and Localized Inflammatory Responses to *Staphylococcus Aureus* Intramammary Infection

Rectal temperatures (Table 4), blood samples parameters (Table 5) and total milk production (Figure 8) were monitored throughout the study as an indicator of a systemic response to *S. aureus* infection.

Animal ID	Temperature					
	TO	T1	T2	T3	T4	T5
1	39,0	39,4	39,5	39,4	41,8	41,0
2	38,7	40,0	38,8	39,2	39,6	40,1
3	38,8	40,0	40,2	39,6	40,7	40,5
4	39,6	39,2	39,0	39,0	40,8	40,2
5	39,5	39,9	39,5	39,8	40,6	40,2
6	39,4	39,5	39,3	39,3	40,0	39,7
7	38,9	39,5	39,0	39,2	39,4	40,1
8	39,5	39,6	39,8	39,9	39,5	40,1
9	39,2	39,6	39,2	39,4	41,0	41,5
10	39,6	39,6	39,6	39,6	41,3	41,4
LSCC (Mean)	**39,3**	**39,6**	**39,4**	**39,5**	**40,2**	**40,6**
HSCC (Mean)	**39,3**	**39,6**	**39,4**	**39,5**	**40,3**	**40,7**

Table 4. Rectal temperatures recorded at each time point as an indicator of a systemic response to *S. aureus* infection.

A trend in elevation of body temperature was apparent from 0 to 18 h post-infection, however, significant increases were only observed at the 24 and 30 h time points. Maximal elevations in temperature were detected 30 h after

infection and reached a peak mean (±D.S.) of 40,2 (±0.5) and 40,7 (±0.6) °C in LSCC and HSCC selected goats (Figure 7). A paired t-test (with threshold for statistical significance set to 0.05) was applied to body temperature of LSCC and HSCC selected goats to verify if the difference at each time point was significant. No significant difference was observed in mean body temperature of LSCC and HSCC goats at different time points.

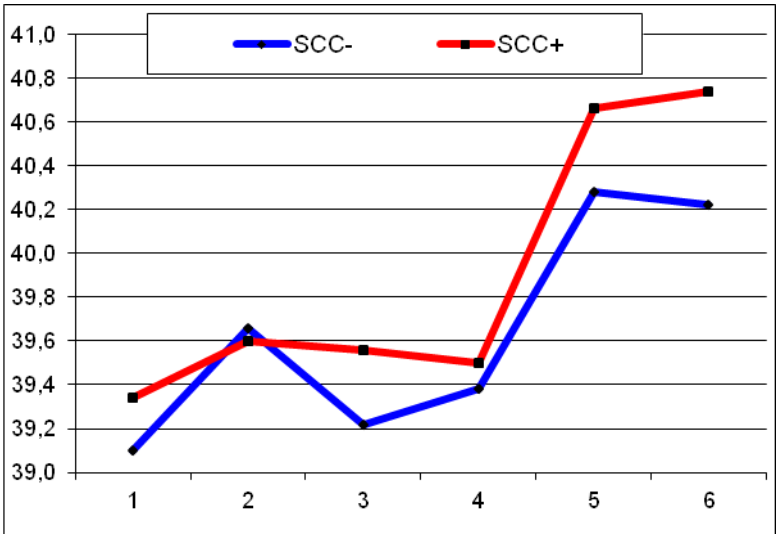


Figure 7. Mean body temperature of LSCC and HSCC goats at at T0, T1, T2, T3, T4 and T5 (0, 6,12, 18, 24 and 30 hours post infection respectively).

Systemic response to intramammary infection (IMI) with *S. aureus* was characterized by a significant decrease of total blood leukocyte (TBL) and of neutrophils (NEU) after 18h post-infection. The TBL and NEU counts were 13,7 and 6 x 10^3 cells/ml in LSCC goats and 11,5 and 4 x 10^3 cells/ml in HSCC goats, respectively. The lowest counts were reached at 30h post-infection, when the TBL and NEU counts were 9,8 and 4,2 x 10^3 cells/ml in LSCC goats and 5,6 and 2 x 10^3 cells/ml in HSCC goats, respectively (Figure 8). A paired t-test (with threshold set to 0.05) was applied to blood samples parameters of LSCC and HSCC goats to verify if the difference at each time point was significant. Blood

samples parameters were not significantly different between LSCC and HSCC goats at different time points

	T0	T1	T2	T3	T4	T5
1	10,54	10,04	9,71	9,35	3,54	3,6
2	19,84	16,03	16,83	17,48	16,4	15,6
5	13,3	14,33	13,55	11,59	10,18	9,67
6	15,97	16,08	15,39	13,91	14,24	12,37
7	8,65	9,11	10,07	9,27	7,91	7,65
LSCC mean	13,66	13,118	13,11	12,32	10,454	9,778
3	13,57	12,99	12,93	13,12	9,18	8,35
4	9,32	8,64	7,84	8,09	6,72	6,01
8	9,99	9,57	11,3	8,76	8,43	6,09
9	10,31	9,48	9,71	8,82	3,34	2,27
10	14,25	15,04	14,02	11,08	5,59	5,19
HSCC mean	11,488	11,144	11,16	9,974	6,652	5,582

Table 5 Total blood leukocyte (TBL) counts of the 10 goats at T0, T1, T2, T3, T4 and T5 (0, 6,12, 18, 24 and 30 hours post infection respectively). Mean values are reported for LSCC and HSCC groups(x 10^3 cells/ml).

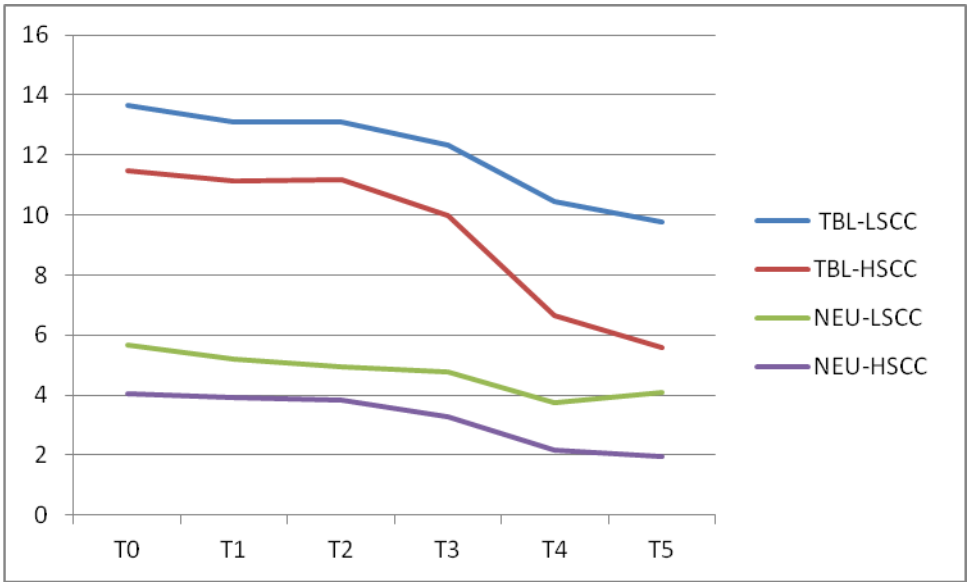


Figure 8. Total blood leukocyte (TBL) and of neutrophils (NEU) counts of LSCC and HSCC goats at T0, T1, T2, T3, T4 and T5 (0, 6,12, 18, 24 and 30 hours post infection respectively) (x 10^3 cells/ml).

Starting mean milk productions of the left udders were 695 ml and 855 ml in LSCC and HSCC goats respectively. A decrease in milk production was

observed from T1 and maximal decrease was reached at 30 h post infection when 245 ml and 210 ml were produced by LSCC and HSCC animals (Figure 9).

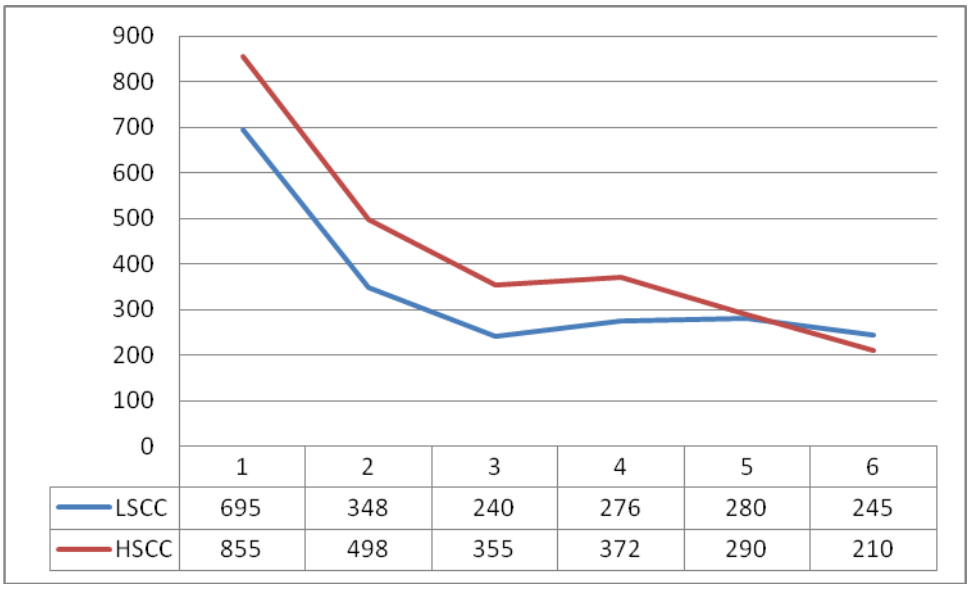


Figure 9. Mean milk production (ml) of LSCC and HSCC group recorded throughout the study.

Changes in milk SCC were monitored throughout the study as an indicator of local inflammation. Mean milk SCCs (x10^3/ml), before *S. aureus* intramammary infection, were 603 and 451 for LSCC left and right udders respectively, and 876 and 517 for HSCC left and right udders respectively (Table 6). After 18 h of infection, initial increases in milk SCC were observed in *S. aureus* infected left udders and elevated levels of somatic cells persisted in the following hours. No significant difference was observed between LSCC and HSCC goats.

	T0	T1	T2	T3	T4	T5
LSCC left	602,8	482,8	396,4	1030	4238,6	4925,4
LSCC right	450,8	221	216	304	487,2	542,2
HSCC left	876	729	466,8	757,6	6365	4768,4
HSCC right	517	1691,8	1661,8	794,8	1438,4	2407

Table 6. Changes in milk SCC monitored throughout the study as an indicator of local inflammation (x10^3/ml)

Relatively to pre-infection (time 0) levels, milk SCC right udders infused with saline remained unchanged throughout the study in LSCC goats, while milk SCC in right udders of HSCC goats increased twofold (Figure 10). A paired t-test (with threshold set to 0.05) was applied to milk SCC of right udders in LSCC and HSCC goats to verify if the changes at each time point was significant.

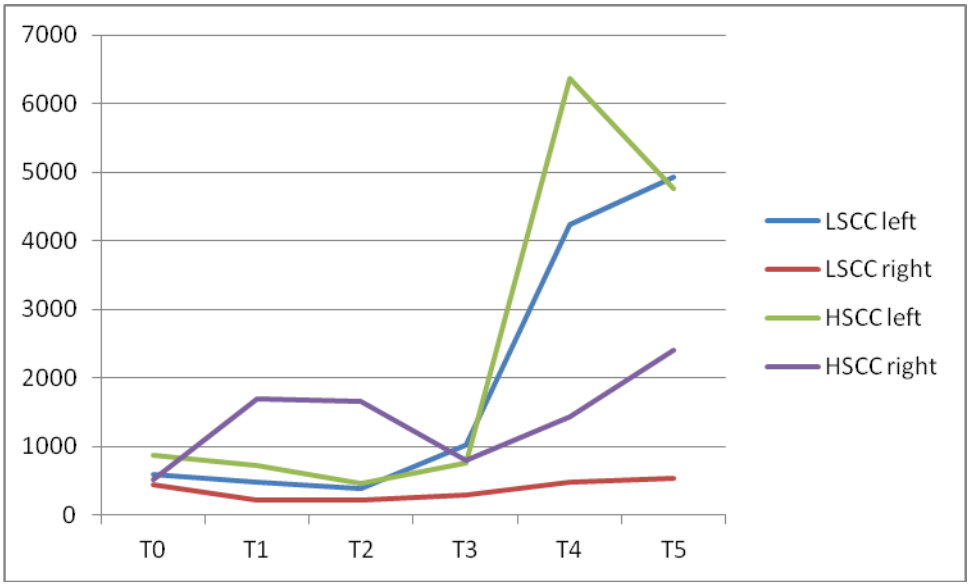


Figure 10. Mean milk SCC (x10^3/ml) of LSCC and HSCC goats in *Staphylococcus aureus*-infected left udders and in PBS-infused right udders at T0, T1, T2, T3, T4 and T5 (0, 6,12, 18, 24 and 30 hours post infection respectively).

16. MICROARRAY ANALYSES

16.1. Milk Samples

According to the statistical analysis, a total of 300 genes were found to be differentially expressed in milk samples between T0 and T4 and 128 genes between T0 and T5, with a p value < 0.01 and log2 fold change > 1.5. Among these, the majority (251 for T4vT0 and 123 for T5vT0) were up-regulated. No

differences were found between T0 and T1, T2, T3. From Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc.), 259 and 127 genes respectively for T4 and T5 of milk samples were mapped or recognized based on annotation to a human or mouse orthologous within the IPA Knowledge base. The complete list of these genes is showed in Appendix (Table 15 and Table 16) and represented in Figure 11. These genes were eligible for generating networks and pathways based on published data across several species, including human, rat and mouse.

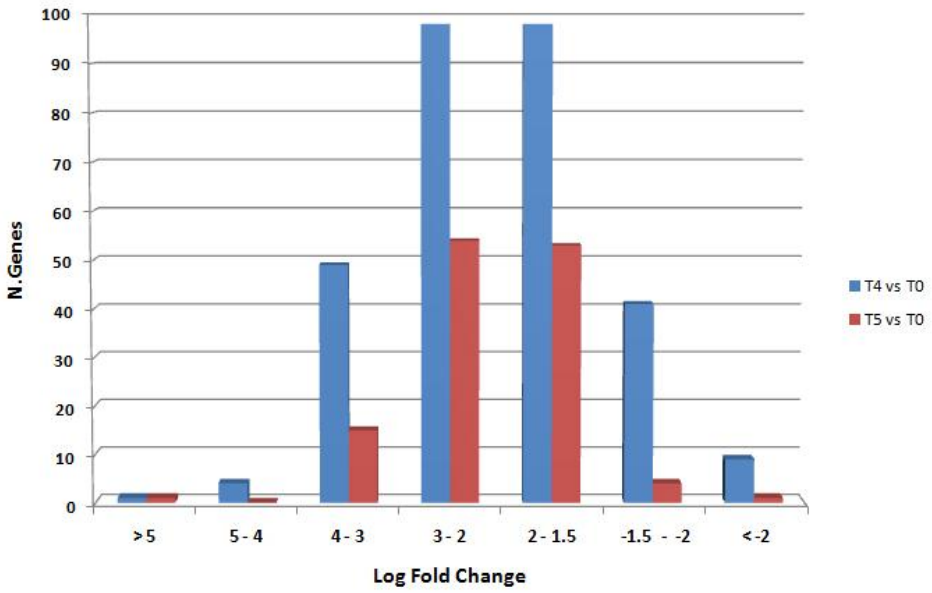


Figure 11. Log fold change distribution of genes differentially expressed in milk samples in T4 and T5 Vs T0 in log fold change categories.

16.1.1 Individual Genes

Tables 15 and 16 show for T4 and T5 the top 10 genes up and down regulated in MSC after IMI with *S. aureus*. The top up-regulated genes (5,65 to 3,16 fold change) plays an important role (i) in immune and inflammatory response (TNFAIP6, BASP1, IRF1, PLEK, BATF3); (ii) in the regulation of innate resistance to pathogens (PTX3); (iii) in the regulation of cell metabolism (CYTH4, SLC2A6). The top down-regulated genes (-1,50 to –2,46 fold) included genes involved in lipid metabolism (ABCG2, FASN), chemokine,

cytokine and intracellular signaling (SPPI), cytoskeleton and extracellular matrix (KRT19).

16.1.2. Canonical Pathway

The top canonical signaling and metabolic pathways within all the differentially expressed genes for T4 and T5 are reported in Appendix Table 17. The results showed an alteration of both immune response and lipid metabolism. These included MIF-mediated Glucocorticoid Regulation, MIF Regulation of Innate Immunity, NF-kB Signaling, IL-10 Signaling and Hypoxia Signaling in Cardiovascular System for T4; Production of Nitric Oxide and Reactive Oxygen Species in Macrophages, LXR/RXR Activation, Toll-like Receptor Signaling, Acute Phase Response Signaling and MIF-mediated Glucocorticoid Regulation for T5. The majority of genes with > 1.5 fold change within these pathways were up-regulated. Between the five pathways, three were related to immune or inflammatory functions such as IL-10 Signaling, which limits the inflammatory response, and Production of Nitric Oxide and Reactive Oxygen Species in Macrophages and MIF-mediated glucocorticoid regulation, which promotes the inflammatory response. Pathway analysis also revealed the activation of Toll-like Receptor Signaling, resulting in stimulation of synthesis of a number of pro-inflammatory cytokines and chemokines in respond to bacterial infection, and the activation of LXR/RXR Activation, involved in inflammation and lipid metabolism.

16.1.3.Network

Within IPA analyses, 19 and 11 networks were identified based on microarray results for T4 and T5, respectively. For T4 the networks included a total of 244 differentially expressed genes involved in pathways and functions including

Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Hematopoiesis, Tissue Development, Antigen Presentation, Cellular Compromise, Cellular Function and Maintenance, Inflammatory Response. For T5 the networks included a total of 109 differentially expressed genes involved in pathways and functions including Inflammatory Response, Digestive System Development and Function, Hepatic System Development and Function, Cell-To-Cell Signaling and Interaction, Tissue Development, Hematological System Development and Function, Kidney Failure, Organismal Injury and Abnormalities, Renal and Urological Disease, Tumor Morphology, Amino Acid Metabolism, Small Molecule Biochemistry, Cell Death, Cellular Compromise.

16.2. Blood Samples

In blood samples a total of 9 genes were found to be differentially expressed between T0 and T5 with a p value < 0.01 and log2 fold change > 1.5 (Appendix Table 18). Eight of them were up-regulated, while only 1 was down-regulated. The complete list is showed in Table 4. No differences were found between T0 and T1, T2, T3, T4.

17. VALIDATION OF MICROARRAY ANALYSES

17.1. Evaluation of Internal Reference Genes in Caprine Milk Somatic Cells.

The expression of ten potential internal reference genes was examined in 30 samples collected in T0, T4 and T5 (Appendix Table 19). The stability of selected control genes was determined using two different specific VBA applets (*geNorm* and *NormFinder*) which produce highly comparable results.

Results from geNorm analysis are shown in Table 7, according to these data the most stable genes appeared to be ACTB and YWHAZ in T0 and T5 samples,

G6PD and TFRC in T4 samples, these genes are characterized by the same average M values within each group: 0,445 and 0,388 in T0 and T5 respectively and 0,527 in T4. On average ACTB, YWHAZ, G6PD, SDHA and TUBB constitute stable genes in all three groups of sample. The least stable genes are PGK1, RPL13A, 18S rRNA. GAPDH and TFRC have variable ranking in different group.

Ranking order	T0		T4		T5	
	Gene symbol	Average M value	Gene symbol	Average M value	Gene symbol	Average M value
1/2	ACTB/ YWHAZ	0,445	G6PD/TFRC	0,527	ACTB/ YWHAZ	0,388
3	SDHA	0,468	TUBB	0,587	TUBB	0,505
4	G6PD	0,517	SDHA	0,624	GAPDH	0,679
5	TUBB	0,646	YWHAZ	0,705	TFRC	0,825
6	PGK1	0,707	ACTB	0,786	G6PD	0,900
7	TFRC	0,756	18S rRNA	0,866	SDHA	0,994
8	18S rRNA	0,818	RPL13A	0,932	18S rRNA	1,057
9	RPL13A	0,858	GAPDH	1,035	RPL13A	1,221
10	GAPDH	0,954	PGK1	1,199	PGK1	1,373

Table 7 Ranking of stability of a set of reference genes after analysis with geNorm software. Mean value of gene expression stability measure (M) in the sample collected in T0, T4 and T5.

The geNorm software suggests that an accurate normalization factor of RT-qPCR data can be calculated by using the two most stably expressed genes (ACTB and YWHAZ for T0 and T5 sample and G6PD and TFRC for T4 sample) (Figure 12) and that the addition of further reference genes will not significantly affect the reliability of the determined normalization factor, yielding a V2/3 value (pair-wise variation between two sequential normalization factors) of 0.141, lower than the default cut-off value of 0.15.

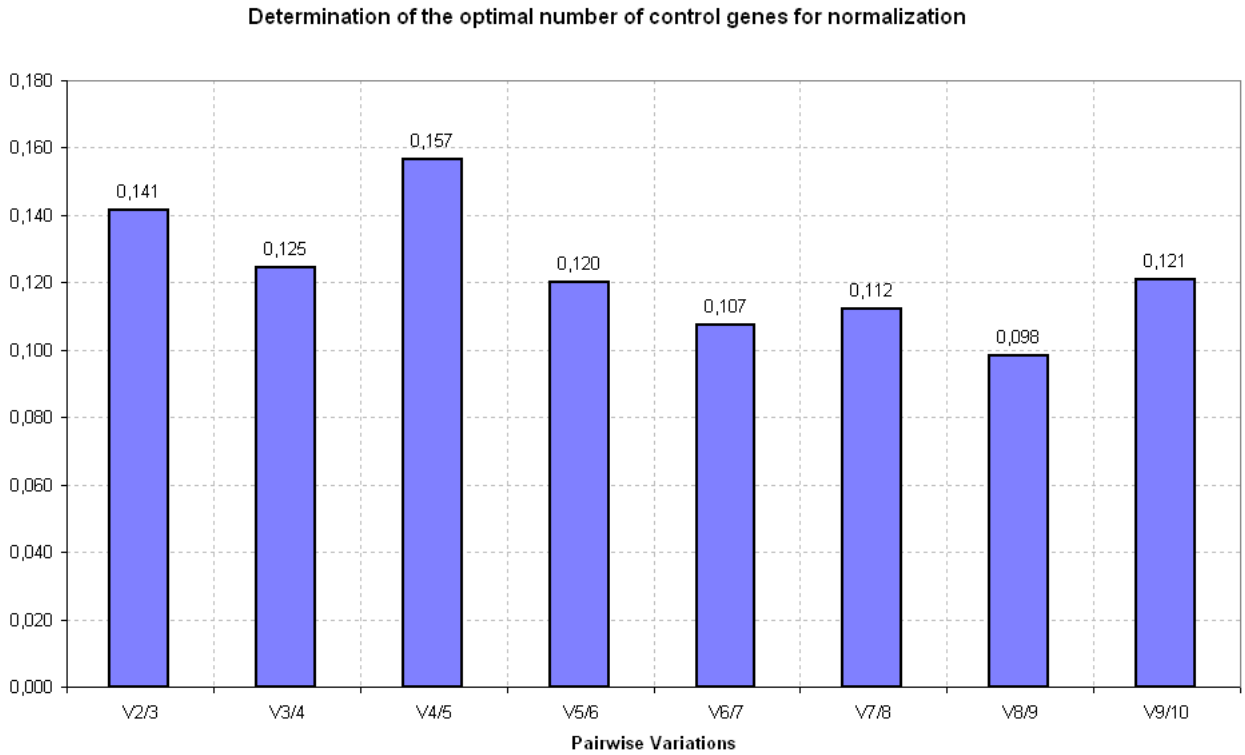


Figure 12. Determination of the optimal number of control genes for normalization calculated on the basis of the pair-wise variation (V) analysis

The results of the NormFinder analysis applied to our data are shown in Table 8 and Table 9. The software allows to estimate not only the overall expression variation of the candidate normalization genes, but also the variation between subgroups of the sample set e.g. pre-infection and post infection samples. According to this analysis the more stable gene both in non-infected and infected samples is G6PD with a stability value (ρ) of 0,170 and the best combination of two genes is G6PD and YWHAZ with a stability of 0,144.

Gene	**Stability value (ρ)**
G6PD	0,170
YWHAZ	0,241
ACTB	0,260
SDHA	0,304
18S rRNA	0,326
GAPDH	0,375

TUBB	0,381
RPL13A	0,492
TFRC	0,598
PGK1	0,626

Table 8. Candidate reference genes for normalization of qRTPCR listed according to their expression stability within all three group of sample calculated by the NormFinder VBA applet.

The intergroup variation of the ten candidate reference genes is shown in Figure 13. The best candidate genes are the ones with an inter-group variance as close to zero as possible (G6PD and YWHAZ). The genes with the maximal intergroup variation are TFRC and PGK1.

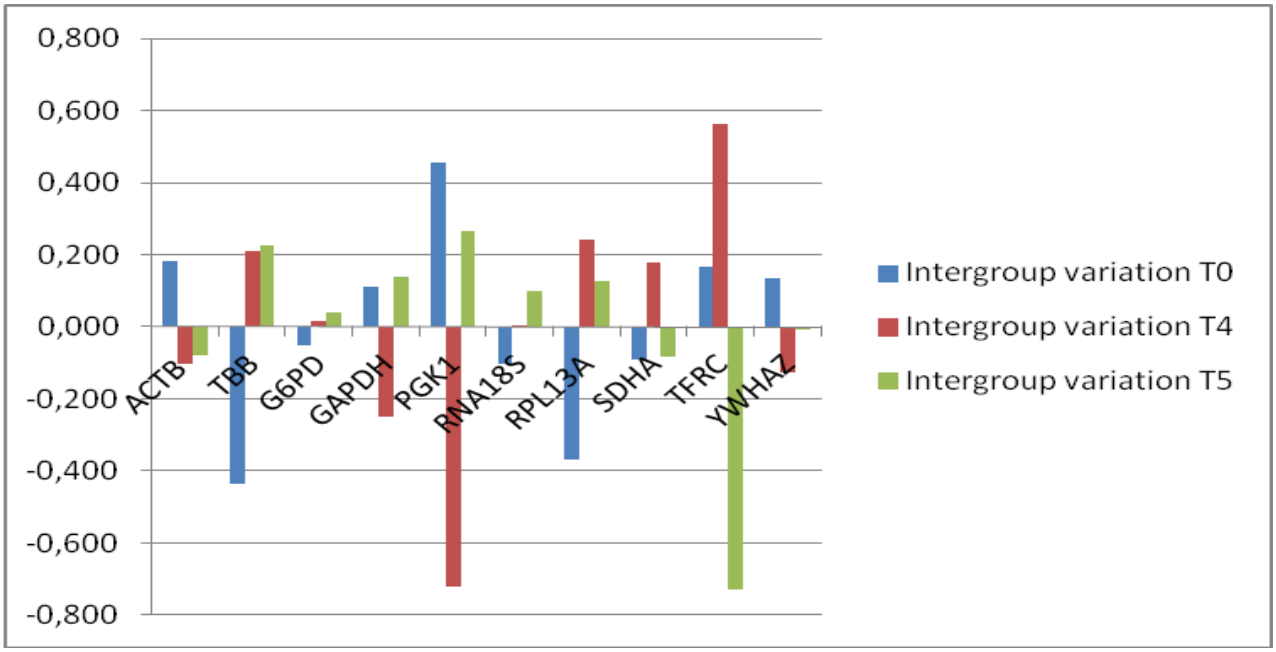


Figure 13. Intergroup variation of the ten candidate reference genes between samples collected in T0, T4 and T5, according to analysis with NormFinder software.

Single group data analysis with NormFinder software showed the intra-group variation of the reference genes (Table 9). In the samples collected in T0 the more stable genes are ACTB and YWHAZ, in the T4 and T5 samples genes

showing the more stably expression are TUBB and G6PD. The least stable genes in all three group of samples are RPL13A, 18S rRNA and PGK1.

T0		T4		T5	
Gene	**Valore di stabilità (ρ)**	**Gene**	**Valore di stabilità (ρ)**	**Gene**	**Valore di stabilità (ρ)**
ACTB	0,036	*TUBB*	0,040	*TUBB*	0,034
YWHAZ	0,037	*G6PD*	0,061	*G6PD*	0,191
G6PD	0,073	*TFRC*	0,113	*ACTB*	0,212
SDHA	0,099	*ACTB*	0,154	*YWHAZ*	0,222
TUBB	0,212	*YWHAZ*	0,202	*TFRC*	0,295
PGK1	0,262	*SDHA*	0,264	*GAPDH*	0,363
GAPDH	0,278	18SrRNA	0,536	*SDHA*	0,463
TFRC	0,302	*GAPDH*	0,592	18SrRNA	0,548
18S rRNA	0,333	*RPL13A*	0,680	*RPL13A*	1,319
RPL13A	0,384	*PGK1*	1,466	*PGK1*	1,536

Table 9. Intragroup variation of the ten candidate reference genes in samples collected in T0, T4 and T5, according to analysis with NormFinder software.

17.2. Real-Time qPCR Validation of the Differentially-Expressed Genes in Somatic Milk Cells

The expression of the genes with the highest difference of expression, as shown by microarray analysis, was evaluated by RT-qPCR (Appendix Table 20).

The normalization factor for the three group of samples (T0, T4 and T5) were calculated after analysis of the two reference genes G6PD and YWHAZ (Table 10).

	Normalization Factor T0	Normalization Factor T4	Normalization Factor T5
capra1	0,920576	0,204126	0,224088
capra2	0,849682	0,095714	0,355402
capra3	0,536602	0,396329	0,462276
capra4	0,376226	0,301635	0,956415
capra5	0,261648	0,313849	0,946055
capra6	0,653250	0,216653	0,034136
capra7	0,220579	0,559945	0,196867
capra8	0,487148	0,201570	0,125961
capra9	0,007164	1,000000	0,094826
capra10	0,320400	0,391980	0,353930

Table 10. Normalization factor calculated after analysis of the three group of sample.

The expression of the top up regulated gene PTX3 (Table 11) and the top down regulated gene SPPI (Table 12) was quantified in sample collected at 0h, 24h and 30 h post infection.

	PTX3 T0	PTX3 T4	PTX3 T5
capra1	0,284570	3,281845	1,712629
capra2	0,754630	0,049836	0,393063
capra3	0,205810	0,926830	0,419641
capra4	0,449280	0,710859	0,614168
capra5	0,528760	0,148351	0,179789
capra6	0,717780	0,081420	0,567736
capra7	0,452940	0,864013	1,203911
capra8	2,052760	0,011956	0,011035
capra9	2,826630	0,103090	2,978186
capra10	0,183177	2,551150	2,825419

Table 11 Normalized expression levels of the PTX3 gene in sample collected in T0, T4 and T5. A normalization factor was used to calculate normalized expression levels.

	SPPI T0	SPPI T4	SPPI T5
capra1	1,086276	0,178321	1,667691
capra2	0,362241	0,568464	0,822499
capra3	0,176536	0,045291	1,225328
capra4	0,297374	0,055531	0,398948
capra5	0,576041	0,406660	1,056618
capra6	0,364577	0,399579	0,460809
capra7	0,341646	0,136460	0,243972
capra8	0,28868	0,678623	1,403450
capra9	0,180067	1,000000	0,437854
capra10	0,272066	0,076483	0,138559

Table 12 Normalized expression levels of the SPPI gene in sample collected in T0, T4 and T5. A normalization factor was used to calculate normalized expression levels.

The expression of the genes PTX3 and SPPI was evaluated by RT-qPCR. The analysis confirmed the significant ($p<0.05$) differential expression levels between the healthy and infected animals.

Results of the expression analysis of the genes PTX3 and SPPI at 24h and 30h after infection are reported in Table 13 and Table 14 respectively.

Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
PTX3	TRG	0,936	58,619	6,383 - 569,751	1,492 - 1.869,690	0,000	UP
YWHAZ	REF	1,0	1,205				
G6PD	REF	0,946	0,830				
SPP1	TRG	1,0	0,263	0,069 - 0,917	0,030 - 1,505	0,002	DOWN

Table 13. Differential expression levels between the healthy and infected animals at 24h after infection. Legend:

P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target

REF – Reference

RT-qPCR confirmed the differential expression of PTX3 and SPPI at T4, according to microarray results (Figure 14).

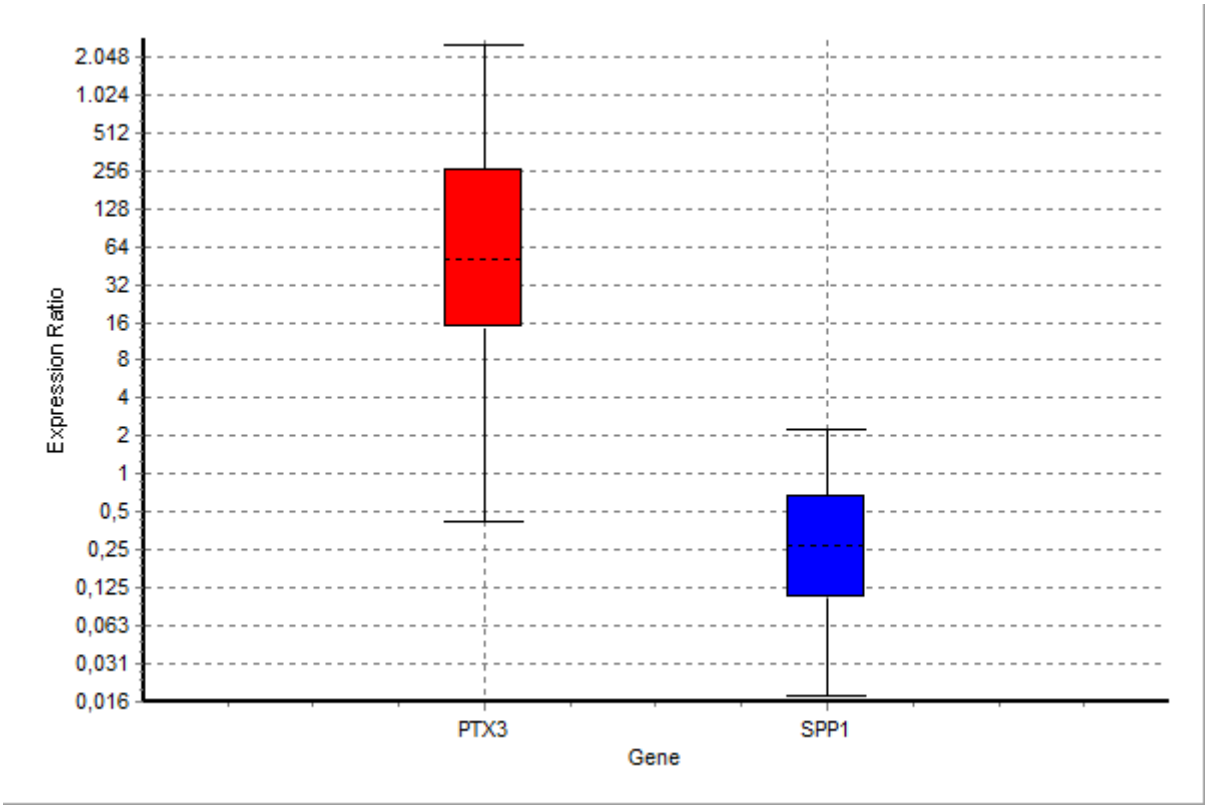


Figure 14. Expression ratio of PTX3 and SPPI between samples collected at T0 and T4. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

PTX3 is UP-regulated in sample group (in comparison to control group) by a mean factor of 58,619 (S.E. range is 6,383 - 569,751) (p = 0.000).

SPP1 is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0,263 (S.E. range is 0,069 - 0,917) (p=0,002).

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
PTX3	TRG	0,936	91,436	20,349 - 631,361	1,176 - 1.682,372	0,000	UP
YWHAZ	REF	1,0	1,328				
G6PD	REF	0,946	0,753				
SPP1	TRG	1,0	0,355	0,140 - 0,937	0,060 - 1,532	0,004	DOWN

Table 14. Differential expression levels between the healthy and infected animals at 30h after infection.Legend:

P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

TRG – Target

REF – Reference

The analysis by RT-qPCR confirmed also the differential expression of PTX3 and SPPI at T5, according to microarray results (Figure 15).

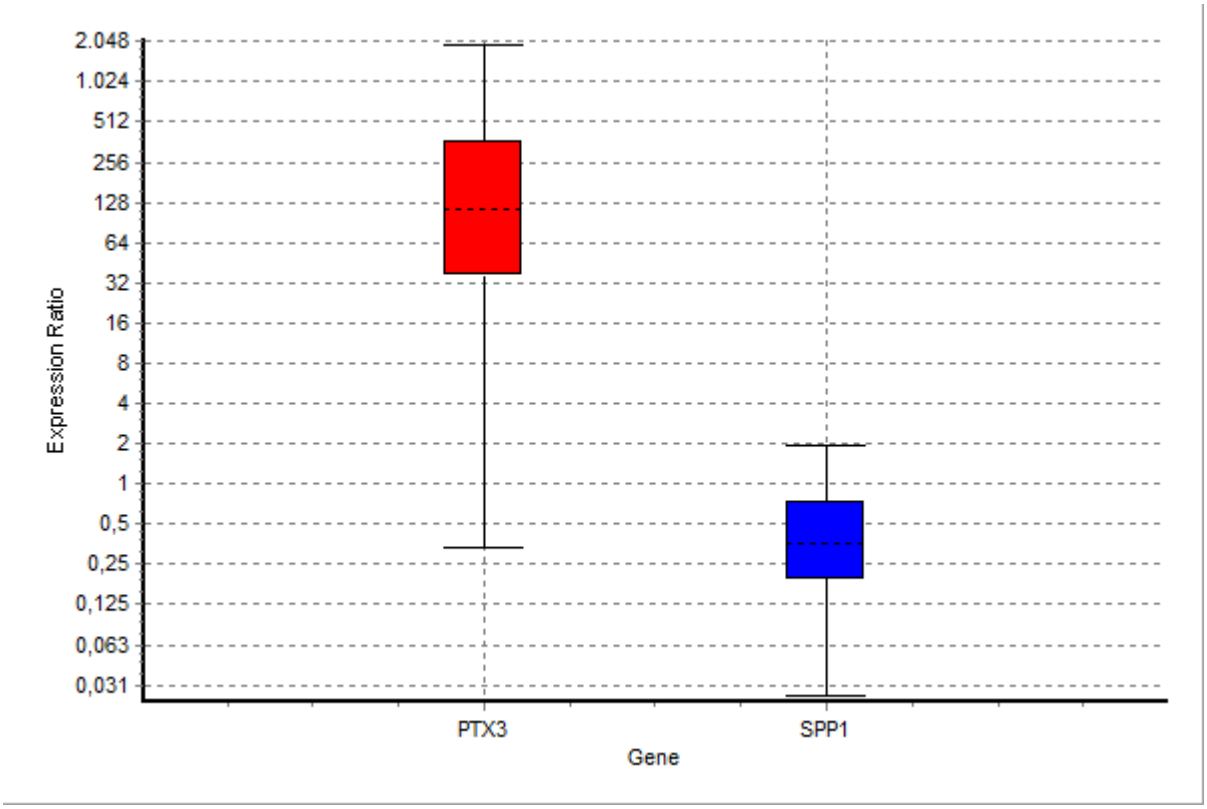


Figure 15. Expression ratio of PTX3 and SPPI between samples collected at T0 and T5. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

PTX3 is UP-regulated in sample group (in comparison to control group) by a mean factor of 91,436 (S.E. range is 20,349 - 631,361) (p = 0.000).
SPP1 is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0,355 (S.E. range is 0,140 - 0,937) (p = 0,004).

DISCUSSION

In the present study, we performed transcriptomic analysis of milk somatic cells (MSC), collected from goats upon mammary challenge with a *S. aureus* strain using a a bovine Combimatrix 90K custom array. To assess the effect of SCS-based selection for resistance or susceptibility to IMI, two group of goats selected for HSCC and LSCC were analyzed. The goats involved in this study were primiparous and they never had IMI by *S. aureus* or other microorganism as demonstrated by repeated microbiological analyses through the lactation period before experimental challenge.

At the moment of the experiment, no enough caprine sequences were available for creating a dedicated array, hence we decided to use the most exhaustive available array for gene expression study in cattle. Up to date, only a small number of studies have analyzed the transcriptome of caprine MSC in healthy and infected glands (Pisoni et al. 2008, 2010). According to previous study the greatest dichotomy in gene expression between infected and non-infected udder halves was observed between 12 and 24 h after infection, corresponding to the greatest proportional increase in bacterial concentration (Pisoni et al. 2010). To confirm that results and to assess difference in early stage of the inflammatory response, in the present experiment we collected samples every 6h, from 0 to 30 hours after infection.

Moreover, to confirm microarray results the expression levels of the two up and down regulated genes were quantified by Real Time qPCR. Quantitative PCR is the method of choice for obtaining accurate quantification of mRNA transcripts although experimental variations can lead to inaccurate results. The use of endogenous reference genes as internal control is commonly accepted as the most reliable approach to normalize RT-qPCR. The study of gene expression in caprine milk somatic cells may be useful to investigate the transcriptional status of the mammary gland of an animal in relation to its genotype, nutritional and pathologic status, and under influence of hormonal factors. We designed 10 PCR assays for commonly employed reference genes belonging to various

functional classes and then ranked their expression stability in goat MSCs samples using the geNorm and Normfinder applets this study provides a validated panel of optimal internal references which may be useful for the identification of genes.

To date, in goats, the most suitable reference genes have been identified only for preantral follicles (Frota et al 2010) and in mammary gland tissue (Finot, 2011). QPCR studies to evaluate gene expression in other tissues and cells (chondrocytes, central nervous system cells, milk somatic cells, germ cells) are reported, but no experiments were carried out in such contexts to identify suitable reference genes and these were arbitrarily selected for the normalization of the data (Abdulmawjood et al. 2005; Pisoni et al. 2010; Ren et al 2011; Vonk et al. 2010).

The first objective was to characterize the host transcriptional early response after IMI infection with *S. aureus* at different time points and to identify some of the genes and molecular mechanisms involved in the genetic basis of the susceptibility/resistance to mastitis, another objective was to develop a set of reference genes for normalizing RT-qPCR data from caprine MSCs.

18. SYSTEMIC AND LOCALIZED INFLAMMATORY RESPONSES TO *S. AUREUS* INTRAMAMMARY INFECTION

As expected and previously described in literature (Pisoni et al. 2010), a significant increase of SCC over 10^6 cells/mL was observed within 24 h post infection with a correspondence decrease of total blood leukocyte and neutrophils. No significant differences were observed for milk SCC between the two groups of animals. Rectal temperatures, blood samples parameters and total milk production were modified as indicators of a systemic response to *S. aureus* infection. No signs of inflammation were found in the right half udder,

considered a semi-autonomous physiological unit (Lutzow et al. 2008). It was found that the response of the two SCC groups was not significantly different in any of local or systemic parameters related to IMI with *S. aureus*.

19. INTRAMAMMARY *S. AUREUS* GROWTH FOLLOWING EXPERIMENTAL INFECTION

The bacteriological count of *S. aureus* recovered from the milk of all 10 experimentally infected left udders peaked at 18h (T3) post-infection and remained relatively constant for the duration of the experiment. Only in the udder of an animal, the *S. aureus* count did not reach the same value of the other goats, although an increasing trend were observed (Figure 6). No significant difference was observed in *S. aureus* counts at different time points in milk from LSCC and HSCC goats. These results suggest that there was no difference in bacterial clearance.

20. GENE EXPRESSION OF MILK SOMATIC CELLS

The microarray analysis clearly indicated that the mammary gland after 24 h since inoculation with *S. aureus* experienced a wide transcriptional response, which encopassed 300 genes.

Few functions were significantly affected such as cell death, cellular movement, cellular growth and proliferation, cell-to-cell signaling and interaction, and lipid metabolism. These functions are intrinsic to general disease response and were reported to be altered in ruminants during the overall response to mastitis (Genini et al. 2011).

Milk somatic cells represent an important cellular component in the innate immune defense of the mammary gland (Rinaldi et al. 2009). Numerous cytokines and chemokines involved in the immune response were up-regulated

in mammary tissue during IMI challenge with *S. aureus*. These proteins are intimately associated with inflammatory processes and are probably essential for the recruitment and activation of neutrophils into the infected tissue (Lutzow et al. 2008). As described previously (Lutzow et al. 2008; Rinaldi et al. 2010) a significant transcriptomic disruption in milk somatic cells becomes evident 24 h (T4) post experimental *S. aureus* infection. The pro-inflammatory cytokines tumour necrosis factor alpha (TNFα), interleukin 1 alpha (IL-1α), interleukin 8 (IL-8) and CD14 antigen (CD14), a pivotal cell surface protein mediating detection of bacterial cell wall components, resulted up-regulated by bacterial infections of mammary tissue. These observations indicate that in goats after recognition of invading pathogens, milk somatic cells are able to initiate a signal to recruit cellular factors of immune defense from the blood to the mammary gland.

Other studies reported that the components of the chemokine signalling and cell adhesion molecule pathways were over-represented after *S.aures* challenge in sheeps (Bonnefont et al. 2011). These pathways play important roles in blood neutrophil arrest and diapedesis across the endothelium (Paape et al. 2003). Furthermore, cytokine-cytokine receptor interactions are also noticeable and cytokines are known to tightly regulate neutrophil functions during inflammatory response (Paape et al. 2003). Thus, pro-inflammatory cytokines lead to the activation of the mitogen-activated protein kinase pathway (MAPK) in neutrophils and promote leukocyte recruitment to inflammation sites (Bonnefont et al. 2011).

Our findings suggest that the alteration of immune response and lipid metabolism are hallmarks of the response to infections causing mastitis.

20.1. Immune Response

The acute phase response is a rapid, non-specific inflammatory response that provides protection against microorganisms by amplification of several cytokines (Ramadori et al. 1999). The early stage response was specifically represented by pathways directly involved in the immune response, such as *IL-10 Signaling* (during T4) and *LXR/RXR activation* (during T5). A close relationship between polyamine regulation, in particular the sub-group spermine, and IL-10 signaling has been previously reported in macrophages (Hasko et al. 2000). Other studies also reported an increase of IL-6 and IL-10 expression during mastitis infection (Swanson et al. 2004; Lutzow et al. 2008). As persistence or over-prolongation of inflammation is harmful for cells (Schroder et al. 2005), the activation of the *IL-10 Signaling* might be a beneficial mechanism adopted by the cells during this stage of mastitis infection to limit and terminate the inflammatory response.

Interleukin-10 is an anti-inflammatory cytokine that blocks NF-kB activity which leads to suppression of pro-inflammatory mediators such as TNF, IL6, and IL1. Expression of 7 out of 78 putative components of the *IL-10 Signaling* pathway present in our microarray platform were moderately but significantly up-regulated.

20.2. Lipid Metabolism

The *LXR/RXR Activation and PPAR (Peroxisome Proliferator-Activated Receptor) Signaling* pathways that resulted altered in T5 are known to be implicated in the regulation of the lipid metabolism (Norata et al. 2005).. *LXR/RXR* is involved in the regulation of lipid metabolism, inflammation, and cholesterol to bile acid catabolism. *PPAR* family consists of PPARα, PPARδ, and PPARγ. They act as ligand activated transcriptional regulators. Their ligands include n-3 and n-6 unsaturated fatty acids and their eicosanoid products which makes the PPARs closely linked to intracellular lipid levels. *PPAR* is highly

expressed in adipose tissue and macrophages and primarily regulates adipogenesis but it interferes also with the transcription of proinflammatory factors such as STAT and NF-κB in macrophages (Ricote et al. 1998). Other studies reported a significantly altered lipid metabolism during the intramammary infections both in cattle and sheep (Moyes et al. 2009; Rinaldi et al. 2010; Genini et al 2011), as previously described, our results seem to suggest that during the early stage response there might be a "general" deregulation of the lipid metabolism and fortify the relevant role of the lipid metabolism during response to infections causing mastitis.

These findings further underline that lipid metabolism is tightly linked to immune response and that lipid antigen presentation might represent an interesting candidate pathway for future work to gain new insights into the host-pathogen interplay in mastitis.

20.3. Top 10 Up-Regulated Genes

According to literature and public websites the description of some genes is not directly referred to caprine genes. Their function descriptions and discussion based on information available for other species, such as *Homo sapiens*.

Both in milk and in blood samples, the most up regulated gene was Pentraxin 3 (PTX3) whose role is widely described in human. PTX3 is the first long pentraxin described, it was originally identified as a cytokine-inducible gene in vascular endothelial cells and fibroblasts (Breviario et al. 1992; Lee et al. 1993). PTX3 expression is rapidly induced in a variety of additional cell types by several stimuli, such as cytokines (e.g., IL-1β, TNF-α), TLR agonists, microbial moieties (e.g., LPS, OmpA, lipoarabinomannans), or intact microorganisms (Garlanda et al. 2005; Bottazzi et al. 2009). Myeloid dendritic cells are a major source of PTX3 that is also expressed by monocytes, macrophages, smooth

muscle cells, kidney epithelial cells, synovial cells, chondrocytes, adipocytes, and alveolar epithelial cells and glial cells as well as fibroblasts and endothelial cells (Moalli et al. 2011). PTX3 binds with high affinity to the complement component C1q, the extracellular matrix component TNFα induced protein 6 (TNFAIP6; also called TNF-stimulated gene 6, TSG-6). PTX3 can interact with a number of different pathogens including selected fungi, virus, and bacteria. A specific binding has been observed to zymosan, *Paracoccidioides brasiliensis,* and conidia from *Aspergillus fumigates* (Garlanda et al. 2002; Diniz et al. 2004). Additional interactions have been reported with some selected Gram-positive and Gram-negative bacteria, including S*taphyloccocus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Streptococcus pneumoniae,* and *Neisseria meningitidis* and with human and murine cytomegalovirus (CMV) and H3N2 influenza virus (Garlanda et al. 2002; Bozza et al. 2006; Gaziano et al. 2004; Reading et al. 2008). The recognition of a microbial component by PTX3 can magnify the inflammatory response with the amplification of the innate response to pathogens (Moalli et al. 2011). PTX3 plasma levels are very low in normal conditions (about 25 ng/mL in the mouse, < 2 ng/mL in humans) but increases rapidly (peaking at 6–8 h after induction) and dramatically (200–800 ng/mL) during endotoxic shock, sepsis and other inflammatory and infectious conditions, correlating with the severity of the disease. Under these conditions, PTX3 is a rapid marker for primary local activation of innate immunity and inflammation but increase in several pathological conditions including infections (Muller et al. 2001; Fazzini et al. 2001; Mairuhu et al. 2005; Azzurri et al. 2005; Latini et al. 2004). PTX3 act as a functional ancestor of antibodies, recognizing microbes, activating complement, and facilitating pathogen recognition by phagocytes, hence playing a nonredundant role in resistance against selected pathogens (Garlanda et al. 2005). PTX3 is stored in a ready-made form in neutrophils and localized in specific granules and is secreted in response to recognition of microbial moieties and inflammatory

signals (Jaillon et al. 2007). In cattle only one study reported PTX3 as one of the most up-regulated genes after IMI with *S. aureus* (Lutzow et al. 2008). Moreover they demonstrated that PTX3 with S100A12 could provide a new class of natural anti-microbial agents that could assist defense of the mammary gland against chronic and subclinical infections.

Our study demonstrated PTX3 involvement in response to *S. aureus* infection in goats first in somatic cells (T4 and T5) then in leukocytes (T5), in both cells type it was the most expressed gene. However, further investigations are necessary to better understand if the increase of PTX3 expression is due to the direct interaction with the pathogen or if it is the consequence of the release of inflammatory mediators. In case of similar in vivo experiments more time points after infections should be considered to better clarify the PTX3 expression course.

20.3.1 Up-regulated genes in the milk samples collected 24 h post infection.

Pleckstrin (PLEK) is the major protein kinase C (PKC) substrate of platelets and leucocytes. Its phosphorylation triggers responses that ultimately lead to platelet activation and blood clot formation. Pleckstrin is highly expressed in human neutrophils. Pleckstrin is rapidly phosphorylated following treatment of neutrophils in response to inflammatory stimuli. Phosphorylation induces a conformational change in Pleckstrin that promotes its interaction with membranes and/or with the cytoskeleton, serving to target proteins or lipids recognized by pleckstrin homology domains to sites where they can contribute to the microbicidal response (Tyers et al. 1988; Cmarik et al. 2000).

The Interferon regulatory factor 1 (IRF1) binds to the upstream regulatory region of type I IFN and IFN-inducible MHC class I genes (the interferon

consensus sequence (ICS)) and activates those genes. Acts as a tumor suppressor.

The neutrophil cytosolic factor 1 (NCF1). The protein encoded by this gene is a 47 kDa cytosolic subunit of neutrophil NADPH oxidase. This oxidase is a multicomponent enzyme that is activated to produce superoxide anion. Mutations in this gene have been associated with Chronic granulomatous disease. Neutrophil cytosolic factor 1 has been shown to interact with Moesin, RelA and Neutrophil cytosolic factor 4 (Wientjes et al. 2001; Grizot et al 2001; Ying et al. 2003).

Solute carrier family 2 (facilitated glucose transporter), member 6 (SLC2A6). The solute carrier (SLC) group of membrane transport proteins include over 300 members organized into 51 families. Solutes that are transported by the various SLC group members are extraordinarily diverse and include both charged and uncharged organic molecules as well as inorganic ions and the gas ammonia. SLC2A6 facilitative glucose transporter; and binds cytochalasin B with low affinity.

The Brain abundant, membrane attached signal protein 1(BASP1) encodes a membrane bound protein with several transient phosphorylation sites and PEST motifs.

The cytohesin 4 (CYTH4) promotes guanine-nucleotide exchange on ARF1 and ARF5. Promotes the activation of ARF through replacement of GDP with GTP.

The tumor necrosis factor, alpha-induced protein 6 (TNFAIP6) is involved in cell-cell and cell-matrix interactions during inflammation and tumorigenesis. The protein encoded by this gene is a secretory protein that contains a

hyaluronan-binding domain, and thus is a member of the hyaluronan-binding protein family. The hyaluronan-binding domain is known to be involved in extracellular matrix stability and cell migration. This protein has been shown to form a stable complex with inter-alpha-inhibitor (I alpha I), and thus enhance the serine protease inhibitory activity of I alpha I, which is important in the protease network associated with inflammation. This gene can be induced by proinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1.

The collagen, type III, alpha 1 (COL3A1) occurs in most soft connective tissues along with type I collagen. This gene encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues such as skin, lung, uterus, intestine and the vascular system, frequently in association with type I collagen.

The basic leucine zipper transcription factor, ATF-like 3 (BATF3) is a negative regulator of AP-1-mediated transcription by heterodimerizing with JUN and binding DNA at 12-O-tetradecanoylphorbol-13-acetate response elements (TRE). Represses IL2 and MMP1 promoter activities.

20.3.2. Up-regulated genes in the milk samples collected 30 h post infection

The S100 calcium binding protein A9 (S100A9) is a calcium-binding protein. It has antimicrobial activity towards bacteria and fungi and is important for resistance to invasion by pathogenic bacteria. Up-regulates transcription of genes that are under the control of NFkB. Plays a role in the development of endotoxic shock in response to bacterial lipopolysaccharide (LPS). Promotes tubulin polymerization when unphosphorylated. Moreover, it promotes

phagocyte migration and infiltration of granulocytes at sites of wounding. It plays a role as a pro-inflammatory mediator in acute and chronic inflammation and up-regulates the release of IL8 and cell-surface expression of ICAM1. Extracellular calprotectin binds to target cells and promotes apoptosis. Antimicrobial and proapoptotic activity is inhibited by zinc ions. It is a member of the S100 family that is highly correlated with somatic cell count (Lutzow, et al. 2008).

Intercellular adhesion molecule 1 (ICAM1). During leukocyte trans-endothelial migration, ICAM1 engagement promotes the assembly of endothelial apical cups through ARHGEF26/SGEF and RHOG activation. In case of rhinovirus infection acts as a cellular receptor for the virus.

The superoxide dismutase 2, mitochondrial (SOD2) destroys radicals which are normally produced within the cells and which are toxic to biological systems.

S100 calcium binding protein A8 (S100A8) The protein encoded by S100A8 is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. This protein may function in the inhibition of casein kinase and as a cytokine. In human this protein has antimicrobial activity towards bacteria and fungi and it is important for resistance to invasion by pathogenic bacteria. It up-regulates transcription of genes that are under the control of NFkB and it plays a role in the development of endotoxic shock in response to bacterial LPS. An up-regulation of this gene is expected in case of mammary gland inflammation. The family of antimicrobial genes including S100A11, S100A12, S100A8, and S100A9 were found to be affected during inflammatory response (Genini et al. 2011). In a recent study in

cattle, microarray analysis revealed that these genes were differentially expressed after 24 h infection (Mitterhuemer et al. 2010).

DNAJB6 gene encodes a member of the DNAJ protein family. DNAJ family members are characterized by a highly conserved amino acid stretch called the 'J-domain' and function as one of the two major classes of molecular chaperones involved in a wide range of cellular events, such as protein folding and oligomeric protein complex assembly. This family member may also play a role in polyglutamine aggregation in specific neurons. Alternative splicing of this gene results in multiple transcript variants; however, not all variants have been fully described. In human it plays an indispensable role in the organization of KRT8/KRT18 filaments and it acts as an endogenous molecular chaperone for neuronal proteins including huntingtin. It has a stimulatory effect on the ATPase activity of HSP70 in a dose-dependent and time-dependent manner and hence acts as a co-chaperone of HSP70 reduces huntingtin aggregation associated with Huntington disease.

The neutrophil cytosolic factor 4, 40kDa (NCF4) is a component of the NADPH-oxidase, a multicomponent enzyme system responsible for the oxidative burst in which electrons are transported from NADPH to molecular oxygen, generating reactive oxidant intermediates. It may be important for the assembly and/or activation of the NADPH-oxidase complex.

The STEAP family member 4 (STEAP4) is a metalloreductase that has the ability to reduce both Fe(3+) to Fe(2+) and Cu(2+) to Cu(1+). It uses NAD(+) as acceptor. It play a role in systemic metabolic homeostasis, integrating inflammatory and metabolic responses. Associated with obesity and insulin-resistance. Involved in inflammatory arthritis, through the regulation of inflammatory cytokines. Inhibits anchorage-independent cell proliferation.

20.4. Top 10 Down-Regulated Genes

20.4.1. Down-regulated genes in the milk samples collected 24 h post infection.

Secreted phosphoprotein 1(SPPI) also known as osteopontin (OPN), bone sialoprotein I (BSP-1 or BNSP), early T-lymphocyte activation (ETA-1), 2ar and Rickettsia resistance (Ric) is a non-collagenous glycoprotein that was first identified in 1986 in osteoblasts. The protein is expressed in bone, although it is also expressed in other tissues, where it as a role as linking protein. OPN is a highly negatively charged, extracellular matrix protein that lacks an extensive secondary structure. Osteopontin has been implicated as an important factor in bone remodelling (Choi et al. 2008), but it has a role also in immune functions, indeed OPN binds to several integrin receptors including α4β1, α9β1, and α9β4 expressed by leucocytes. These receptors have been well-established to function in cell adhesion, migration, and survival in these cells. OPN is expressed in a range of immune cells, including macrophages, neutrophils, dendritic cells, and T and B cells, with varying kinetics. OPN is reported to act as an immune modulator in a variety of manners (Wang et al. 2008). Firstly, it has chemotactic properties, which promote cell recruitment to inflammatory sites. It also functions as an adhesion protein, involved in cell attachment and wound healing. In addition, OPN mediates cell activation and cytokine production, as well as promoting cell survival by regulating apoptosis (Wang et al. 2008).
Acts as a cytokine involved in enhancing production of interferon-gamma and interleukin-12 and reducing production of interleukin-10 and is essential in the pathway that leads to type I immunity (Wang et al. 2008).

ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2). Xenobiotic transporter that may play an important role in the exclusion of xenobiotics from

the brain. May be involved in brain-to-blood efflux. Appears to play a major role in the multidrug resistance phenotype of several cancer cell lines. When overexpressed, the transfected cells become resistant to mitoxantrone, daunorubicin and doxorubicin, display diminished intracellular accumulation of daunorubicin, and manifest an ATP-dependent increase in the efflux of rhodamine 123.

CD24 molecule (CD24). Modulates B-cell activation responses. Signaling could be triggered by the binding of a lectin-like ligand to the CD24 carbohydrates, and transduced by the release of second messengers derived from the GPI-anchor. Promotes AG-dependent proliferation of B-cells, and prevents their terminal differentiation into antibody-forming cells.

Keratin 19 (KRT19).It is involved in the organization of myofibers. Together with KRT8, helps to link the contractile apparatus to dystrophin at the costameres of striated muscle.

Exosome component 2 (EXOSC2). Component of the exosome 3'->5' exoribonuclease complex, a complex that degrades unstable mRNAs containing AU-rich elements (AREs) within their 3' untranslated regions. Required for the 3'processing of the 7S pre-RNA to the mature 5.8S rRNA. Has a 3'-5' exonuclease activity.

Histone deacetylase 10 (HDAC10). It is responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Histone deacetylation gives a tag for epigenetic repression and plays an important role in transcriptional regulation, cell cycle progression and developmental events. Histone deacetylases act via the formation of large multiprotein complexes.

Chromosome 5 open reading frame 56 (C5ORF56).Changes in the structure of chromosome 5 are associated with certain forms of cancer and conditions related to cancer. These changes are typically somatic. Deletions in the long (q) arm of the chromosome have been identified in a form of blood cancer known as acute myeloid leukemia (AML).

E74-like factor 5 (ETS-domain transcription factor, ELF5). Transcriptionally activator that may play a role in regulating the later stages of keratinocytes terminal differentiation.

Midline 2 (MID2). The protein encoded by this gene is a member of the tripartite motif (TRIM) family. The TRIM motif includes three zinc-binding domains, a RING, a B-box type 1 and a B-box type 2, and a coiled-coil region. The protein localizes to microtubular structures in the cytoplasm.

RNA binding motif protein 5 (REG3G). Might be a stress protein involved in the control of bacterial proliferation.

20.4.2. Down-regulated genes in the milk samples collected 30 h post infection.

Fatty acid synthase (FASN). Fatty acid synthetase catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH. This multifunctional protein has 7 catalytic activities and an acyl carrier protein.

Cyclin B2 (CCNB2). It is assential for the control of the cell cycle at the G2/M (mitosis) transition.

21. GENE EXPRESSION IN BLOOD CELLS

To our knowledge this is the first gene expression study on caprine white blood cells performed at different time points after IMI with *S.aureus*.

Our intention was to identify new genetic and subsequently chemical markers easily detectable during *S.aureus* infection in goats. First changes in blood gene expression occurred at 30h post-infection and overlapped with the lowest TBL and NEU counts . From the statistical point of view, no significant data was found in previous time points after IMI. So we considered this changes in gene expression as the earliest signals of the disease in blood. From 24h to 30h post infection only nine genes revealed to be differentially expressed and only one of them was down-regulated. According to literature and public websites the description of some genes is not directly referred to caprine genes. Their function descriptions and discussion based on information available for other species, such as *Homo sapiens*.

The PTX3, DNAJB6, S100A8 genes were found to be deregulated both in MSC and in blood, a description about them has been reported above in the text.

21.1. Top 10 Up-Regulated Genes in the Blood Samples Collected 30 h Post Infection.

The trophoblast Kunitz domain proteins (TKDPs) represent such a placenta-specific multigene family, which is apparently restricted to the ruminant ungulates and is hence of relatively recent origin. Each member of this family is characterized by the presence of a carboxyl-terminal Kunitz domain, which, in most contexts functions as a serine peptidase inhibitor, preceded by one or more amino-terminal N-domains of similar sequences but of unknown function (MacLean et al. 2004). These proteins are evolving very rapidly and have an intriguing expression pattern in which they are abundantly expressed in the trophoblast only around the time of implantation (Chakrabarty et al. 2006). So

far, the function of TKDPs in placental development is speculative, and experiments indicate that some members have lost their ability to inhibit proteases. TKDPs may modulate ion channels (MacLean et al. 2004).
The role of TKDP3 is unclear in our study, we need more information to explain the up-regulation of this gene in white blood cells at a time point that it does not coincide with the time of implantation. There are no enough published information about its expression in other tissues in addition to placenta.

The EGF-like module-containing mucin-like hormone receptor-like 1 precursor (EMR1) gene encodes a protein that has a domain resembling seven transmembrane G protein-coupled hormone receptors (7TM receptors) at its C-terminus. The N-terminus of the encoded protein has six EGF-like modules, separated from the transmembrane segments by a serine/threonine-rich domain, a feature reminiscent of mucin-like, single-span, integral membrane glycoproteins with adhesive properties. EMR1 is a highly specific marker for eosinophils in humans (Hamann et al. 2007) and it could be involved in cell-cell interactions. Eosinophils play an important role in host defense mechanisms in parasitic infestation and pathogenesis of allergic, immunological, and malignant disorders. Increased number of eosinophils in an inflamed tissue is essential for the development of the late and chronic phases of allergic inflammation (Gleich 2000). In this study caprine leukocytes EMR1 is up regulated, probably in response to the pathogen invasion, however further investigations are needed to understand whether specifically caprine eosinophils show an up-regulation of this gene after *S. aureus* infection, or whether other blood cellular subpopulations expressed this gene.

The Homo sapiens activin A receptor, type IB (ACVR1B) gene encodes an activin A type IB receptor. Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-beta (TGF-beta)

superfamily of structurally related signaling proteins. Wenz et al. (2010) performed an observational study to determine the potential associations between cow factors, clinical mastitis etiology, and concentrations of select acute phase proteins and cytokines in milk from affected quarters of cows with clinical mastitis. The study demonstrated that the concentrations of TGF-α and TGF-β in milk were associated only with season. Our study was concentrated in 30 hours and no seasonal variation was considered, but ACVR1B was up-regulated.

The protein encoded by transglutaminase 1 (TGM1) is a membrane protein that catalyzes the addition of an alkyl group from an akylamine to a glutamine residue of a protein, forming an alkylglutamine in the protein. This protein alkylation leads to crosslinking of proteins and catenation of polyamines to proteins. This gene contains either one or two copies of a 22 nucleotides repeat unit in its 3' UTR. Mutations in this gene have been associated with autosomal recessive lamellar ichthyosis and nonbullous congenital ichthyosiform erythroderma (NCIE). In human it catalyzes the cross-linking of proteins and the conjugation of polyamines to proteins. It is responsible for cross-linking epidermal proteins during formation of the stratum corneum. For this gene further studies are required to find a role in mastitis response.

The protein encoded by Cluster of differentiation 14 (CD14) gene is a surface antigen that is preferentially expressed on monocytes/macrophages. It cooperates with other proteins to mediate the innate immune response to bacterial lipopolysaccharide. In cattle membrane CD14 has been identified on PMN, monocytes and macrophages (Paape et al. 1996, 2002). LPS binding protein binds LPS with high affinity, and the resulting LPS-LBP complex is recognized by mCD14 and causes activation of those phagocytes (Wright et al.1990). In our study CD14 expression is modulated by *S. aureus* infection,

even if further investigations are necessary to better understand its role in this kind of infection.

21.2. Down-Regulated Genes in the Blood Samples Collected 30 h Post Infection.

AMICA1 gene is involved in transmigration of leukocytes through epithelial and endothelial tissues. Expressed at the plasma membrane of polymorphonuclear leukocytes (Moog-Lutz et al. 2003). Cell adhesion molecules also play an important role in the immune response to virus infection, including inflammation or antigen presentation (Kesson et al. 2002). This gene resulted to be down-regulated in our study, it might mean that at 30h post-infection polymorphonuclear leukocytes in mammary gland were so highly concentrated to not recall others.

22. TRANSCRIPTOME DIFFERENCES BETWEEN TWO ANIMAL GROUPS

The second objective of this study was to search differences in this immune response by using two animal groups belonging to caprine families with extreme SCC breeding values. In accordance with the top canonical pathway analysis the altered molecular and cellular functions identified by IPA (i.e. antigen presentation, cell death, cell to cell interaction, inflammatory response and cellular movement) reflected an intensification of the immune response to mastitis infection.

Elimination of the invading pathogens in the area of the mammary alveoli is crucial to prevent the establishment of an infection in the udder. Pathogens can only multiply in sufficient quantity to become resident in the gland if the defense mechanisms fail in this compartment. There are several lines of defense

in the udder. It is well known that macrophages and certain effectors cells of immune function, such as the polymorph nuclear neutrophil granulocytes, are resident in the udder and cooperate in killing of pathogens. An infection of the udder grossly alters the metabolism of the gland. Synthesis of the main milk proteins, caseins for instance, is blocked entirely within 24-36h following an infection. This indicates that the expression of health will be altered as consequence of an infection and many of the regulated genes will be irrelevant to the immune defense, and just reflect gross alterations of the metabolism within the gland. According to our results 24h after infection, 300 genes were altered, mainly affecting lipid metabolism and immune response, that could be a confirmation of a general alteration of the metabolism within the gland. As described in the results section, from the physiological point of view and gene expression analyses, no differences were found in the response of the two SCC groups (HSCC and LSCC). Probably, this could be due to the early stage of IMI observed in this experiment. Although in milk samples 128 genes were altered 30h after infection, only ten genes were found to be differentially expressed in blood at T5, indicating an early stage of the systemic response. Sampling at later stages of the infection could possibly reveal some difference in the response to infection between the two SCC groups and in the efficiency to counteract the pathogens, that could reflect a group-specific resistance to mastitis. And more, since the fact that some *S. aureus* strains could induce subclinical mastitis in contrast to others that could induce mild and even severe clinical mastitis, the intensity of the IMI response was similar between the two caprine families.

In a recent study on trascriptomic analysis of milk somatic cells in sheep, 95 genes were more expressed 12 h after *S.aureus* infection (Bonnefont et al. 2011). The main functions of the genes expressed at a higher level were associated with the immune response: hematopoiesis, cell mediated immune response, cell death, immunological disease and inflammatory response. Two networks were mainly affected, one which is characterised by cell-to-cell

signalling and interaction and cell-mediated immune response, the T cell receptor signalling pathway and the major histocompatibility complex – MHC – pathway are highly represented. In a second network, defined by inflammatory disease and response and haematological disease, both the IL1 receptor pathway and the TNF pathway are well represented. Although none of the differentially expressed gene was shared with our study, some affected networks were the same. In particular the Toll-like signaling pathways was found to be up-regulated in mastitis resistant sheep (Bonnefont et al. 2011), similar results were described in mastitis resistant cows (Griesbeck-Hilch et al. 2009).

24.EVALUATION OF INTERNAL REFERENCE GENES IN CAPRINE MILK SOMATIC CELLS

To confirm microarray results, milk samples were analized by RT-qPCR. The study of gene expression in caprine MSCs may be useful also to investigate the transcriptional status of the mammary gland of an animal in relation to its genotype, nutritional and pathologic status, and under influence of hormonal factors. Quantitative PCR is the method of choice for obtaining accurate quantification of mRNA transcripts although experimental variations can lead to inaccurate results. The use of endogenous reference genes as internal control is commonly accepted as the most reliable approach to normalize RT-qPCR. Hence anaother of this study was to develop a set of reference genes for normalizing RT-qPCR data from caprine MSCs, since no data are available in literature. To date, in goats, the most suitable reference genes have been identified only for preantral follicles and mammary tissue (Frota et al 2010; Finot et al. 2010). QPCR studies to evaluate gene expression in other tissues and cells (chondrocytes, central nervous system cells, milk somatic cells, germ cells, etc) are reported, but no experiments were carried out in such contexts to identify suitable reference genes which were arbitrarily selected (Abdulmawjood

et al. 2005; Pisoni et al. 2010; Vonk et al. 2010; Ren et al 2011). We designed 10 PCR assays for commonly employed reference genes belonging to various functional classes and then ranked their expression stability in goat MSCs samples using the geNorm and Normfinder applets.

GeNorm provides a ranking of the tested genes, based on their expression stability, determining the two most stable reference genes or a combination of multiple stable genes for normalization. In the set suited, candidate reference genes were ranked according to the gene-stability measure (M, average pair-wise variation of a particular gene with all other control genes), from the most stable (lowest M values) to the least stable (highest M values) (Table 7). All studied genes reach a high expression stability below the default limit of M = 1.5 (Vandesompele et al. 2002), although the M value are lower, less than 1, in samples at T0. Based on M average value ranking, the most stable genes appeared to be ACTB and YWHAZ in sample at T0 and T5, and G6PD and TFRC in samples at T4; these genes are characterized by the same average M values within each group: 0,445 and 0,388 in T0 and T5 respectively and 0,527 in T4. M value. On average ACTB, YWHAZ, G6PD, SDHA and TUBB constitute stable genes in all three groups of sample. TUBB is the most stable gene in infected animals with comparable M values. The least stable genes are PGK1, RPL13A, 18S rRNA. GAPDH and TFRC have variable ranking in different group. It is also remarkable that the two ribosomal proteins (18S rRNA and RPL13A) exhibit a similar behavior (ranking together in the bottom of the list in all three group of samples) and are characterized by highly comparable average M values (Table 7).

NormFinder is another VBA applet based on an algorithm for identifying the optimal normalization genes among a set of candidates. It ranks the set of candidate genes according to their expression stability value (ρ) in a given sample set and a given experimental design (Andersen et al. 2004). Lower values are assigned to the most stable genes. The algorithm is rooted in a

mathematical model of gene expression and uses a solid statistical framework to estimate not only the overall expression variation of the candidate normalization genes, but also the variation between sample subgroups of the sample set (e.g. healthy and infected samples). Taking into account the analysis of the three group of sample singly (Table9), the ranking appears to be similar to the one previously determined using geNorm. The most stable genes in samples at T0 results ACTB and YWHAZ, the ones in sample at T4 are TUBB G6PD and TFRC showing reverse position respect geNorm results. Similar results were obtained in samples at T5 were TUBB G6PD and ACTB still occupy the highest positions but with ranking position inverted respect geNorm results. Least stable genes according to NormFinder analysis were similar to geNorm and PGK1, 18S rRNA and TFRC are defined as the least reliable controls.

According to the evaluation of the variation between sample subgroups by NormFinder, G6PD, followed by YWHAZ, is the most reliable control gene for normalization of data relative to healthy and infected animals (Table8). These two candidate genes are the ones with an inter-group variance as close to zero as possible. The genes with the maximal intergroup variation are TFRC and PGK1(Figure 9).

In previous study (Finot et al. 2011) on caprine mammary tissue the most stable reference genes were the two ribosomal protein gene Ribosomal protein large, P0 (RPL0) and 18S rRNA. The latter gene ranked as one of the least stable genes both in our study and in other evaluation in goat preantral follicles (Frota et al. 2010), in these tissue the most stable gene resulted ACTB and the Ubiquitin. The few studies focused on gene expression analysis by RT-qPCR in caprine tissue (Frota et al. 2010; Finot et al. 2011) did not test G6PD and YWHAZ, hence no comparisons are possible.

In a recent study on trascriptomic analysis of milk somatic cells in sheep (Bonnefont et al. 2011), the stability of seven reference genes was checked and

after analysis by geNorm software, the most stable genes selected for the normalizatione of RT-qPCR were RP19, HPRT, SDH and GAPDH.

Our results demonstrate the importance of validating reference genes under experimental conditions in different species and tissue, as the choice of the reference gene in data normalization impacts on interpretation of the results and can lead to misinterpretation. According to our evaluation, we recommend using a panel of reference genes including G6PD and YWHAZ genes which are to be relevant in caprine MSC gene expression profiling.

CONCLUSIONS

Resistance to mastitis is the consequence of a fine-tuning of immune and inflammatory processes in a complex network of cell and gene interactions. Our study has highlighted some specific genes and gene pathways associated with response to *S. aureus* mastitis in dairy goats. Results obtained from the transcriptomic analysis in goats confirmed gene expression studies performed in other species, such as cow and sheep, indeed there was an inverse relationship between immune response and lipid metabolism. A lists of significant and biological relevant differentially expressed genes in MSC was identified. The list provides relevant information for the identification of candidates for the genetic basis underlying resistance to mastitis. It paves the way for further genetic and mechanistic studies. In particular PTX3 gene had the higher log fold change value both in milk and in blood and it might represent a candidate for the genetic basis underlying resistance to mastitis. In our study no differences were found in the inflammatory response to pathogens of the two SCC groups (HSCC and LSCC). Probably, this could be due to the early stage of IMI observed in this experiment and sampling at later stages of the infection could possibly reveal some difference in the response to infection between the two SCC groups and in the efficiency to counteract the pathogens, that could reflect a group-specific resistance to mastitis. Further investigations with additional time points might also provide a wider overview on the possible interaction between the local and systemic response. Moreover the results gave some insight into the differential response of the host to IMI according to the mastitis causing pathogen and its genetic background.

From a technical point of view, in our study the bovine CustomArray 90K was demonstrated to be useful for the evaluation of gene expression in somatic cells and blood of goats infected by *S. aureus*.

Finally, this study provides a validated panel of optimal internal references genes which may be useful for the identification of genes differentially expressed by RT-qPCR in caprine MSCs, with the potential of identifying

specific markers and gene pathways associated with complex infectious diseases such as mastitis. Moreover, the panel could be used to normalized data from analysis of MSCs by RT-qPCR to investigate the transcriptional status of the mammary gland of an animal in relation to its genotype or nutritional status, and under influence of hormonal factors.

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APPENDIX

Table 15: List of the top 10 up-regulated genes in milk due to intramammary infection with *S. aureus* in sample collected 24 h (T4) and 30 h (T5) after infection.

Gene Symbol	**Gene Name**	**log Fold Change**
T_4		
PTX3	Pentraxin 3, long	5.659
PLEK	Pleckstrin	4.490
IRF1	interferon regulatory factor 1	4.378
NCF1	neutrophil cytosolic factor 1	4.090
SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6	3.973
BASP1	brain abundant, membrane attached signal protein 1	3.934
CYTH4	cytohesin 4	3,907
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	3.813
COL3A1	collagen, type III, alpha 1	3.802
BATF3	basic leucine zipper transcription factor, ATF-like 3	3.760
T_5		
PTX3	Pentraxin 3, long	5.346
S100A9	S100 calcium binding protein A9	3.805
ICAM1	intercellular adhesion molecule 1	3.744
SOD2	superoxide dismutase 2, mitochondrial	3.596
PLEK	Pleckstrin	3.443

S100A8	S100 calcium binding protein A8	3.419
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	3.258
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	3.194
NCF4	neutrophil cytosolic factor 4, 40kDa	3.183
STEAP4	STEAP family member 4	3.166

Table 16: List of the top 10 down-regulated genes in milk due to intamammary infection with *S. aureus* in sample collected 24 h (T4) and 30 h (T5) after infection.

Gene Symbol	**Gene Name**	**log Fold Change**
T_4		
SPP1	secreted phosphoprotein 1	-2.469
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-2.129
CD24	CD24 molecule	-2.080
KRT19	keratin 19	-2.073
EXOSC2	exosome component 2	-1.958
HDAC10	histone deacetylase 10	-1.935
C5ORF56	chromosome 5 open reading frame 56	-1.911
ELF5	E74-like factor 5 (ets domain transcription factor)	-1.878
MID2	midline 2	-1.877
REG3G	RNA binding motif protein 5	-1.862
T_5		
FASN	fatty acid synthase	-2.154

CCNB2	cyclin B2	-1.502

Table 17. Most significant affected IPA canonical pathways.

Five most significant canonical pathways identified with IPA using the significantly affected genes for T_4 and T_5. The identified canonical pathways are listed from the lowest to the highest p-value, and are reported with the involved genes and the corresponding ratio (# of genes involved/ # of known genes in the pathway).

Canonical pathway (IPA)	**Genes**	**P-value**	**Ratio**
T_4			
MIF-mediated Glucocorticoid Regulation	*TLR4, NFKBIA, CD14, PTGS2, NFKBIB, NFKB1*	4.67E-06	6/41
MIF Regulation of Innate Immunity	*TLR4, NFKBIA, CD14, PTGS2, NFKBIB, NFKB1*	1.8E-05	6/49
NF-κB Signaling	*TLR4, IL1A, TGFBR1, RIPK1, NFKBIA, MYD88, BMP2, RELB, TNFAIP3, NFKBIB, NFKB1*	2.46E-05	11/176
IL-10 Signaling	*IL18RAP, IL1A, NFKBIA, CD14, ARG2, NFKBIB, NFKB1*	4.47E-05	7/78
Hypoxia Signaling in the Cardiovascular System	*HSP90B1, UBE2H (includes EG:7328), NFKBIA, UBE2B, HIF1A, NFKBIB, UBE2L6*	5.4E-05	7/71
T_5			
Production of Nitric	*TLR4, PPP1R3D, NFKBIA,*	1.98E-	9/189

Oxide and Reactive Oxygen Species in Macrophages	*ARG2, NCF4, NFKB1, RHOH, IRF1, SIRPA*	06	
LXR/RXR Activation	*TLR4, IL18RAP, FASN, CD14, ARG2, NFKB1*	1.85E-05	6/93
Toll-like Receptor Signaling	*TLR4, NFKBIA, MYD88, CD14, NFKB1*	2.72E-05	5/55
Acute Phase Response Signaling	*SOD2, C3, NFKBIA, MYD88, OSM, SERPINA1, NFKB1, SAA1*	3.59E-05	8/183
MIF-mediated Glucocorticoid Regulation	*TLR4, NFKBIA, CD14, NFKB1*	8.09E-05	4/41

Table 18. List of the 9 up- and down-regulated genes in blood due to intramammary infection with *S. aureus* 30h after infection

Gene Symbol	**Gene Name**	**Gene description**	**log fold Change**
Up-regulated			
PTX3	ENSBTAG00000009012	Pentraxin-related protein PTX3 precursor (Pentaxin-related protein PTX3)	4.975
DNAJB6	gnl|UG|Bt#S26165666	Bos taurus DnaJ (Hsp40) homolog, subfamily B, member 6 (DNAJB6), mRNA	2.809
S100A8	ENSBTAG00000012640	Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Neutrophil cytosolic 7 kDa protein) (P7) (BEE11)	2.352
TKDP3	ENSBTAG00000014345	Trophoblast Kunitz domain protein 3 (Fragment)	2.351
EMR1	ENSBTAG00000007901	EGF-like module-containing mucin-like hormone receptor-like 1 precursor (Cell surface glycoprotein EMR1) (EMR1 hormone receptor)	2.327
ACVR1B	gb|CO881044.1|CO881044	Homo sapiens activin A receptor, type IB (ACVR1B), transcript variant 1, mRNA	1.727
TGM1	ENSBTAG00000003920	transglutaminase 1	1.661
CD14	ENSBTAG0	Monocyte differentiation antigen	1.578

	0000015032	CD14 precursor (Myeloid cell-specific leucine-rich glycoprotein)	
Down-regulated			
AMICA1	ENSBTAG0 0000023283	AMICA1 protein	-1.691

Table 19. Expression data of the ten candidate reference genes obtained by RT-qPCR analysis from samples collected in T0, T4 and T5. The Cq values were transformed to quantities using the comparative Ct method.

	ACTB	TUBB	G6PDH	GAPDH	PGK1	18S rRNA	RPL13A	SDHA	TFRC	YWHAZ
capra1	0.53961	0.21210	0.39228	0.46	0.36349	0.23340	0.12414	0.38423	0.93303	0.34868
capra2	0.87661	0.26395	1	0.43398	0.47303	1	1	0.70888	0.44442	0.54337
capra3	0.49655	0.19589	0.56748	0.40155	0.30145	0.34216	0.26981	1	0.35355	0.40895
capra4	0.21613	0.14251	0.34319	0.22869	0.22376	0.30884	0.15822	0.32574	0.13213	0.28126
capra5	0.57038	0.40071	0.37044	0.33937	0.22531	0.64585	0.30566	0.54577	1	0.65975
capra6	1	0.37324	0.73203	1	1	0.34925	0.30779	0.98633	0.78458	1
capra7	0.30355	0.07954	0.26268	0.15510	0.09087	0.55573	0.21022	0.34181	0.21169	0.16267
capra8	0.60710	1	0.71363	0.74255	0.97942	0.57504	0.16724	0.48552	0.80107	0.45062
capra9	0.24485	0.10507	0.12005	0.03687	0.08021	0.20924	0.07130	0.23091	0.17194	0.13774
capra10	0.42632	0.11917	0.53588	0.11819	0.10438	0.58695	0.37893	0.34181	0.14762	0.27168
capra1t4	0.96594	0.50532	0.43434	0.63986	0.21169	0.7404	0.15604	0.55715	1	0.66896
capra2t4	0.17075	0.21636	0.32824	0.05402	0.00724	0.79818	0.45376	1	0.47963	0,1387
capra3t4	0.66434	0.72273	1	0.41476	0.40895	0.57898	0.38156	0.94644	0,8409	0.64171
capra4t4	1	1	0.92645	0.68707	1	0.35892	0.56644	0.76462	0.59874	1
capra5t4	0.29321	0.45148	0.48091	0.12609	0.10511	0.18886	0.49312	0.95297	0.58642	0.34151
capra6t4	0.25174	0.69918	0.64864	0.14167	0.07911	0.49144	1	0.84195	0.93303	0.35849
capra7t4	0.52851	0.53283	0.73671	0.37396	0.14865	1	0.46009	0.59275	0.65067	0.32086
capra8t4	0.15177	0.20655	0.55321	0.058	0.03039	0.60321	0.95264	0.45323	0.61985	0.16043
capra9t4	0.16043	0,2622	0.22261	0.06908	0.01003	0.50161	0.39777	0.49907	0.35111	0.17075
capra10t4	0.91383	0.61239	0.40497	1	0.19211	0.53342	0.48297	0.64822	0.56253	0.13397
capra1t5	0.35849	0.322	0.15886	0.13985	0.03665	0.50851	0.36098	0.3235	0.12414	0.23005
capra2t5	0.11582	0.2121	0.42885	0.0828	0.09278	0.26214	0.84674	0.81347	0.04803	0.125
capra3t5	0.6783	0.75204	0.5603	0.55496	0.70222	0.40312	0.71698	0.81909	0.30145	1
capra4t5	1	0.73724	0.61252	0.5959	0.95264	0.34925	0.5905	0.44703	0.21316	0.99309
capra5t5	0.63288	1	1	1	1	0.60734	0.25174	1	1	0.63288
capra6t5	0.05633	0.07954	0.23574	0.03809	0.15177	0.1333	0.03716	0.29786	0.06983	0.05041
capra7t5	0.27932	0.4485	0.40497	0.71891	0.95264	0.47819	1	0.16596	0.09875	0.17434
capra8t5	0.13397	0.15329	0.308	0.05096	0.01252	0.48147	0.77378	0.22619	0.07695	0.09739
capra9t5	0.27357	0.15846	0.23424	0.1602	0.02272	0.2499	0.0364	0.10394	0.03768	0.19211
capra10t5	0.21022	0.28203	0.20624	0.26368	0.16494	1	0.10584	0.32129	0.05954	0.16724

Table 20 . Expression data of the two most stable reference genes (G6PD and YWHAZ) and of the top up (PTX3) and down (SPPI) regulated genes obtained by RealTime qPCR analysis. Data from samples collected in T0, T4 and T5 are reported. The Cq values were transformed to quantities using the comparative Ct method.

	G6PD	**YWHAZ**	**PTX3**	**SPPI**
T0				
capra1	0.84746	1	0.26197	1
capra2	1	0.72196	0.64120	0.30779
capra3	0.65695	0.43830	0.11044	0.09473
capra4	0.31194	0.45376	0.16903	0.11188
capra5	0.26268	0.26062	0.13835	0.15072
capra6	0.66962	0.63728	0.46889	0.23816
capra7	0.24805	0.19615	0.09991	0.07536
capra8	0.37758	0.62851	1	0.14063
capra9	0.01261	0.00407	0.02025	0.00129
capra10	0.40497	0.25349	0.05869	0.08717
T4				
capra1	0.06272	0.66434	0.66991	0.03640
capra2	0.07640	0.11991	0.00477	0.05441
capra3	0.24648	0.63728	0.36733	0.01795
capra4	0.16400	0.55478	0.21442	0.01675
capra5	0.25284	0.38958	0.04656	0.12763
capra6	0.23276	0.20166	0.01764	0.08657
capra7	0.54970	0.57038	0.48380	0.07641
capra8	0.24805	0.16380	0.00241	0.13679
capra9	1	1	0.10309	1
capra10	0.23128	0.66434	1	0.02998
T5				
capra1	0.21980	0.22846	0.38378	0.37371
capra2	0.76054	0.16608	0.06528	0.29118
capra3	0.44555	0.47963	0.19399	0.56644
capra4	0.91473	1	0.58740	0.38156
capra5	1	0.89502	0.17009	1
capra6	0.09307	0.01252	0.01938	0.01573
capra7	0.36113	0.10732	0.23701	0.04803
capra8	0.33034	0.04803	0.00139	0.17678
capra9	0.13898	0.06470	0.28241	0.04152
capra10	0.48399	0.25882	1	0.04904