AN ABSTRACT OF THE THESIS OF

Fernanda T. da Rosa for the degree of Master of Science in Animal Science presented on December 8, 2016.

Title: Role of Peroxisome Proliferator-Activated Receptor Gamma on Prevention/Cure of Mastitis.

Abstract approved: _____

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Mastitis is a major endemic disease in dairy cows resulting in significant economic losses for the dairy industry. The peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that is able to bind and be activated by natural (e.g., fatty acids) and synthetic (e.g. thiazolidinedione) compounds. PPARγ plays important roles in adipocyte differentiation, inflammation, and re-epithelialization in monogastric. In ruminants, PPARγ may play a role in milk fat synthesis. The aim of this study was to assess the role of PPARγ in host response to mammary infection and milk fat synthesis in ruminants. Our hypothesis is that activation of PPARγ improves the host response to mastitis and increases milk fat yield. By using a synthetic PPARγ agonist in dairy goats in combination with intramammary infection to induce subclinical mastitis, the objectives of the present experiments were to test if activation of PPARγ improves

1) the response to mastitis and 2) milk fat production. To achieve our objectives we performed two *in vivo* experiments (Experiments 1 and 2).

In Experiment 1, 24 Saanen lactating goats with a low body condition score and getting a low-energy diet without vitamin supplementation received a daily intrajugular injection of either 8 mg of 2,4-thiazolidinedione (TZD) per kg of BW or saline (as a control) and, after a week of TZD injection, an intramammary infusion (IMI) of either *Streptococcus uberis* to induce subclinical mastitis or saline used as a control (6 goats/group). Milk yield and components, body weight, rectal temperature, leukocyte phagocytosis, blood metabolic and inflammation parameters plus insulin, adipocyte size by histology, and expression by RT-qPCR of PPARγ target genes in adipose tissue obtained through biopsy and in mammary epithelial cells (MEC) isolated from milk were assessed. In MEC, expression of *CCL2* and *IL8* was also measured. Data were analyzed by GLIMMIX of SAS with Mastitis, TZD, and Time and all interactions as main effects and goat as random effect. Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively. The induction of mastitis was successful achieved as indicated by >5-fold increase of milk somatic cells count (SCC) in goats receiving *Strep. uberis* and by 30% decrease of % polymorphonuclear leukocytes in blood. The SCC in milk were overall lower in TZD-treated goats. Mastitis induction but not TZD decreased milk yield and production of milk fat. Goats receiving *Strep. uberis* had increased concentrations of glucose, triglycerides, and non-esterified fatty acids (NEFA) in blood after IMI. NEFA was not affected in TZD goats, which did not receive *Strep. uberis*. Inflammatory markers increased in blood of all goats but the increase of haptoglobin was overall lower in TZD treated goats. Indicators of liver activity, including albumin, paraoxonase, and cholesterol, overall decreased after IMI but cholesterol did not decrease in TZD-treated goats. The bactericidal myeloperoxidase was higher

in TZD-treated goats after mastitis. Insulin sensitivity was not affected by TZD or mastitis. Adipocytes size increased over time and was higher in TZD goats not receiving *Strep. uberis*. Subclinical mastitis increased expression of *CCL2* and prevented a decrease in expression of *IL8*. MEC from TZD-treated goats tended to have higher expression of *PPARG, FASN* and *SCD1* after 3 weeks of TZD treatment. Neither mastitis nor TZD affected the expression of genes in adipose tissue. Overall the data of Experiment 1 indicated that the subclinical mastitis model was successfully achieved. The treatment with TZD decreased somatic cells in milk, improved the response of liver, decreased the severity of inflammation, and increased the killing capacity of neutrophils after IMI. The data suggested a more lipogenic adipose tissue in TZD-treated goats but also some active, although minor, nutrigenomic effect of TZD on MEC that may have counteracted the competition of lipid substrates between mammary and adipose tissue. Blood metabolic data suggested that goats responded to *Strep. uberis* intramammary infusion similar to dairy cows in negative energy balance. Data obtained from Experiment 1 indicated that TZD aids with mastitis response. TZD had some effect on milk fat synthesis but, overall, had a smaller-than-expected nutrigenomic effect probably also due to the low body condition and low energy in the diet of the goats. Thus, the effect of PPARγ on milk fat synthesis is still unclear.

The rationale to perform Experiment 2 stemmed from the possibility that the limited nutrigenomic response observed in Experiment 1 was due to a potential dietary deficiency. Subsequent *in vitro* work demonstrated that TZD is a strong activator of PPAR but only in the presence of 9-*cis*-retinoic acid, a metabolite of vitamin A and the activation of PPARγ obligate heterodimer Retinoic-X-Receptor (RXR). Therefore, we hypothesized that continuous activation of PPARγ by TZD in dairy goats supplemented with adequate amount of vitamin A improves the inflammatory response to subclinical mastitis. In order to test this hypothesis we used 12 Saanen

multiparous goats in early lactation. Goats received a diet that met the NRC requirements, including vitamin A, and a daily injection of 8 mg TZD per kg of BW (n=6) or saline (n=6; CTRL). Following 14 days of treatment, all goats received an IMI of *Strep. uberis* to induce subclinical mastitis in the right half of the udder with the left half used as control. Metabolic, inflammation, and oxidative-status profiling in blood including 20 parameters was performed. Milk yield, SCC, rectal temperature and leukocytes phagocytosis were measured. Expression of several PPARγ target genes and genes involved in inflammation was measured in MEC, macrophages isolated from milk, and liver tissue. Data were analyzed by GLIMMIX of SAS with treatment (TRT) and Time and TRTxTime interaction as main effects and goat as random effect. For milk and SCC, mammary half was also included in the main effect (including interactions). Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively. Milk yield decreased after IMI but the decrease was larger in TZD-treated goats. SCC increased after IMI but was not affected by TZD administration. Milk fat decreased after IMI in all halves except in the untreated half of TZD-treated goats. In blood within 2 days from IMI, ceruloplasmin, haptoglobin, and glucose were increased while Zn was decreased. These data confirmed successful induction of sub-clinical mastitis and a status of slight inflammation after IMI. None of the parameters in blood was affected by TZD with the exception of a lower bilirubin concentration and a tendency for higher haptoglobin in TZD vs. CTRL after IMI, indicating a more robust response of the liver to inflammation. The stronger inflammation was also supported by a tendency for higher reactive oxygen metabolites in TZD vs. CTRL group after IMI. We also detected a tendency for a higher globulin in TZD vs. CTRL indicating a better adaptive immune system. Leukocyte phagocytosis was strongly reduced by TZD treatment. None of the genes measured were affected by TZD in liver. In milk macrophages

and MEC, expression of inflammatory genes was higher compared to control in halves receiving *Strep. uberis*, whereas no effect of TZD was observed with the exception of a lower *SCD1* in TZD-treated goats compared to CTRL. We conclude that, contrary to our hypothesis, in goats receiving NRC recommended amount of vitamin A, TZD had a minor effect on the response to mastitis with a likely better liver response, but a lower phagocytosis and minor effect on expression of genes.

Considering both *in vivo* experiments, we can conclude that TZD has an important effect on inflammatory response in dairy goats receiving low energy diet without vitamins supplementation but the effect disappears for the most part in goats receiving adequate feeding, including vitamin A. Furthermore, the lack of effects on expression of PPARγ target genes does not support TZD being a strong PPARγ agonist in dairy goats.

Role of Peroxisome Proliferator-Activated Receptor Gamma on Prevention/Cure of Mastitis.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Fernanda T. da Rosa, Author

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LIST OF ABBREVIATIONS

APP	Acute phase response
+ APP	Positive acute phase protein
- APP	Negative acute phase protein
BCS	Body condition score
BHBA	β-hydroxybutyric acid
bMEC	Bovine primary mammary epithelial cells
BW	Body weight
DMI	Dry matte intake
ECM	Energy corrected milk
Hp	Haptoglobin
IMI	Intramammary infusion
LCFA	Long-chain fatty acids
LdNR	Ligand-dependent nuclear receptors
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
MEC	Mammary epithelial cell
MPO	Myeloperoxidase
NCBI	National Center for Biotechnology Information
NEFA	Non-esterified fatty acids
NR	Nuclear receptor
PMN	Polymorphonuclear
PON	Paraoxonase
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
PPRE	PPAR response element

LIST OF ABBREVIATIONS (Continued)

PUFA	Polyunsaturated fatty acids
QUICKI	Quantitative Insulin Sensitivity Check Index
ROMt	Total reactive oxygen metabolites
RQUICKI	Revised QUICKI
RT	Rectal temperature
RTqPCR	Reverse Transcription quantitative Polymerase Chain Reaction
RXR	Retinoic-X-Receptor
SCC	Somatic cell count
TAG	Triacylglycerol
TF	Transcription factor
TLRs	Toll-like receptors
TZD	Thiazolidinedione
γGT	Gamma-glutamyl transferase

CHAPTER I

INTRODUCTION

Mastitis is an inflammation of the mammary gland and the major endemic disease of dairy animals. Mastitis treatment and control is one of the largest costs to the dairy industry, costing the US dairy industry approximately $2 billion dollars annually (Cha et al., 2011), including costs of antibiotics (which in a long term can cause antibiotic resistance) and also there is a cost associated with the reduced overall performance of the infected animals (i.e. early culling; reduction in milk yield) (Hogeveen et al., 2011; Awale et al., 2012). The development of mastitis generates an immune response to bacterial invasion of the teat canal but can also occur as a result of chemical, mechanical, or thermal injury to the udder of the animal. Mastitis is a multifactorial disease that can be present in a herd sub-clinically, where few, if any, symptoms are present in the infected animals, making subclinical mastitis a big challenge. Practices such as close attention to milking hygiene, the culling of chronically-infected animals and good housing management contribute to lower the mastitis incidence (Leitner et al., 2004); however proper animal nutrition is pivotal to prevent mastitis (Awale et al., 2012).

From a nutritional standpoint proper levels of antioxidants present in a diet can enhance the response to mastitis (Heinrichs et al., 2009). Nutrition can also prevent mastitis through a nutrient-gene interaction. Nutrients can affect biological functions of tissues by changing the transcription of genes via activation or inhibition of transcription factors (TF) (Desvergne and Wahli, 2006; Afman et al., 2006).

Among TF, the peroxisome proliferator-activated receptor (PPAR)-α, -β/δ, and –γ are the ones with greatest nutrigenomic potential (Bionaz et al., 2015). PPARγ modulates adipocytes differentiation and macrophage development and function (Chinetti et al., 2000), playing an

important role in the anti-inflammatory response (Kushibiki et al., 2001) and in the control of milk fat synthesis in dairy cows (Kadegowda et al., 2009) and dairy goats (Shi et al., 2013). The PPAR isotypes, specifically, PPARα and PPARγ are the most studied TF from the nutrigenomic standpoint in ruminants due to their capability to be activated by long chain fatty acids (Bionaz et al., 2013; Bionaz et al., 2015). The nutrigenomic role of these two PPAR isotypes in ruminants has been clearly demonstrated *in vitro* (Bionaz et al., 2013; Bionaz et al., 2015) but specific nutrigenomic studies *in vivo* are lacking. It is well known that in monogastrics PPARγ responds to synthetic agonists (various thiazolidinedione) such as rosiglitazone (Lehmann et al., 1995; Deeks et al., 2007; Malinowski et al., 2000), a response which has been also demonstrated in ruminants (Bionaz et al., 2013).

Therefore, our hypothesis is that the activation of PPARγ by 2,4-thiazolidinedione (a putative PPARγ agonist) prior to and during induced mastitis in lactating dairy goats improves the host response to mastitis and increases milk fat synthesis.

This thesis includes two *in vivo* experiments with lactating dairy goats with the following objectives: to investigate the effect of PPARγ activation on (1) the inflammatory response, (2) milk fat synthesis.

CHAPTER II

LITERATURE REVIEW

1 Mastitis

Mastitis is defined as an inflammatory condition of one or more quarters of the mammary gland in response to an injury, almost always caused by microorganisms (Rinaldi et al., 2010). With an annual cost of approx. $2 billion (or 11% of total U.S. milk production), it is the most costly disease in the dairy industry (Viguier et al., 2009). Mastitis-related losses are associated with reduction in milk yield, permanent damage to udder tissue, costs of veterinary care and medicines, discarded milk, increase in culling and associated animal replacement rates and also, due to the use of antibiotics to treat the disease, it might cause antibiotic resistance (Oliver et al., 2012).

Mastitis is a multifactorial disease (see Figure 1) related to the milk production system and environment. Thus, conditions such as incorrect use of udder washes or cleaning compounds, wet teats, frostbite, failure to prep cows, among others, contribute to increased incidence of mastitis. As the pathogens some mastitis causing organisms are normally found in feces and bedding materials and feed, a clean and dry environment is a crucial factor to prevent mastitis.

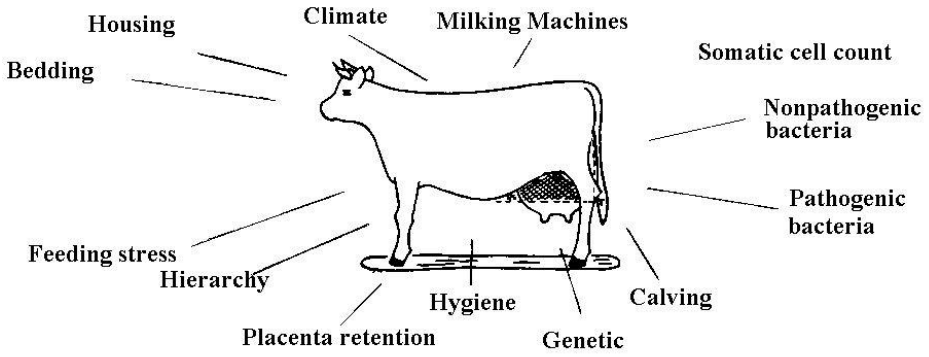

Figure 1. Factors influencing the incidence of mastitis in dairy animals. Adapted from Ecological Agriculture Projects, 1997.

Mastitis develops if a pathogen enters the teat canal. The teat end (streak canal) serves as the body's first line of defense against infection (Figure 2), by which the cells lining the teat canal produce keratin, a fibrous protein with lipid components that have bacteriostatic properties. The keratin together with immune cells, such as macrophages and B cells, in the teat canal form a physical and chemical barrier against bacteria (Capuco et al., 1992). However, if the pathogens can evade the immune response, pathogens multiply in the mammary gland at which they begin to damage the mammary epithelium (Sordillo et al., 2002). Once bacteria multiply, they produce toxins, enzymes, and, upon death, release cell-wall components that stimulate the production of numerous mediators of inflammation by mammary epithelium and leukocytes present in the udder. The magnitude of the inflammatory response is influenced by the causative pathogen, stage of lactation, and several animal-related factors, including age, immune status, genetics, and nutritional status (Harmon et al., 1994).

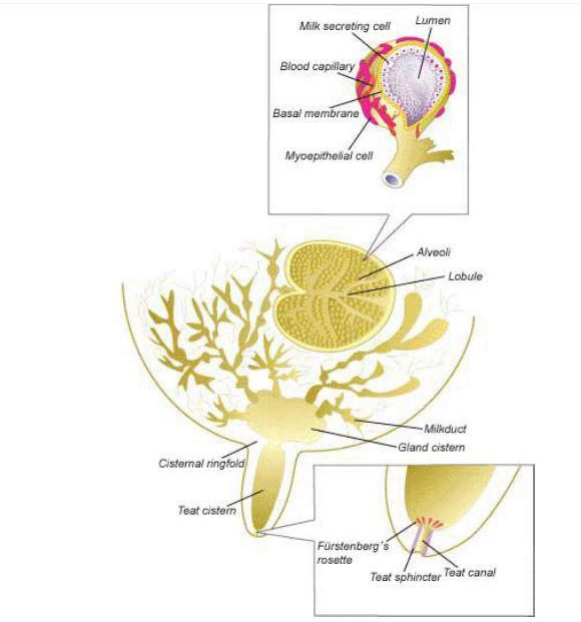

Figure 2. Schematic udder anatomy. Adapted from Delaval, Inc. 2006.

Classically, microorganisms causing mastitis have been classified into two types: contagious and environmental pathogens; mastitis has been classified based on the severity of the infection as clinical and subclinical (Burvenich et al., 2003). Contagious pathogens can survive within the host in the mammary gland, causing mild inflammation and are typically spread from animal to animal at or around milking. In addition, contagious pathogens can induce chronic mastitis, which refers to a long-term udder infection that can persist from one lactation to another (Awale et al., 2012). Environmental pathogens are present in the animal environment (feces, soil, bedding, or water), such as *Escherichia coli* (*E.coli*). *E.coli* is the most common pathogen isolated from clinical mastitis (Bradley et al., 2002). *Streptococcus uberis* (*Strep. uberis*) is a gram-positive bacteria causing subclinical infections (Zadoks et al., 2009). *Strep. uberis* has been detected as the most prevalent pathogen of subclinical mastitis in dairy herds (Kromker et al., 2014).

1.1 Clinical mastitis

Clinical mastitis presents visual signs such as decreased milk production with a clear change in milk appearance and/or physical signs of injury in the udder (redness, swelling, heat and pain) (National Mastitis Council, 1996). The clinical mastitis can be divided into three types: 1) peracute mastitis, characterized by a severe inflammation of the udder, causing fever and loss of body weight in the infected animals; 2) acute mastitis, characterized by moderate to severe inflammation of the udder, but less severe than the peracute form; 3) subacute mastitis, characterized by mild inflammation, with no visible changes in the udder but visual signs in the milk (Awale, 2012).

1.2 Subclinical mastitis

The subclinical mastitis is the dominant form of mastitis in dairy cows, with prevalence ranging from 20 to 50% in a given herd (Pitkala et al., 2004). Wilson and collaborators (1997) estimated that subclinical mastitis can cost from $180 up to $320 per case in a dairy herd with a prevalence of 45% of subclinical mastitis. In dairy goats, incidence of subclinical mastitis range between 5 and 30% (Contreras et al., 2007).

The pathogens responsible for most of the subclinical cases of mastitis in goat herds are the gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Strep. uberis* (Jayarao et al., 1999). *S. aureus* is a contagious pathogen, whereas *Strep. uberis* is an environmental pathogen (Zadoks and Fitzpatrick, 2009). *Strep. uberis* is a type of bacteria that can colonize in both, animals and their environment (Kromker et al., 2014).

Usually, subclinical mastitis goes undetected for a period of time, until specific measurements are made in milk samples, such as the count of somatic cells (SCC). The somatic cells in milk are composed mainly of white blood cells (75% of SCC) and a small proportion of

epithelial cells. The somatic cells are measured on farm indirectly using systems such as California Mastitis Test (CMT) (Shearer and Harris, 1992). Briefly, CMT is an indirect estimation of the SCC in milk, by which a detergent breaks down the cell membrane of somatic cells and releases of nucleic acids. The latter with the detergent forms a viscous gel. The increase in viscosity is proportional to the presence of nucleated cells, i.e., leukocytes. A score based on the viscosity of the milk after the addition of the bromocresol-purple solution has been developed. A score of 2> or 3> are indicative of mastitis (Awale et al., 2012). In Table 1 an interpretation of a CMT in goat milk is presented.

Table 1. Interpretation of California Mastitis Test (CMT) score on dairy goat milk. Adapted from Shearer and Harris, 1992.

CMT score	Description	Average of neutrophils/mL
0	No reaction	68,000
Trace	Slight slime	268,000
1	Apparently slime, without formation of gel	800,000
2	Formation of a gel	2,560,000
3	Formed gel adhered to the bottom of the cup	≥ 10,000,000

Subclinical mastitis is always associated with an increase in the SCC in milk, which is commonly used as a milk quality standard. However, SCC in dairy goats were reported to increase in late lactation and in older goats, even in the absence of intramammary infection (Wilson et al., 1995). Moreover, Lerondelle et al., (1992) showed that there are certain factors such as vaccination that leads to an increase in SCC in goats. As in dairy cows, high milk SCC affects the milk composition in goats, but normal goat milk has a higher cell count than normal milk from cows (Raynal-Ljutovac et al., 2007). This fact influences the milk regulatory grade A standards, by which in dairy cows the SCC legal limit is 750,000/mL in a bulk tank, but in goat milk the limit is 1,000,000/mL, due to the fact that goat milk can reach a SCC of 750,000/mL and still be normal (Shearer and Harris, 1992).

2 Immune response to intramammary infection (IMI)

An inflammatory response in the mammary gland is initiated when a pathogen enters the streak canal and migrates into the gland cistern (Alnakip et al., 2014). Once pathogens are in the mammary gland, the second line of defense is leukocytes of the innate immune system, consisting of neutrophils, macrophages, natural killer cells and dendritic cells. In addition, the mammary epithelial cells (MEC) contribute to the defense against bacterial infection due to their capability to recognize invading pathogens and to release pro-inflammatory factors (Rainard and Riollet, 2006).

A pathogen recognition process by the pattern recognition receptors (PRRS) initiates the activation of the immune response within the mammary gland (Elazar et al., 2010). These receptors include the toll-like receptors (TLRs) that are able to recognize pathogen-associated molecular patterns (PAMPs) such as lipoteichoic acids (LTA; a PAMP of gram-positive bacteria presents in the cell wall of gram positive bacteria, such as *Strep. uberis* and *S. aureus*) and lipopolysaccharides (LPS; a PAMP of gram-negative bacteria, such as *E. coli*). TLRs are expressed on resident macrophages in the mammary gland and on MEC (Strandberg et al., 2005), by which both resident macrophages and MEC trigger the innate immune defense (Tizard, 2004). At least ten different TLRs have been identified in humans, and cattle (Tizar, 2004; Werling et al., 2006, respectively). The most studied TLRs in ruminants are TLR4, which recognizes LPS, and TLR2, that recognizes LTA. Once a TLR recognizes the invading pathogen, a signal process starts towards activation of genes encoding cytokines and chemokines. For instance, upon a mastitis infection, TLRs stimulation on resident macrophages and on MEC in the mammary gland induce the activation of the transcription factor NFκB and cytokine production, such as Interleukin 8 (IL8), for the recruitment of other immune cells from the blood

into the mammary gland, mainly polymorphonuclear leukocytes (PMN) such as neutrophils (Sordillo and Daley, 1995).

One particularity of macrophages cells is that they have other accessory molecules that can recognize microbial patterns, such as the cluster of differentiation 14 (CD14) which is present in the macrophage surface and can bind LPS released by gram-negative bacteria. The binding of CD14 with LPS, induces the former to binds TLR4. The CD14/TLR4 complex then activates macrophages to produce cytokines (Netea et al., 2004). This pathogen-recognition process activates the nuclear factor NFκB. In turn, this stimulates the expression of genes coding for pro-inflammatory cytokines, such as interleukin 1 (IL1), interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα), and chemokines, such as IL8 and C-C Motif Chemokine Ligand 5 (CCL5). IL1 and TNFα, in turn, induce the inflammatory reaction of the liver (Bannerman et al., 2004; Eckersall et al., 2006). This is characterized by the induction of the acute phase response (APR), with increase hepatic synthesis of positive acute phase proteins (+APP) such as serum amyloid A (SAA), ceruloplasmin (CP) and haptoglobin (Hp), while the production of negative acute phase proteins, (-APP) such as albumin and apolipoproteins, decreases (Trevisi, 2008).

Taking together, the key players for a faster resolution of mastitis (schematic summary in Figure 3) are pathogen recognition, local mammary immune defense, neutrophils recruitment, and the cross-talk of pro-inflammatory signal molecules between the mammary gland and liver.

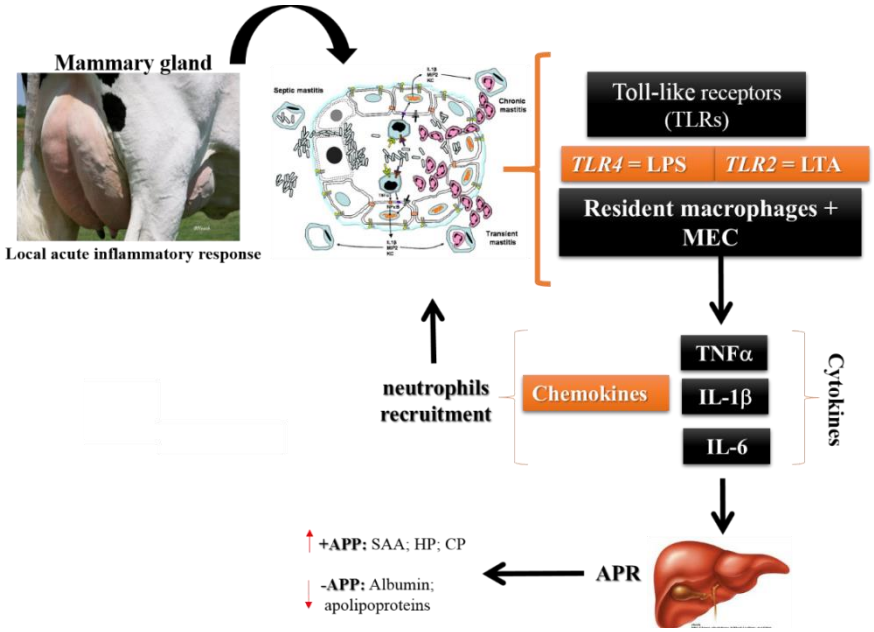

Figure 3. Model for the activation of the innate-immune system in the mammary gland tissue upon an intramammary infection and the molecular communication with the liver. Adapted from: Kuby Immunology, 2000.

2.1 Cytokines and chemokines

Cytokines are signal molecules responsible, among other messengers, for the intercellular communication during an inflammatory condition (Schijns and Horzinek, 1997). Cytokines play an essential role in the regulation of the immune response. For example, neutrophils are cells "ready" to attack, but they need to be activated in order to move into the infected area to phagocytize invading pathogens. This activation is due to cytokines such as TNFα, which is a protein produced by macrophages and MEC during the early phase of inflammation. Together with TNFα IL1, mainly the IL1β produced by macrophages and MEC, provide neutrophil adherence (Tizard, 2004).

Followed by TNFα and IL1β, IL6 is another cytokine produced by macrophages, triggering the movement of monocytes from blood into the site of infection. It was suggested that IL6 from macrophages is the mediator of the transition from the early stage of inflammation,

whereas the neutrophils and MEC are the major cells of defense against mastitis infections at the later stage of inflammation, which is mostly a macrophage-meadiated process (Ovideo-Boyso et al., 2006). In addition, IL6 is one of the main cytokines responsible for the regulation of the hepatic APR (Slebodzinski et al., 2002). Another important cytokine is the interleukin-12 (IL12), which regulates the T lymphocytes differentiation, activating the adaptive immune system (Hornef et al., 2002). In summary, cytokines modulate the leukocytes influx into the mammary gland upon an infection, with certain cytokines also regulating the neutrophils migration (Paape, et al., 2003; Zhao and Lacasse, 2008)

Chemokines are chemo-attractants for specific cell populations. They are produced by macrophages and mast cells. In general, at least 50 chemokines have been identified and they are classified based on the spacing of their cysteine residues. The CC (α family) presents two cysteine residues together, while the CXC (β family) presents the cysteine residues separated by one other amino acid (Tizard, 2004).

IL8 (also known as CXCL8) as well as CXCL2 (macrophage inflammatory protein-2), are chemokines secreted by macrophages that trigger the migration of neutrophils into the site of infection (Lay and Gallo, 2009). Also, IL8 can activate neutrophils to phagocytize the invading pathogens. Furthermore, activated neutrophils produce one chemokine attractant for macrophages, the monocyte chemoattractant protein-1 (CCL2).

2.2 Neutrophils and macrophages

Neutrophils compose 20–30% of blood leukocytes in cattle and sheep (Tizard, 2004). The same or higher % of neutrophils was reported for goats (Daramola et al., 2005). In healthy mammary glands, less than 10% neutrophils are present among somatic cells present in milk; however, during mastitis neutrophils are the predominant cell type in milk, resulting in up to

90% of the total somatic cells. The large increase in neutrophils is due to the large influx of these cells into the mammary gland undergoing inflammation (Paape et al., 2003).

The neutrophils capability to defend tissues against microbial invasion relies on 5 steps: margination, migration, phagocytosis, respiratory burst, and degranulation (Burton and Erskine, 2003).

- *Margination* is when the circulating blood neutrophils detect an infection in underlying tissues, where the cytokines and chemokines secreted by macrophages or MEC turn on cellular adhesion molecules present at the neutrophils plasma membrane such as L-selectin (CD62L) and this promotes the neutrophils binding to the endothelium and facilitate their migration towards the infection site (Ovideo-Boyso et al., 2006).
- During *migration*, neutrophils cross the blood vessel wall passing between endothelial cells by diapedesis and they are guided towards the infection area by increasing concentrations of proinflammatory molecules (TNFα, IL1β, IL6, IL8), in a process called chemotaxis (Burton and Erskine, 2003). It was reported that upon an intramammary infection in bovine it takes from 2 to 4 hours to detect neutrophils migration into the mammary gland, while the peak of neutrophils in milk is observed after 8–12 hours post infection (Persson et al., 1992; Schuster et al., 1996).
- Once neutrophils reach the infection site and become activated (i.e. by TNFα; IL8), they kill bacterial pathogens through *phagocytosis* (Persson et al. 1993). In this process, specialized receptors on neutrophils are able to recognize invaders and initiate the process of engulfment of the invading pathogen, i.e., phagocytosis.
- During phagocytosis, neutrophils consume large amount of oxygen molecules, resulting in the *respiratory burst* (i.e., increased production of reactive oxygen species) and the release of antimicrobial molecules (Alnakip et al., 2014). In the respiratory burst pathway, the hydrogen

peroxide (H_2O_2) is converted to anti-bacterial compounds via myeloperoxidase (MPO) activity (Tizard, 2004). MPO (primary granule) is the most significant enzyme in neutrophils and enhances the oxidative killing capacity of these immune cells.

- Secondary granules present in the cytoplasm of neutrophils contribute to finish the pathogen killing process (*degranulation*). For instance, lysozyme destroys the bacterial cell walls (peptidoglycans of the cell wall of Gram-positive and Gram-negative bacteria); lactoferrin that binds iron, making iron unavailable to bacteria, preventing bacterial growth; and collagenase that degrades connective tissue (Rainard and Riollet, 2006).

Macrophages are mononucleated cells and monocytes in blood are the precursor forms of them. After entering the tissue or milk they mature to macrophages. The resident macrophages in the mammary gland are the ones responsible for the detection of invading pathogens (Tizard, 2004). Also, the ability of macrophages to release chemoattractants that facilitate the migration of neutrophils is essential in the early stage of inflammation (Hoeben et al. 2000; Wittmann et al. 2002). However, when neutrophils are dying they release defensins and other peptides that are chemo-attractants to monocytes and macrophages. Also, they release the chemokine CCL2 that attracts macrophages to the site of infection. Therefore, macrophages can phagocyte the invading pathogens by a similar process to neutrophils phagocytosis (Rainard and Riollet, 2006). In summary, macrophages participate in the initiation and in the resolution of inflammation.

Macrophages present two different pathways of activation. The classical activation encompasses toll-like receptors and the soluble cytokine interferon gamma (IFNγ); the alternative activation encompasses IL4 and IL13. Whereas the classical activation results in proinflammatory cytokine production, the alternative activation results in anti-inflammatory cytokines and induce tissue repair (Tizard, 2004).

2.3 Transcriptional response to mastitis

Resident macrophages, members of the innate immune system, serve as the first line of defense against invading pathogens in the mammary gland. Lewandowska-Sabat et al. (2013) demonstrated *in vitro* that treatment with *S. aureus* induced the activation of macrophages by the up-regulation of the tumor necrosis factor receptor superfamily member 5 (Cluster of differentiation 40 or CD40), which plays a role in the toll-like receptor signaling in inducing the innate immunity.

Transcriptomic studies of Taraktsoglou et al. (2011) and Franchini et al. (2006) demonstrated that bovine macrophages stimulated with LPS from *E. coli* and LTA from *S. aureus* increased mRNA expression of *TLR4* and *TLR2*, respectively, after 24 hours treatment. In addition, the work of Taraktsoglou et al. (2011) showed that stimulation of macrophages with LPS also resulted in increased expression of pro-inflammatory genes such as *TNF* and *IL10*. Transcriptomics analysis of SCC isolated from the milk of Holstein cows experiencing subclinical mastitis indicated an increased abundance of mRNA of pro-inflammatory cytokines, such as interleukin 6 and 8, (*IL6*, *IL8*) (Peli et al., 2003). Regarding to mastitis response, Genini et al., (2011) reported a large meta-analysis of microarray data generated from goat mammary tissue challenged with *S. aureus*, concluding that both clinical and subclinical mastitis activate pro-inflammatory pathways, i.e. up-regulating cytokines genes, and, at the same time, down-regulate the expression of genes related to lipid metabolism in mammary tissue.

2.4 Relationship between hepatic transcriptome and mastitis response

The liver has a strong response during intramammary infection as consequence of proinflammatory cytokines produced by the immune cells. IL1β, IL6 and TNFα serve as signal molecules to the hepatic tissue in order to produce positive acute phase proteins. The liver

transcriptome is highly affected by intramammary infection. Studies with hepatic tissue collected through biopsy in dairy cows infused with LPS resulted in up-regulation of genes associated with the acute phase response and in contrast, a down-regulation of genes related to lipid metabolism (Jiang L, et al., 2008). The latter could be that the pro-inflammatory cytokines (i.e. IL1β, IL6 and TNFα) produced by the immune cells stimulated the expression of genes encoding acute phase proteins and repressed genes coding for metabolic-related pathways in the liver.

Loor et al (2011) published a review where the authors re-analyzed data from the study of Jian et al. (2008). Among many categories of pathways analyzed in this data set, the metabolic-related pathways were the most inhibited from 3 hours to 12 hours after the LPS challenge, whereas the cholesterol pathway was strongly inhibited. As plasma cholesterol is a well-known biomarker of liver function (Bertoni et al., 2008), it is clear that upon an inflammatory condition the liver sustains the production of positive acute phase proteins that can compromise the usual activity, including metabolism (e.g. plasma cholesterol and negative acute phase proteins reduced).

As the liver for ruminants is the central organ for lipid metabolism (i.e., oxidation of fatty acids and synthesis of very low density lipoprotein) and gluconeogenesis, supporting the lactose synthesis in the mammary gland, a repartitioning of gene expression in the liver in response to inflammation, i.e. mastitis, do not only affect the liver as an organ but has a strong negative effect on the whole animal, in particular the mammary gland of lactating animals, negatively affects the overall animal performance.

3 Nutrition and mastitis

Mastitis can affect the animal well-being, milk quantity and quality (Halasa et al., 2007, Hogeveen et al., 2011, Awale et al., 2012). Therefore, there is a need for an approach to prevent mastitis and to reduce the use of antibiotics as a treatment, which it is not permitted in organic farming in the U.S. (Ruegg, 2009). Among the several strategies to prevent the disease, the dietary approaches can also be effective in improving the animal response to mastitis.

4 Nutrigenomic effect via transcription factor

Metabolic regulation relies on three main types of control. The first is the allosteric control, which plays a role in the metabolic pathways controlling key enzymes. The second is the post-translational modifications, such as proteolytic cleavage, phosphorylation, sumoylation, acetylation and glycosylation. The third type of control is the metabolic homeostasis in complex organisms via transcriptional regulation.

Transcriptional regulation is under control of transcriptional regulatory factors (TFs). TFs are proteins that can enter the cell nucleus and bind specific DNA sequences (response elements) located in the enhancer regions of the genes. When this binding happens, it can affect mRNA expression of target genes. There are a variety of transcription factor families involved in metabolic regulation. However, the nuclear receptor (NR) family has a predominant role in the regulation of the metabolism. Among NRs, the ligand-dependent nuclear receptors (LdNR), plays a special role in the control of metabolism by also dietary compounds. LdNR are activated by specific dietary compounds that bind a ligand pocket of LdNR regulating their transcription activity. LdNR include the peroxisome proliferator-activated receptor (PPAR) and vitamin-specific LdNR, such as vitamin D receptor activated by vitamin D3 and retinoid X receptors (RXR) and retinoic acid receptors (RAR) activated by retinoic acids, metabolites of the vitamin A (Minucci et al., 1997).

PPAR can be activated by dietary fatty acids (Burris et al., 2013; Jump et al., 2013), known as natural ligands for PPAR, but it can also be activated by synthetic ligands, such as the thiazolidinedione (TZD) family of molecules. As in monogastrics, long-chain fatty acids (LCFA) are among the most potent natural ligands of PPAR in ruminants. PPAR are normally located in the nucleus of the cell but PPAR can also be present in the cytoplasm (Patel et al., 2005). In

several species, including bovine, there are three PPAR isotypes identified: PPARα (gene symbol *PPARA*), highly expressed in liver, kidney and intestine; PPARβ/δ (gene symbol *PPARD*), ubiquitously expressed throughout the body; and PPARγ (gene symbol *PPARG*), highly expressed in white adipose tissue and also, relatively abundant in mammary tissue (Bionaz et al., 2013) (Figure 4).

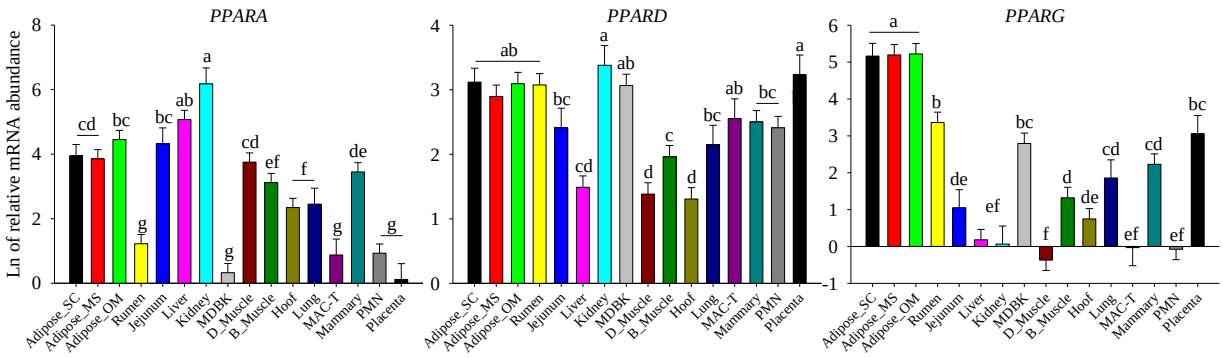

Figure 4. Relative mRNA abundance of each PPAR isotype in 14 different bovine tissues and cells. Adipose: SC = subcutaneous; MS = mesenteric; OM = omental. D_muscle = semitendinosus muscle; B_muscle = skeletal muscle of beef cattle. PMN = blood polymorphonuclear leukocytes. MDBK = Madin-Darby Bovine Kidney. MACT = bovine mammary alveolar cells. Adapted from Bionaz et al., (2013).

The three PPAR isoforms present a similar structure. They possess four functional domains (Figure 5): 1) A/B domain is responsible for PPAR phosphorylation; 2) C domain is the DNA binding domain (DBD); 3) D domain is the hinge region (for cofactors); and 4) E domain is the ligand-binding domain (LBD), which is responsible for ligand specificity and promotes the cofactor recruitment required for the transcription (Kota et al., 2005). Both DBD and LBD are highly conserved among the three PPAR in monogastrics (Escher and Wahli, 2000). PPAR is functional when it forms a heterodimer with RXR. After the heterodimer formation, the PPAR DBD binds to the major groove of the DNA at the PPAR response element – PPRE (the specific DNA sequence recognized by the heterodimer PPAR-RXR) in the promoter region of the PPAR

target genes. The PPAR/RXR binding on DNA produce a covalent modification of the PPAR structure, resulting in the recruitment of several co-factors or co-activators, which allow up-regulation or down-regulation of the expression of target genes (Figure 6).

The consensus sequence of the PPRE is composed of two hexametric core binding motifs directionally aligned with a single nucleotide space (AGGTCANAGGTCA; where N is the variable nucleotide base). PPAR cavity binding is composed of 34 amino acid residues, which were demonstrated to be ~80% conserved in humans and rodents (Zoete et al., 2007). In bovine, the similarity between PPAR isotypes is variable with PPARα and PPARβ/δ being more conserved (59.4%) than with PPARγ (52.4%) (Bionaz et al, 2012). The alignment analysis between the overall conservation of PPAR isotypes in bovine is shown in Table 2.

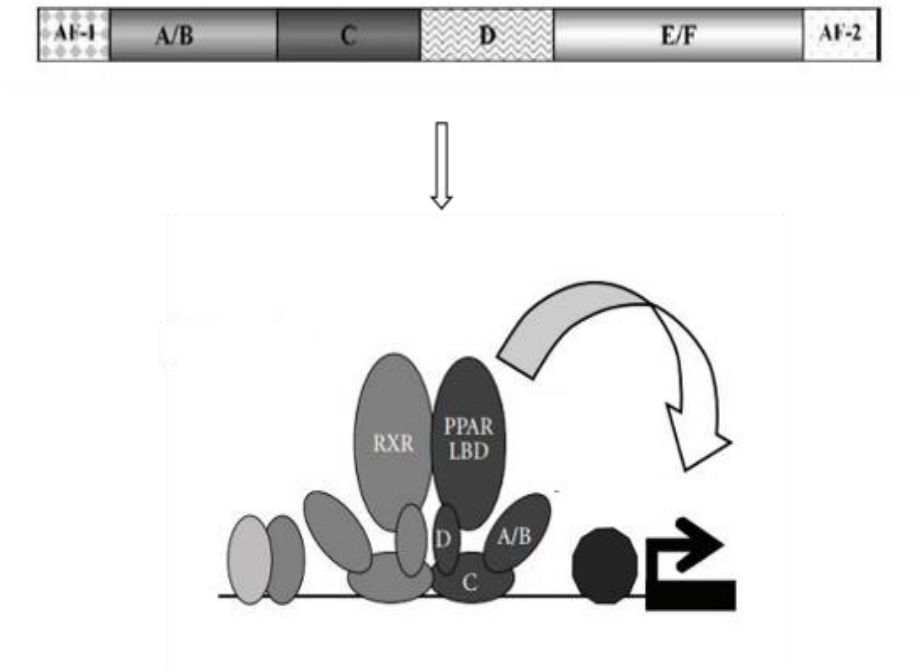

Figure 5. Schematic representation of the functional domains of PPAR. A/B domain (the N-terminal) responsible for PPAR phosphorylation and contains the ligand-independent activation function 1 (AF-1); C domain is the DNA binding domain (DBD); D domain is implicated the region for cofactors; E/F domain is the ligand-binding domain (LBD) containing the ligand-dependent AF- 2. Adapted from Kota et al., (2005) and from Bugge and Mandrup (2010).

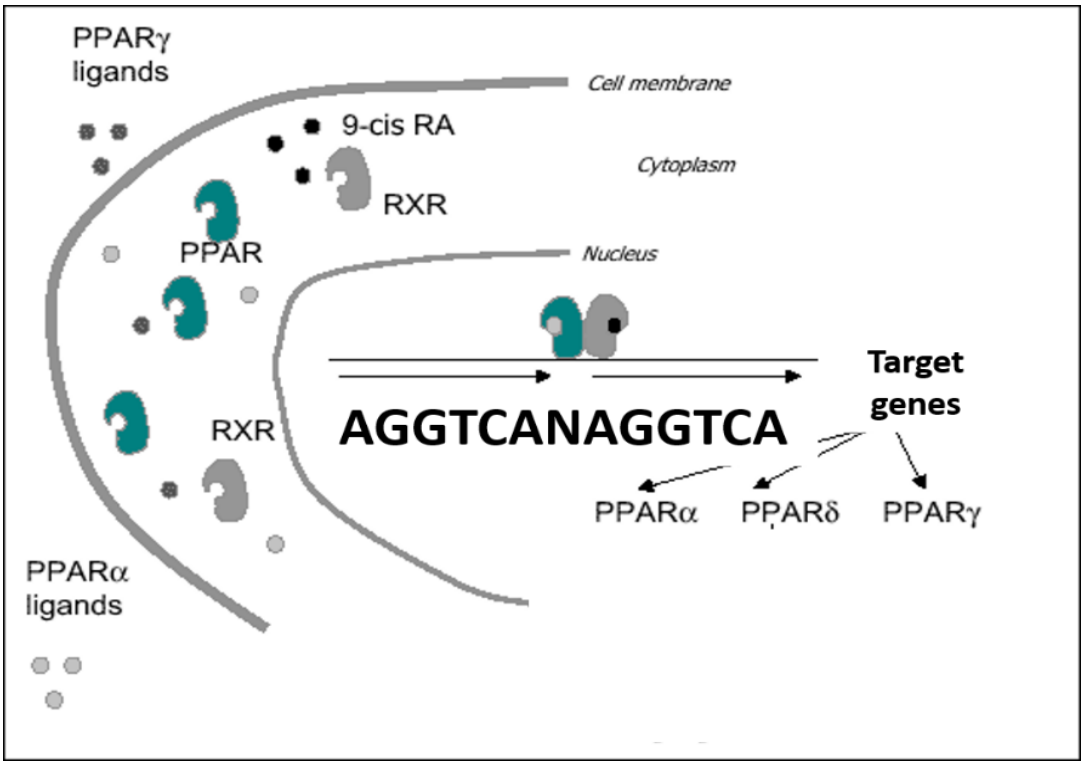

Figure 6. Schematic representation of PPAR/RXR heterodimer binding to the PPAR response element (AGGTCANAGGTCA) on DNA. PPAR = peroxisome proliferator-activated receptor; RXR = retinoid X receptor. Adapted from Sugawara et al., (2010).

Table 2. Comparative alignment analysis between bovine PPARα, PPARγ, and PPARδ: alignment analysis between Bos taurus PPARα (NP_001029208.1), PPARγ (NP_851367.1), and PPARβ/δ (NP_001077105.1) proteins. Adapted from Bionaz et al., 2012.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	PPARα	470	2	PPARγ	505	54
1	PPARα	470	3	PPARβ/δ	441	60
2	PPARγ	505	3	PPARβ/δ	441	55

In non-ruminants the capability of PPAR to bind and be activated by LCFA, especially polyunsaturated fatty acids (PUFA), is well established (Escher and Wahli, 2000). The potency of activation is affected by type of LCFA and there is a dose-response dependence (Bragt et al., 2008). PPAR can bind saturated, monounsaturated and polyunsaturated fatty acids (Jump, 2008);

up to date, linoleic acid, linolenic acid, arachidonic acid, and leukotriene have been demonstrated to be the strongest PPAR natural agonists in monogastrics (Bionaz et al., 2013).

In ruminants most of the studies performed so far were done in bovine by *in vitro* assays using bovine epithelial cells; few *in vivo* experiments were performed in sheep and goats (see Table 3). PPARs in ruminants can be activated by synthetic agonists. It has been demonstrated that ruminant PPAR respond to Wy-14643 and fenofibrate (PPARα agonists), various TZDs (PPARγ agonists) and GW501516 (PPARβ/δ agonist) (Bionaz et al., 2013). Contrary to monogastrics, PPAR in ruminants are potently activated by saturated LCFA, such as palmitate and stearate at a concentration of 100μM (Kadegowda et al., 2009).

Table 3. PPAR activation in ruminants by long chain fatty acids (LCFA).

	Agonist effect on PPAR isotype			
LCFA	PPARα	PPARγ	PPAR β/δ	References
16:0	strong	strong	No information	Kadegowda et al., 2009; Bionaz et al., 2012.
18:0	strong	strong	No information	Kadegowda et al., 2009; Bionaz et al., 2012.
18:2	weak	No information	No information	Kadegowda et al., 2009; Bionaz et al., 2012.
20:0	moderate	No information	No information	Kadegowda et al., 2009; Bionaz et al., 2012.
20:4n-6	moderate	No information	moderate	Sheldrick et al., 2007; Riahi et al., 2010.
20:5n-3	moderate	moderate	No information	Kadegowda et al., 2009; Bionaz et al., 2012.
22:6n-3	weak	No information	No information	Kadegowda et al., 2009; Bionaz et al., 2012.

The three PPAR isotypes have multiple functions in mammals. It has been well-stablished in monogastrics, including humans, that PPARγ plays a role in adipogenesis and insulin sensitivity. PPARα is essential for fatty acid catabolism in the liver, whereas the PPARβ/δ plays a role in the skeletal muscle and heart. The PPARβ/δ received recently more attention due to discover of novel functions, such as skin repair and overall metabolic control (Sun et al., 2014). In ruminants, studies on PPARβ/δ function are scant; however, recently

Bionaz and collaborators (2013, 2015) discussed the potential effect of PPARβ/δ in glucose uptake by mammary tissue in bovine, and Zhou et al., (2014) demonstrated that PPARβ/δ can play a role in lipid peroxidation in the mitochondria.

The main interest in studying PPAR isotypes in ruminants is due to their important roles in lipid metabolism and inflammatory response. Among the three PPAR isotypes, PPARγ has been the most studied in ruminants (Bionaz et al., 2013).

5 Peroxisome proliferator-activated receptor gamma (PPARγ)

In non-ruminants, it has been demonstrated that activation of PPARγ has anti-inflammatory effects (Varga et al., 2011) and plays a crucial role in the regulation of lipid and glucose metabolism (Desvergne et al., 2006). In particular, PPARγ is the major regulator of adipogenesis and lipogenesis (Tontonoz and Spiegelman, 2008a) and has insulin sensitizing effects (Feige et al., 2006). The same functions appear to also be conserved in sheep and bovine (Bionaz et al., 2013). In bovine PPARγ is principally expressed in mammary gland, and all adipose tissues and plays pivotal roles in regulating lipid metabolism (Bionaz et al., 2013).

PPARγ can be activated by thiazolidinedione-derived molecules, (Eldor et al., 2013, Janani & Kumari, 2015). Among those, rosiglitazone and pioglitazone have been widely used in the treatment of type 2 diabetes to ameliorate insulin sensitivity in humans (Mooradian et al., 2002).

PPARγ can be activated by dietary fatty acids. Data from Kadegowda et al. (2009) using bovine MacT cells and Wangsheng et al., (2014) using primary goat mammary epithelial cells indicated that palmitate and stearate at concentration of 100μM, induced PPARγ activation. The above studies plus several other studies reported by Bionaz and collaborators (2013, 2015) strongly indicated that PPARγ in ruminants has a stronger response to saturated rather than unsaturated LCFA. However, to our knowledge there are not published studies in ruminants that demonstrate *in vivo* PPARγ activation by dietary LCFA.

5.1 PPARγ and its role on milk fat synthesis

PPARG is highly expressed in adipose tissues, including mammary tissue (Bionaz et al., 2013) (Figure 4). The expression of *PPARG* and the PPARG coactivator 1 (PPARGC1A) increased 2.5- and 3-fold, respectively during lactation in bovine mammary tissue and can be a

putative transcription factor controlling the network of genes coding for proteins involved in milk fat synthesis (Bionaz and Loor, 2008). These findings together, indicated a role of PPARγ in controlling milk fat synthesis, which was supported by subsequent *in vitro* studies performed in bovine mammary alveolar cells treated with rosiglitazone at concentration of 10μM (Kadegowda et al., 2009); and in goat mammary epithelial cells treated with 50μmol/L of rosiglitazone, whereas PPARγ affected the expression of genes related to triacylglycerol synthesis and lipid droplets (Shi et al., 2013).

The role of PPARγ on milk fat synthesis is likely ruminant-specific (Bionaz et al., 2013). Peterson et al., (2004) suggested that the regulation of milk fat synthesis might be controlled by a common transcription factor. Later on, Kadegowda et al., (2009) suggested that PPARγ in bovine mammary cells up regulate the mRNA expression of PPARγ lipogenic target genes, such as *ACACA*, *FASN*, and *SCD*. Findings from the work of Kadegowda et al., (2009) support PPARγ likely being the central regulatory point for milk fat synthesis, considering also the finding from the same author that PPARγ controls the transcription of the Sterol Regulatory Element Binding Transcription Factor 1 (*SREBF1*) (Figure 7).

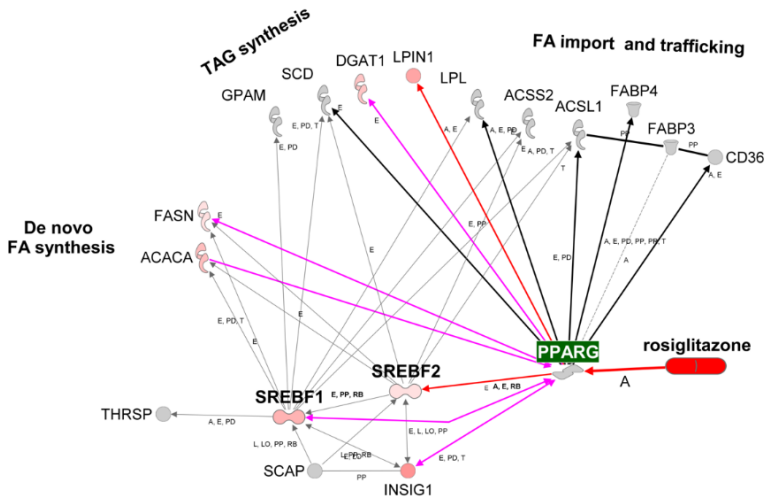

Figure 7. Genes studied in response to peroxisome proliferator-activated receptor-γ (*PPARG*) activation via rosiglitazone in bovine mammary epithelial cell line (MacT). Genes with a purple and red arrow were upregulated by rosiglitazone. *LPIN1* = lipin 1; *THRSP* = thyroid hormone responsive (SPOT14 homolog); *SREBF2* = sterol regulatory element-binding transcription factor 2; *INSIG1* = insulin-induced gene 1; *SCAP* = SREBF chaperone; *SREBF1* = sterol regulatory element-binding transcription factor 1; *DGAT1* = diacylglycerol O-acyltransferase homolog 1; *AGPAT6* = 1-acylglycerol-3-phosphate O-acyltransferase 6; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *SCD* = stearoylcoenzyme A desaturase; *FASN* = fatty acid synthetase; *ACACA* = acetyl-coenzyme A carboxylase; *ACSS2* = acyl-coenzyme A synthetase short chain family member 2; *ACSL1* = acyl-coenzyme A synthetase long-chain family member 1; *CD36* = the fatty acid translocase CD36 molecule [thrombospondin receptor]; *FABP3* = fatty acid-binding protein 3; *LPL* = lipoprotein lipase; *FABP4* = fatty acid-binding protein 4. From Kadegowda et al., 2009.

5.2 PPARγ and its role in adipose tissue

In monogastrics, PPARγ activation is required for adipose tissue differentiation (Rosen and MacDougald, 2006). In ruminants, Torri and colleagues (1998) demonstrated that PPARγ affects the regulation of adipogenesis and insulin sensitivity due to the observed adipogenic differentiation in vascular stromal cells from bovine adipose tissue. In addition, as for monogastrics, PPARγ enhances insulin sensitivity in ruminants, as demonstrated in dairy steers i.v. injected with the putative PPARγ activator 2,4-thiazolidinedione (2mg/Kg BW/d) (Kushibiki et al. 2001). More recently, a research group at Cornell University demonstrated a strong effect of a daily intrajugular injection of 2,4-thiazolidinedione (2.0 or 4.0 mg/Kg of BW) on subcutaneous adipose tissue in dry dairy cattle (Smith et al., 2007; Smith et al., 2009;

Schoenberg and Overton, 2011; Schoenberg et al., 2011). These studies demonstrated that the use of 2,4-thiazolidinedione lowered plasma NEFA concentration after parturition. Another study performed by Ji et al. (2012) also supported the effect of PPARγ on insulin sensitivity in subcutaneous adipose tissue. In that study dairy cows were fed with a moderate energy diet (1.47 Mcal/Kg of DM) during the close-up period. The treatment led to an up-regulation of *PPARG* and *ADIPOQ* (the insulin-sensitizing adipokine) likely driven via PPARγ activation after calving (at least 7 d postpartum), providing evidence that PPARγ helped adipose tissue to retain insulin sensitivity.

In a fetal reprograming study conducted in sheep it was demonstrated that rosiglitazone s.c. intrafetal administration (4.28 mg/fetus/d) in pregnant sheep resulted in a significant increase in expression of *LPL* (lipoprotein lipase; responsible for the uptake of fatty acids into adipose tissue) in the fetal perirenal adipose tissue (Muhlhausler et al., 2009). As *LPL* is a PPARγ target gene and rosiglitazone is a PPARγ agonist, this finding emphasizes potential strong effect of PPARγ on adipose tissue. In summary, the few data available suggest that PPARγ play important roles in the adipose tissue of ruminants, that appear to also be conserved in monogastrics.

5.3 PPARγ and inflammatory response

PPARγ is expressed in various immune system-related cell types, particularly in antigen-presenting cells such as macrophages. In these cells, PPARγ does not only regulate genes related to lipid metabolism, but also immunity and inflammation (Glass et al. 2010). The first demonstration that PPARγ may play an anti-inflammatory role in ruminants was carried out by a Japanese group who s.c injected human recombinant TNFα (rbTNF; 2.5μg/Kg) plus i.v. injection of 2,4-thiazolidinedione (TZD; 2 mg/Kg).They observed that TZD treatment partially reversed insulin resistance caused by TNFα in dairy steers (Kushibiki et al., 2001). Furthermore, in a

study by Lutzow et al. (2008), bovine epithelial cells (bMEC) isolated from Holstein cows at peak of lactation were treated with the PPARγ natural agonist prostaglandin J2 (PGJ2) at a concentration of 10μM and the mRNA expression of selected genes was examined after 48 hours of treatments. The results demonstrated increased expression of *IL8* and Chemokine (C-X-C Motif) ligand 6 (*CXCL6*) (Figure 8). These genes are involved in the pro-inflammatory signaling and a robust production is desirable for a quick response to intramammary infection. Thus, these findings demonstrate a potential role of PPARγ activation in enhancing the response to mastitis. A discussion of a role for PPARγ in treatment of mastitis is available in Mandard and Patsouris (2013). In addition, in monogastrics a role of PPARγ in wound re-epithelialization (Gurtner et al., 2008) has been clearly demonstrated which may be relevant for mammary epithelial regeneration after damage due to mastitis, allowing for a full recovery of the mammary gland.

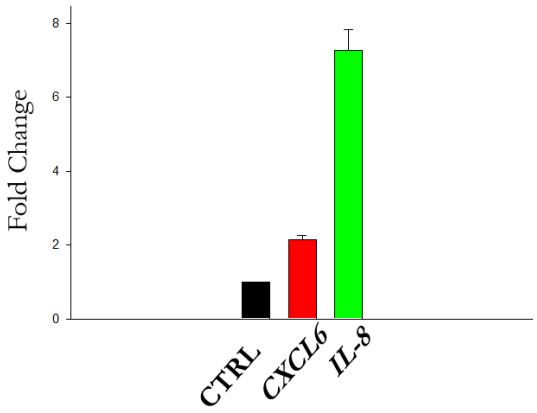

Figure 8. *CXCL6* and *IL8* mRNA fold change compared to CTRL (untreated cells) in bovine primary mammary epithelial cells incubated with 15d-PGJ2 (10mm) for 48h and stimulated with LPS for 24h. Adapted from Lutzow et al., (2008).

CHAPTER III

EXPERIMENT 1: ROLE OF PEROXISOME PROLIFERATOR ACTIVATOR RECEPTOR GAMMA ON MILK FATTY SYNTHESIS AND INFLAMMATORY RESPONSE IN LACTATING GOATS UNDERGOING SUBCLINICAL MASTITIS

1 Introduction

Mastitis is an inflammatory response of the udder to infection (usually bacteria) with detrimental consequences to animal well-being and milk production and quality (Awale et al., 2012; Halasa et al., 2007; Hogeveen et al., 2011) . It has been estimated that the cost of mastitis in the US dairy industry is ca. $2 billion annually or 11% of total U.S. milk production, with an average of ca. $172/cow/year (Jones, 2009).

Mastitis may be clinical or sub-clinical. Sub-clinical mastitis can be detected only by the measurement of somatic cells in milk, which increases several folds compared to milk from healthy mammary gland. The high somatic cells count during subclinical mastitis negatively affects milk quality but also reduces milk production. It has been estimated that subclinical mastitis costs $130/cow per year (Halasa et al., 2007). Considering approx. 9 million cows in US, the cost can be over $1 billion/year. Therefore, prevention of subclinical mastitis is a priority in the dairy industry.

From a nutritional standpoint it is becoming evident that adequate levels of dietary antioxidants, particularly vitamin E, selenium, zinc, and vitamin A reduce the incidence of environmental mastitis in dairy cows (Awale et al., 2012, Erskine, 1993, Heinrichs et al., 2009). Besides providing protection from oxidative stress, several of these compounds enhance the mammary response to mastitis (Heinrichs et al., 2009). However, nutrition can help to prevent mammary infections not only by aiding the immune function nut also by decreasing damaging peroxides, through a nutrient-gene interaction. It is becoming increasingly evident that nutrient

compounds can profoundly alter the host's response via the transcriptome (Arevalo-Turrubiarte et al., 2012, Lichtenstein et al., 1998). Nutrigenomics is the discipline that studies the effects of nutrition on the transcriptome (Bionaz et al., 2015) and it is revolutionizing the field of nutrition (Mutch et al., 2005). Dietary compounds can affect the transcriptome *via* binding of transcription factors (TF) (Bionaz et al., 2015). Peroxisome Proliferator-Activated Receptors (PPARs) are among TF the most interesting from a nutrigenomic standpoint (Bionaz et al., 2013, Bionaz et al., 2015).

PPARs are nuclear receptors and members of the nuclear hormone receptor superfamily that work as TF. PPARs form a heterodimer with retinoid-X receptor (RXR) and when activated by natural or synthetic agonists, modulate transcription by binding to a specific DNA sequence termed PPAR response element (PPRE) inducing the transcription of target genes (Desvergne et al., 2006). Three PPAR isotypes denominated PPARα, PPARβ/δ, and PPARγ have been identified in several species, including bovine (Bionaz et al., 2013). Among the three PPAR isotypes the PPARγ is highly expressed in bovine white adipose tissue but its expression is also relatively abundant in mammary tissue (Bionaz et al., 2013). Long-chain fatty acids (LCFA) are among the most potent agonists of bovine PPAR opening up the possibility of dietary interventions to improve the response to diseases in dairy cows *via* activation of PPAR (Bionaz et al., 2013).

In non-ruminants, PPARγ has anti-inflammatory effects (Varga et al., 2011). Furthermore, PPARγ plays a crucial role in the regulation of lipid and glucose metabolism (Desvergne et al., 2006) by regulating adipogenesis and lipogenesis (Tontonoz and Spiegelman, 2008a) and by insulin-sensitizing effects (Feige et al., 2006). The same functions appear to be conserved also in ruminant PPARγ (Bionaz et al., 2013).

In mammary tissue of dairy cows expression of PPARγ is up-regulated from pregnancy to lactation (Bionaz and Loor, 2008b) indicating a role of this nuclear receptor in controlling milk fat synthesis. This was supported by *in vitro* studies performed in bovine and goat (Kadegowda et al., 2009, Shi et al., 2013, Zhu et al., 2014) but not in mouse (Vyas et al., 2014), suggesting that the role of PPARγ in milk fat synthesis is likely ruminant-specific (Bionaz et al., 2013).

Besides a role on the regulation of milk fat synthesis, PPARγ may have also a positive role on the host's response to mammary infection, as reviewed recently (Bionaz et al., 2015). Particularly, in bovine primary mammary epithelial cells (bMEC) the activation of PPARγ by several agonists caused down-regulation of several pro-inflammatory cytokines and increased expression of the Chemokine (C-C Motif) Ligand 2 and Tumor Necrosis Factor alpha (*TNFA*) (Lutzow et al., 2008). In contrast, activation of PPARγ by a natural agonist markedly enhanced the expression of both interleukin 8 (*IL8*) and chemokine (C-X-C motif) ligand 6 (*CXCL6*) and had no effect on other cytokines (Lutzow et al., 2008). IL8 is the strongest chemo attractant for neutrophils and higher production is desirable for a quick response to intramammary infection (Loor et al., 2011, McClenahan et al., 2006). In addition, in monogastrics all PPAR isotypes play an important role in wound re-epithelialization (Gurtner et al., 2008) which might be relevant for mammary epithelial regeneration after damage due to mastitis, allowing for a full recovery of the mammary gland.

Our long-term hypothesis is that modulation of PPAR activity in dairy animals by LCFA can improve the overall health and performance (Bionaz et al., 2013). The objectives of this study were to test if TZD-induced activation of PPARγ prior and during induced subclinical mastitis 1) improves the response to mastitis and 2) affect milk fat synthesis in dairy goats.

2 Material and methods

2.1 Experiment design and animal management

The Institutional Animal Care and Use Committee (IACUC) of Oregon State University approved all procedures for this study (protocol #4448). Twenty four lactating Saanen goats (mean±SD; age 5.1±0.6 year old, 3.6±0.6 lactations, 156±14 day in milk, 68.1±7.6 kg of BW, 1.6±0.5 body condition score [1-5 scale]) negative to a milk bacterial analysis were purchased from a commercial farm (Tumalo Farm, Bend, Oregon, USA) and housed in the Oregon State University Sheep Center facility. After one week of adaptation the goats were assigned to treatments in a randomized block design (body condition score, milk yield and milk components) and housed in 4 separated pens (6 goats/pen). Animals were fed twice a day (8AM and 6PM) with a similar diet as in the original commercial farm with ad-libitum hay (approx. 50% orchard hay and 50% alfalfa). The goats received approx. 150 g of a commercial goat grain mix (Kountry Buffet, PayBack, Harrisburg, OR) during milking. The goats were milked once a day at 8 AM in a stanchion using a portable milking machine. Teats of the goats were pre-dipped before milking and post-dipped after milking using 0.5% iodine solution.

2.2 Treatments

After the week of adaptation, goats were fitted with an indwelling jugular catheter (Cat#017376, Henry Schein, USA) with an extension (Cat#005642, Henry Schein). Originally the catheter was kept in place and hidden from the goats using elastikon stretch tape (Cat#000925, Henry Schein); after one week a Bandage-VetWrap 4'' was used for practicality. The catheter was flushed twice a day using heparinized saline (2 iu/mL). The day after catheter insertion, the goats receive- daily injections of 8 mg/kg of BW of 2,4-thiazolidinedione in 10 mL of saline (n=12; TZD; Cat# 375004, SigmaAldrich, USA) or 10 mL of sterile physiological

saline (n=12; CTR; VINV-SALN-1000, Henry Schein) throughout the whole study (20 days). The daily injection was performed at 12PM. The dose of TZD was decided upon the reported efficacy of 4 mg/kg BW in dairy cows (Smith et al., 2007) and the faster drug clearance typical of dairy goats (Lo et al., 1985) that requires typically doubling the dose of drugs for this species compared to bovine.

2.3 Intramammary infusion of Streptococcus uberis

In order to induce subclinical mastitis, after one week of TZD or saline injection half of the goats in each treatment received an intramammary infection (IMI) of $1.7x10^8$ *Streptococcus uberis* (*Strep. uberis*) in 10 mL sterile physiological saline (cat# VINV-SALN-1000, Henry Schein, USA) in each of the two mammary halves (the groups were named mastitis control or MCTR and mastitis TZD or MTZD) following the protocol by Lasagno et al. (2012). The remaining two groups received an intramammary infusion of 10 mL sterile physiological saline (the groups were named control or CTRL and control TZD or CTZD) in both halves. The pre-dosed aliquots of *Strep. uberis* in 1.5 mL sterile vials was provided by the laboratory of Peggy Dearing, College of Veterinary Medicine, Oregon State University. Prior to intramammary infusion, the teat ends were carefully cleaned with individual moistened towels and disinfected with swabs containing 70% ethanol. The infusion was performed with the aid of a disposable sterile urinary catheter (TomCat, USA). After IMI, each mammary half was thoroughly massaged upward into the gland cistern for 30 s to distribute the inoculation dose.

The *Strep. uberis* used in the present experiment was isolated from a mastitic cow by the laboratory of Peggy Dearing but the strain had not been determined. In order to determine the strain DNA was isolated from the bacteria using a DNA Clean & Concentrator-5 (cat# D4013,

Zymo Research, Tustin, CA) and submitted for Sanger sequencing at the Center for Genome Research and Biocomputing at Oregon State University. The sequencing result was blasted against the *Strep. uberis* strain 0140J genome using the BLASTN 2.4.0 at National Center for Biotechnology Information. The sequenced had 89% identity with *Strep. uberis* strain 0140J (Appendix I.).

2.4 Measurements and sample collection

Milk was collected aseptically from both halves of the mammary for bacterial analysis at the time of purchase, 3 days prior IMI, just before IMI, and 24 h post-IMI. Before sampling, the teat was treated with teat dipping solution and cleaned using disposable paper towels. The orifice of the teat was disinfected with swabs containing 70% ethanol and approx. 1 mL of milk was collected in a sterile 1.5 mL tubes. The samples were immediately put on ice and shipped within 4 hours to the Ag Health Laboratories, Inc (Sunnyside, WA) for a bacterial culture blood agar plate. All the samples were negative prior IMI. To ascertain SCC status of the mammary gland a California Mastitis Test was performed at the last milking prior IMI.

Milk yield was recorded daily throughout the experiment. Milk samples were collected for components analysis 5 days prior to treatment assignment and then the day before starting TZD injection, the day before IMI and 1, 2, 3, 5, 7, and 12d post-IMI. Samples were shipped with a preservative (Bronopol) to the Willamette National Dairy Herd Information Association (Salem, OR) to measure somatic cell count (SCC), lactose, fat and protein. The energy corrected milk (ECM) was calculated using the equation (0.327 × kg of milk) + (12.95 × kg of fat) + (7.65 × kg of protein) (Hultquist and Casper, 2016).

Rectal temperature was checked daily using a rectal thermometer prior to and after IMI and every hour during the first 6h post-IMI and approx. every 8 h until 114h post-IMI. Body weights (BW) was recorded weekly throughout the study.

Blood samples were collected prior to the morning feeding from the jugular vein using a 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ). The blood was collected just before starting the TZD injection (i.e., 7 days prior IMI or baseline), 2 days prior IMI (5 days into TZD injection), every day during the first 3 days post-IMI (i.e., 1d or 24h post-IMI, 2d, and 3d post-IMI) and at 6 and 12 d post-IMI. Samples were collected into evacuated tubes (10 mL, BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) containing either serum clot activator or sodium heparin. After blood collection, tubes containing sodium heparin were placed on ice, while the tubes with clot activator were kept at room temperature (approx. 30 min) until centrifugation by a C3 Select centrifuge (LW Scientific) and frozen at −20°C until analysis.

2.5 Blood Metabolites and inflammatory markers

Aliquots of plasma and serum were shipped in dry ice to the Istituto di Zootecnica, Universitá Cattolica del Sacro Cuore, Piacenza, Italy, for metabolic and inflammatory profiling. Blood samples were analyzed for 19 parameters. These included the metabolic parameters glucose, cholesterol, urea, calcium, magnesium, non-esterified fatty acids (NEFA), triacylglycerol (TAG), β-hydroxybutyric acid (BHBA), and retinol and the inflammatory-related parameters albumin, haptoglobin (Hp), ceruloplasmin, paraoxonase (PON), myeloperoxidase (MPO), total bilirubin, total reactive oxygen metabolites (ROMt), zinc, and tocopherol plus the liver enzyme gamma-glutamyl transferase (γGT). The analyses were performed using a clinical

auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA) and HPLC following procedures described previously (Bionaz et al., 2007, Trevisi et al., 2012).

2.6 Insulin, QUICKI, and RQUICKI

Concentration of insulin was analyzed using a commercial ELISA assay kit from NeoScientific (Cat# GTI0011) following the manufacturer instruction. The Quantitative Insulin Sensitivity Check Index (QUICKI; (Katz et al., 2000) and the Revised QUICKI (RQUICKI; (Holtenius and Holtenius, 2007)) were calculated as following:

$$QUIKI = 1 / [\log(\text{fasting insulin } \mu U/mL) + \log(\text{fasting glucose mg/dL})]$$

$$RQUIKI = 1 / [\log(\text{fasting insulin } \mu U/mL) + \log(\text{fasting glucose mg/dL}) + \log(\text{NEFA mmol/l})]$$

2.7 Phagocytosis and % granulocytes

The phagocytic capacity of leukocytes isolated from 100 μL heparinized whole blood was determined using Phagotest kit (Glycotope Biotechnology, Heidelberg, Germany) following the manufacturer's instructions but using half of the amount of each reagent. The use of half of each reagent had approximately 5% consistent reduction of phagocytosis (Figure S1). The % granulocyte was assessed by using side scatter and forward scatter after gating on eukaryotic cells on FL2 (i.e., DNA staining solution or nucleated cells; Figure S2). Percentage phagocytosis on all leukocytes, polymorphonucleated cells (PMN), and monocytes were determined.

2.8 Adipose biopsy

Subcutaneous adipose tissue was collected through biopsy procedure from alternate sides of the tail-head 1 d before and 7 d after IMI. The biopsy was performed as previously described (Schmitt et al., 2011) with modifications. Briefly, the clipped tail-head area was thoroughly scrubbed with povidone scrub (Henry Schein Inc., USA) and 2% lidocaine (Henry Schein Inc.,

USA) was injected subcutaneously on the area of incision. A 4 to 5-cm incision was made to expose the tissue. This was collected using sterile forceps and scalpel blade or Metzenbaum scissors. The incision was closed using non-absorbable suture. Biopsied tissue was placed in a sterile petri dish containing gauze sprayed with RNAse Zap (ThermoFisher, USA). Connective tissue and large vessels were dissected out using a sterile scalpel blade and the tissue was washed using a sterile physiological saline solution with the aid of a disposable sterile syringe. The cleaned tissue was dissected in 3 pieces. Two pieces were transferred to 2.0 ml self-standing cryovials (cat#, 26-201, Genesee Scientific, USA) and put in a foam box with dry ice for transport to the laboratory and stored at -80°C until analyses. The other piece was put in a 1.5 mL tube containing 10% neutral buffered formalin (#16004-126, VWR, USA) to be fixed for histological analysis.

2.9 Mammary epithelial cells isolation

The mammary epithelial cells (MEC) were isolated from 50 mL of milk using magnetic sorting. The milk sample was collected in 50 mL sterile tubes (cat# 89004-364, VWR, USA) and immediately preserved on ice until isolation (approx. 1h later). Tubes were centrifuged at 1,000×g at 4°C for 10 min to separate the fat and pellet the cells. Cells were washed twice with 10 mL of sterile PBS and centrifuged (500×g at 4°C for 5 min). Before the last wash the cells were counted using a MOXI Z Mini Automated Cell Counter (Orflo Technologies, USA). The final pellet was re-suspended in 500 μL of a PBS solution plus 0.1% bovine albumin and transferred in a 1.5 mL tube pre-wetted with the PBS+0.1% albumin solution. An antibody against the epithelial-specific marker mucin 1 (NBP1-60046, Novus Biologicals, USA)(Stingl et al., 2001) conjugated with Pierce Protein AG Magnetic Beads (ThermoFisher, USA) was added (1 μL/10^6 cells). Samples were incubated for 30 min in ice in a shaker and cells were washed

once using PBS and immediately isolated using an autoMACS separator (Miltenyi Biotec, USA). The positive and the negative separated cells were stored at -80°C until RNA extraction. The evaluation of the enrichment of mammary epithelial cells was performed on 5 positive and 5 negative cells from 5 random milk samples measuring expression of casein kappa (*CSN3*) and lactalbumin (*LALBA*) on MUC1 positive and MUC1 negative cells. Overall, the use of MUC1 antibody tended to enrich for cells expressing higher amount of mammary-specific genes casein κ (*CSN3*) and lactalbumin (*LALBA*) plus mucin 1 (*MUC1*) (Figure S3).

2.10 RNA isolation and Reverse Transcription quantitative Polymerase Chain Reaction (RTqPCR)

RNA extraction was performed using RNA Clean & Concentrator-5 (cat# R1013, Zymo Research, USA) following the vendor protocol. Prior to RNA extraction, the adipose tissue was disrupted using a Bullet Blender Next Advance (Laboratory Instruments, USA). RNA was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed using a 2100 Bioanalyzer Instrument (Agilent, USA) by the Center for Genome Research and Biocomputing at Oregon State University. For the MEC the 260/280 ratio was 1.9±0.4 (mean±SD) and the RIN was 4.7±2.1, with a range from 1 to 9.2. For the adipose tissue the 260/280 ratio was 1.8±0.2 (mean±SD) and the RIN was 4.0±2.0, with a range from 1 to 9.0.

Primers were designed and tested using Primer Express 3 as previously described (Bionaz and Loor, 2007) (Table 4) with modifications. Briefly, *Capra hircus* specific sequences were searched in the National Center for Biotechnology Information (NCBI at https://www.ncbi.nlm.nih.gov/) and blasted against the sheep genome in the University of California Santa Cruz Genome Browser (https://genome.ucsc.edu/) in order to determine the

exon-exon junctions. Primer-pairs were blasted using NCBI BLASTN tool. Amplicon from each primer-pair was cleaned using DNA Clean & Concentrator™-5 (Zymo Research. USA) and sent to the Center for Genome Research and Biocomputing at Oregon State University for sequencing. Results of sequencing are available in Table 5.

Despite the low RIN numbers we proceeded with the RT-qPCR analysis for all the internal control genes. Six potential internal control genes (ICG) were tested [i.e., glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S9 (*RPS9*), ubiquitously-expressed transcript (*UXT*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*), glucose-6-phosphate dehydrogenase (*G6PD*) and mitochondrial ribosomal protein L39 (*MRPL39*)] based on prior publications (Bionaz and Loor, 2007, Cremonesi et al., 2012) using geNorm (Vandesompele et al., 2002). For adipose tissue a more reliable normalization factor was obtained (V-value = 0.227) by using 5 ICG (all the ICG tested except *MRPL39*). For the MEC 3 ICG (*GAPDH*, *RPS9*, and *YWHAZ*; V-value = 0.264) were used to calculate the normalization factor. Target transcripts measured were related to lipid synthesis, PPARγ activation, and inflammation. In particular we measured in both MEC and adipose tissue the abundance of PPARγ transcript (*PPARG*) and the PPARγ putative targets Lipoprotein Lipase (*LPL*) and Fatty Acid Synthase (*FASN*). In adipose tissue we also measured transcription of Acetyl-CoA Carboxylase Alpha (*ACACA*), Sterol Regulatory Element Binding Transcription Factor 1 (*SREBF1*), and Tumor Necrosis Factor alpha (*TNFA*). For MEC we measured transcription of *MUC1*, Interleukin 8 (*IL8*), and Chemokine (C-C Motif) Ligand 2 (*CCL2*). The RT-qPCR analysis was performed as previously described (Bionaz and Loor, 2007) with some modifications. Briefly, RevertAid (ThermoFisher, USA) was used as reverse transcriptase following manufacturer's indication and Power SYBR Green Master Mix

(ThermoFisher, USA) was used for the qPCR. The PCR reaction was performed in a 7900HT (Applied Biosystems, USA) in MicroAmp® Optical 384-Well Reaction Plate (Applied Biosystems, USA). The reaction was as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15s at 95°C followed by 1 min at 60°C. A dissociation curve was performed (gradient from 95C to 60°C to 95°C) to check for amplicon quality. Final qPCR data were obtained by using a 6-point 2-fold dilution standard curve. The RT-qPCR was performed following MIQE guidelines (Bustin et al., 2009).

Table 4. Features of the primers pairs used to analyze gene expression by RTqPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.	bp[1]
ACACA	GACCTGCTTCAATCCAAGAGGTT	CAAGCTACCATGCCAATCTCATT	XM_005693156	200
CCL2	AAATTCAATAAGAAGATCCCCATACAG	CCTAGGATGGTCTTGAAAATCACA	XM_005693218	100
CSN3	GAGCTGACGGTCACAAGGAAA	CTGGGCACCCAAAAATGG	NM_001285587	90
FASN	GGTGAACTGTCTCCGGAAAGAG	TCGGGCTTGTCTTGTTCCA	NM_001285629	100
GAPDH	CCATCTTCCAGGAGCGAGATC	CCAGCCTTCTCCATGGTAGTG	XM_005680968	100
IL8	CAACGGAAAAGAGGTGTGCTTA	GGATCTTGCTTCTCAGCTCTCTTC	XM_005681749	90
LALBA	GAATTAACTACTGGTTGGCCCATAA	CAGAAAGAGGACAGAAGCAGCAA	NM_001285635	90
LPL	CTTGGAGATGTGGACCAGCTAGT	GGTACGCCTTACTTGGATTTTCTTC	NM_001285607	100
MRPL39	CAGTATGAAGTGTCAGCGGTTCA	AATGTGCTCTTAAGTGTGCAGGTAGA	XM_005674737.1	100
MUC1	TCTTTTCGCATTATAAACCTCCAGTT	GCAAAATCACATCCAAAATGCTT	XM_005677405.1	100
PPARG	TCCGCTCCGCACTACGA	TGATTGCACTTTGGTACTCTTGGA	NM_001285658.1	100
RPS9	AGGTCTGGAGGGTCAAATTCAC	CAGGGCATTACCTTCGAACAG	XM_005709411	100
SCD1	GGCGTTCCAGAATGACGTTT	AAAGCCACGTCGGGAATTG	NM_001285619	100
SREBF1	ACCGACATAGAAGACATGCTTCAG	GCCTTCAAGTGAGGAGCTCATC	NM_001285755	100
TNFA	CTCTTCTGCCTGCTGCACTTC	AAGATGACCTGAGTGTCTGAACCA100	NM_001286442	100
UXT	AGCGGGACTTGCAAAAGGT	AGCTTCCTGGAGTCGCTCAA	XM_005700842	100
YWHAZ	TGGTGATGACAAGAAAGGGATTG	TCTGATAGGATGTGTTGGTTGCA	XM_005689196.1	100

[1]Amplicon size in base pairs (bp).

Table 5. Sequencing results of PCR amplicon obtained from the primer-pairs used for this experiment.

Gene	Sequence
CCL2	GAGAGTCACCACCAGCAAGTGTCCCAAAGAAGCTGTGATTTTCAAGACCATCCTAGGA
SCD1	GGGATCGTGCACNACAAGTTTTCAGAAACGGATGCTGATCCCCACAATTCCCGACGTGGCATTACAG
MUC1	TTCTGAACCCAACCAGCTACTATCAGGAGCTGAAGAGAAGCATTTTGGATGTGATTTTGCA
PPARG	CTGACGATGGTTGCAGATTATAAGTATGACCTGAAGCTCCAAGAGTACCAAAGTGCAATCAA
FASN	CGGCGGATCGGTGCGTCCTGGTGTCTAACCTTAACAGCACGTCCCCCATCCCTGAGATAGCCCCGAAG TCCTTGGAGCTGCAGAAGGTGCTCCAGAGTGACCTGGTGATGAACGTCTACCGTGATGGGGCTTGGGG AGCATTCCGCCACTTCCTACTGGAACAAGACAAGCCCGAA
IL8	GCGAGTTGTGCAGGCATTTTTGAGAGAGCTGAGAAGCAAGATCC
ACACA	GGGTCNTAGGCACATACATATATGACATCCCAGAAATGTTTCGGCAGTCCCTGATCAAACTCTGGGAA TCTATGTCCTCTCAAGCATTCCTTCCACCGCCCCCTCTGCCTTCAGACATACTGACGTACACTGAGCTC GTGTTGGATGATCAAGGTCAACTGGTTCACATGAACAGGCTTCCAGGAGGAAATGAGATTGGCATGGT AGCTTG
SREBF-1	AGACGCGACTTACCGGGCCTGTTTGACCCGCCCTACGCTGGGGGTGGAGCAGGGACCACAGACCCTGC CAGTCCCGATGTCAGCTCCCCAGGCAGCCTGTCCCCACCTCCTTCCACGATGAGCTCCTCACTTGAAGG CAAA
LPL	GGCAGCGTTCGTTCTCTCTTGATTGACTCTCTGTTGAATGAAGAAAATCCAAGTAAGGCGTACAGNG
LALBA	TGGTGAGAAGCCATAGTACAAACAATGACAGCACAGAATATGGACTCTATCA
CSN3	GAAATTTTCTAGTGTGACTATCCTGGCATTAACCCTGCCATTTTTGGGTGNCCAGAAA
YWHAZ	GTTCAGCAGCATACCAAGAAGCTTTTGAATCAGCAAAAAGGAAATGCAACCAACACATCCTATCAGA ACCNT

2.11 Histological analysis of the adipose tissue

Adipose tissue samples preserved in 10% neutral buffered formalin were immersed in 15% and then in 30% sucrose diluted in PBS before cutting in cryostat sections of 15-µm-thick at a temperature of -27°C. Prior to H&E staining, sections were washed in PBS buffer followed by a rinsed in water for 30 s. Next, sections were incubated with Harris' hematoxylin (VWR, US, Cat. No. 95057-858) solution for 3 min and rinsed afterwards with warm water for 25 s. The sections were submerged in eosin solution and rinsed with PBS for 1 min. Tissue was washed in 80% ethanol for 1 min and finally immersed in xylene for 10 s and imaged with a 10× magnification objective using a Leica DM6000 microscope (Leica Microsystems Inc., IL, USA). The analysis of adipocytes size was performed using CellProfiler 2.1.1 software

(http://www.cellprofiler.org) (Kamentsky et al., 2006). Besides average area of adipocytes, the frequency of increased range of area and diameters of adipocytes was measured to estimate the formation of new adipocytes (i.e., small size) and formation of large adipocytes, i.e., accumulating large amount of stored triglycerides.

2.12 Statistical analysis

Prior to statistical analysis data were checked for outliers using PROC REG of SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). Data with a studentized t >3.0 were removed. No normally distributed data was assessed using PROC UNIVARIATE of SAS. Data with a Shapiro-Wilk test with P<0.01 and statistic <0.90 and a Kolmogorov-Smirnov P<0.01 were considered non normally- distributed and were log- transformed prior to statistical analysis. Data were analyzed with the PROC GLIMMIX procedure of SAS 9.2. Fixed effects in the model were treatment (i.e., 2,4-TZD or Z), time (T), mastitis (M), with all interactions as fixed effects and goat was considered as random effect. Due to the irregular timeline of each measurement, the covariance structure SP (POW) for repeated measures was used for analysis. Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

In order to account and correct for differences between groups at baseline (for blood parameters and milk yield was -7 day relative of IMI [d] and for milk composition was -8 d), a statistical analysis of Z, M, and Z×M was performed for the milk and blood metabolic parameters. If a difference with P<0.2 in any of the parameters analyzed was observed, data for each sample were corrected arithmetically to obtain the same average between groups at baseline. This was performed by adding to each sample the difference between the averages at baseline between the group of that sample and the CTRL group (i.e., all data were corrected by the CTRL group). This approach was chosen compared to the classical co-variate at baseline in

order to have the average-corrected data with standard error at baseline for each group. The corrected dataset was used for the statistical analysis.

The statistical analysis of overall mean adipocytes size was performed using the above model. In order to evaluate the effect in each range of adipocyte areas or diameters, the statistical analysis using the above model was performed for each range. When range and time were combined, the model included TRT, Mastitis, Time, and Range an all interactions as main effects.

3 Results

Two animals in the MTZD group were removed from the study because they ceased to produce milk after IMI and piked a fever (>41°C) compared to the other animals. For this reason we had only n=4 for the MTZD groups. The effect however was not due to an interaction between TZD injection and IMI because in a follow-up study performed in the same animals to validate this effect, 2 out of 6 animals in the control group ceased milk production after IMI while all of the TZD-treated animals continued to milk (data not shown). The reason for agalactia in these two animals in TZD group remains unclear. Five days post-IMI a few goats had issues with the catheter (either the catheter was dislodged or was chewed by other goats) and needed to be removed. In order to keep all the treatments consistent we removed the catheter from all the animals and we injected the 2,4-TZD directly into the jugular vein until the end of the trial.

3.1 Rectal temperature and body weight

The rectal temperature overall declined in the first 5 hour post-IMI in all groups but increased significantly afterwards until 44 hours post-IMI especially in the animals treated with *Strep. uberis* (Figure 9). The rectal temperature tended to remain higher in the MCTR group compared to the other groups until it reached a significantly higher values compared to all the other groups at 114h post-IMI (Figure 9). The MTZD group returned to temperature similar to the CTRL and CTZD groups within 68 h post-IMI. Overall the CTZD group had a more stable and lower temperature compared to all the other groups, including CTRL especially at 44 h post-IMI where a significant increase in body temperature was observed for the CTRL animals. Body weight was not affected by TZD or mastitis (Figure 10).

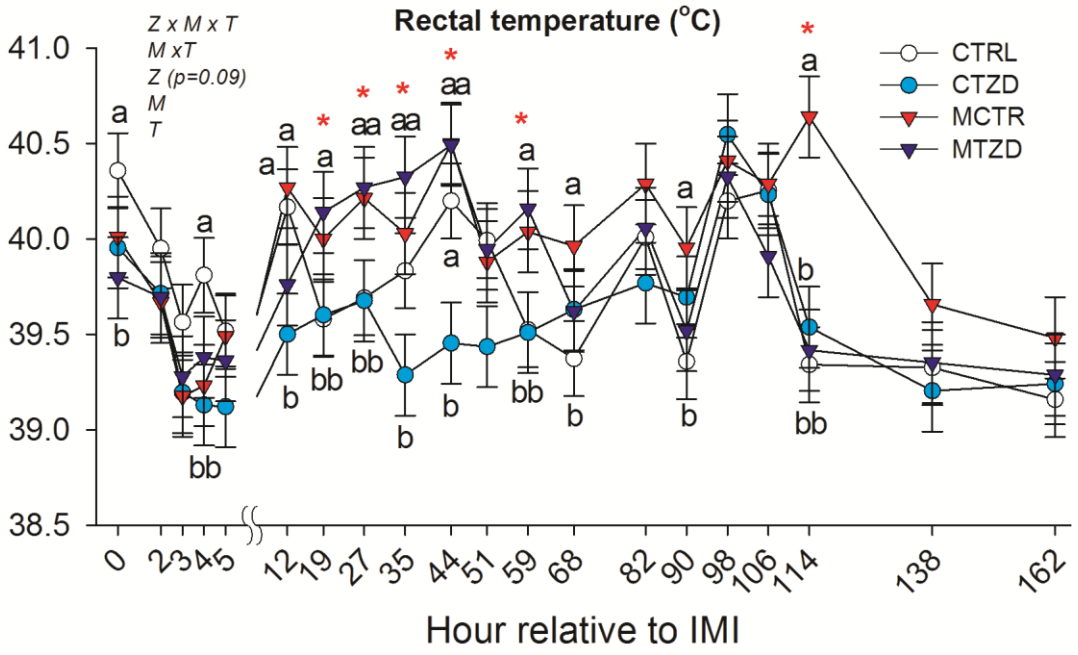

Figure 9. Rectal temperature measurements from 2 to 162 hours and milk production (Kd/day) and somatic cell count (SCC) from -8 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, time 0, d 0), during daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) are denoted with *.

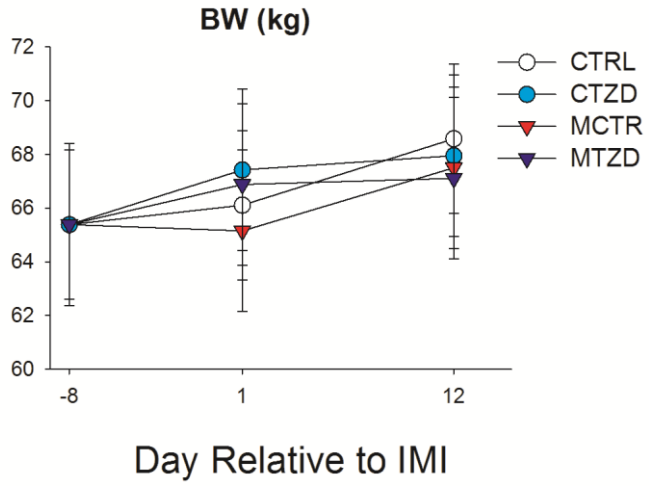

Figure 10. Body weight (BW) measurement from -8 to 12 d relative to intramammary infusion of Streptococcus Uberis (IMI, d 0), during daily 2,4-thiazolidinedione (TZD) or saline i.v. injections.

3.2 *Milk yield and composition*

SCC were overall significantly higher in the groups receiving IMI (i.e., MTZD and MCTR) compared to the groups receiving intramammary infusion of saline at the beginning of the trial, but the differences disappeared just before IMI (Figure 11). Due to IMI the MTZD and MCTR groups had a significant larger increase in SCC (12.5 and 9.7-fold, respectively from -1 to 2 day post-IMI) than the control groups (1.3 for CTRL and 1-fold for CTZD) (Figure 11). The concentration of SCC remained larger in MCTR and MTZD compared to CTRL and CTZD until the end of the trial (Figure 11). A tendency for a Z×M×T was detected due to an overall lower SCC for the animals receiving TZD, especially for the CTZD group during the first 2 days post-IMI.

The milk yield at baseline was 1.12±0.35 kg/d. A significant effect of M×T was detected due to an overall decrease of milk yield by the goats treated with *Strep. uberis* compared to goats receiving intramammary saline (Figure 11). A tendency for the interaction Z×M×T (P = 0.08) was detected on milk yield (Figure 11). *Strep. uberis* intramammary infusion decreased milk yield in the MCTR group, while the MTZD had only a non-significant numerical decrease in milk yield.

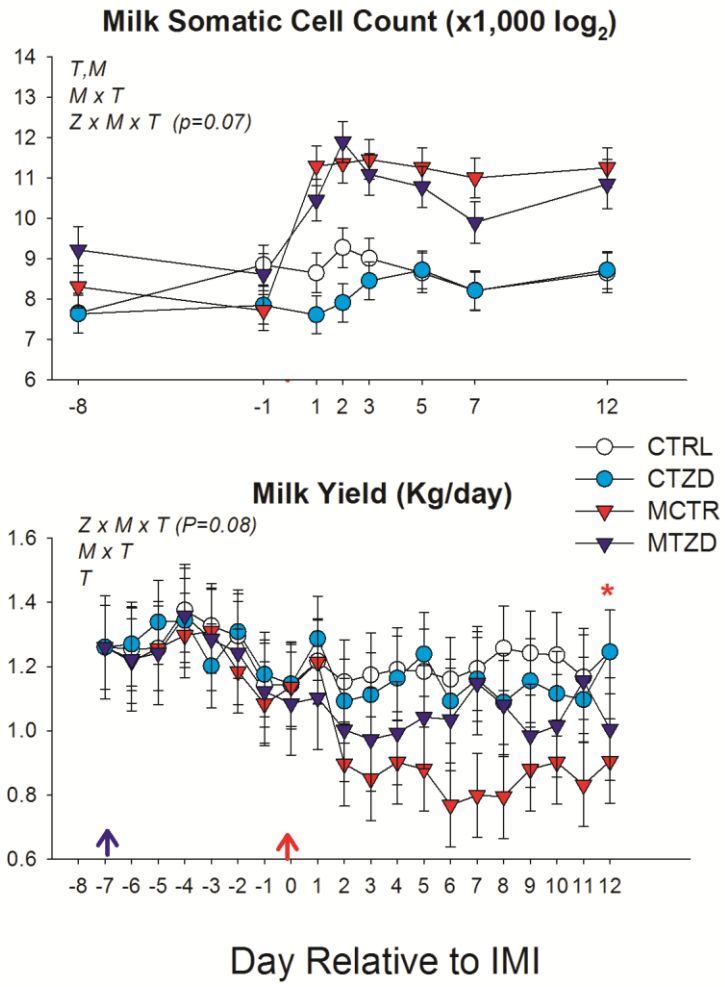

Figure 11. Somatic cell count (cells/mL) and milk yield measurements from -8 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) are denoted with *.

Despite the significant difference observed at -8d, which would have prompted us to correct the data based on the criteria utilized in the present work, we decided to show the original SCC data, due to the practical importance of the SCC data for the farmers; however, when the data were adjusted at -8 d, the MCTR had an overall larger increase in SCC (12.5-fold from -1 to 2 days post-IMI) compared to all the other groups (<1.4-fold), including MTZD (10.5-fold) (Figure 12).

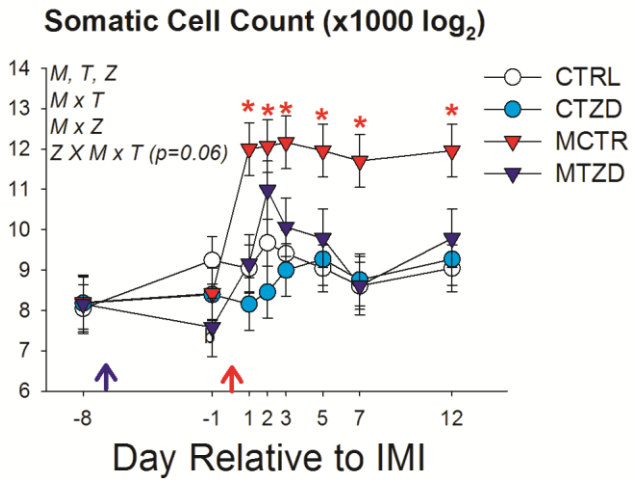

Figure 12. Measurements of somatic cell count (SCC; cells/mL) corrected (-8d) from -8 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, d 0), during daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) are denoted with *.

The other milk parameters (Figure 13) were not affected by IMI or TZD with the exception of % protein, which was affected by the interaction of M×T (driven by the higher SCC) and % of milk lactose which was higher for TZD treated animals after IMI (Figure 13). Even though not statistically significant, the milk fat yield was numerically lower in MCTR after IMI (Figure 13). The ECM was not significantly affected by treatments but a tendency for M×Z×T was detected due to a decrease in ECM in MCTR but not in the other groups (Figure 14).

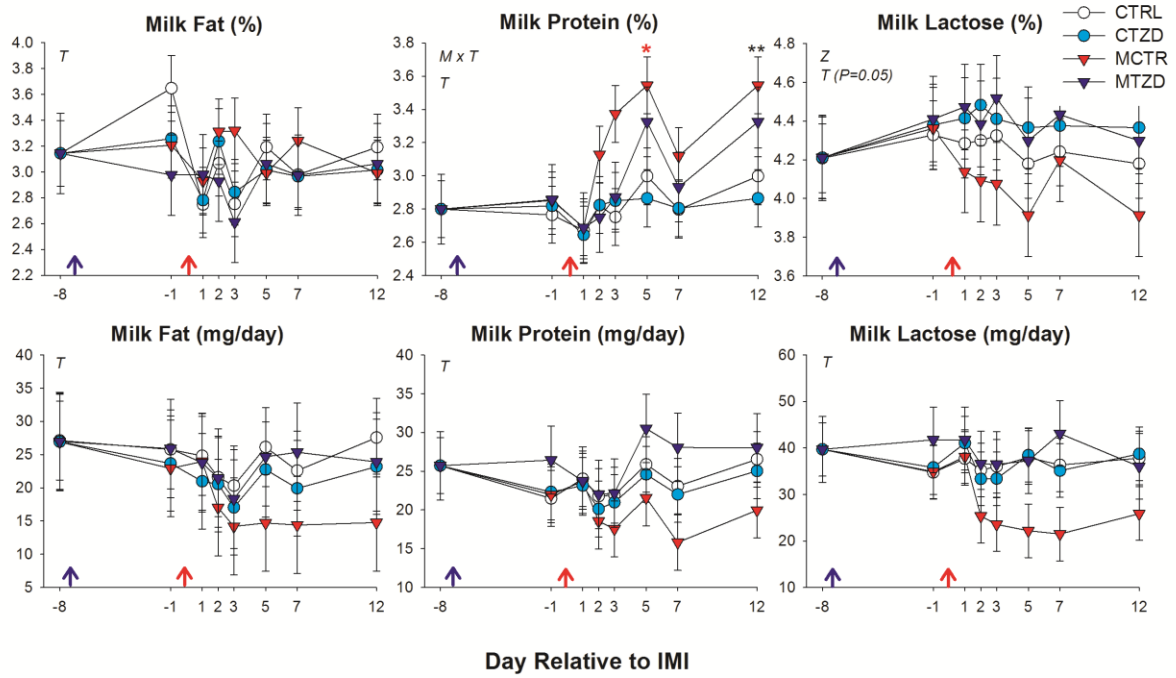

Figure 13. Milk parameters from -8 to 12 d relative to intramammary infusion of Streptococcus Uberis (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) are denoted with *****.

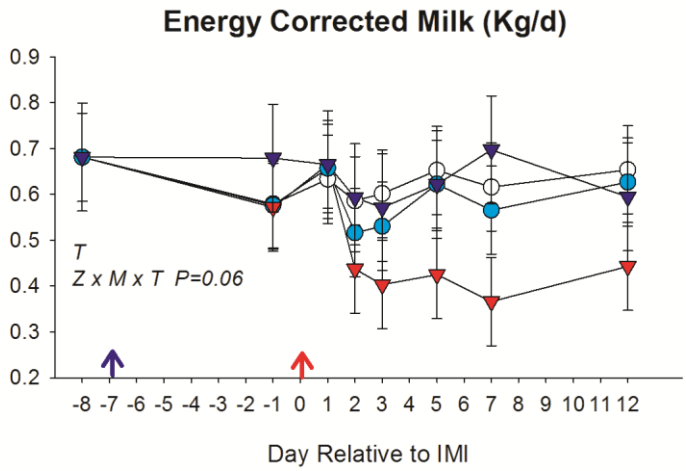

Figure 14. Energy corrected milk measured from from -8 to 12 d relative to intramammary infusion of Streptococcus Uberis (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections.

3.3 Blood metabolic parameters

Glucose concentration was not affected by TZD but overall higher glucose was detected after IMI in animals receiving *Strep. uberis* (Figure 15), mostly due to a large increase in glycemia in MCTR group while the group MTZD did not have any difference from the non-mastitis groups (Figure 15). We observed an overall increase in NEFA due to IMI in all groups except CTZD (Figure 15). Overall NEFA tended to be lower in TZD-treated animals. The BHBA was affected by time and by Z×T and had a tendency (P=0.07) for a full interaction. All animals had a decrease in BHBA after IMI and an overall lower value for TZD was observed at 12 d post-IMI. The concentration of triacylglycerol (TG) was not affected by TZD or Z×T but tended to be higher (P = 0.06) in mastitis treated animal post-IMI. A trend (P=0.09) for TZD, mastitis, and full interaction Z×M×T resulted from MTZD group with a greater urea concentration than the other groups on d 1, 6 , and 12 post-IMI (Figure 16). After IMI in all groups α-tocopherol decreased significantly with no difference between groups (Figure 16). Administration of TZD had no effect on plasma insulin concentrations, QUIKI, or RQUIKI, although a numerically larger QUIKI was detected for TZD-treated vs. control goats (Figure 17). A significant effect of mastitis on insulin detected by the statistical analysis was also observed prior IMI; thus, it cannot be considered biologically relevant.

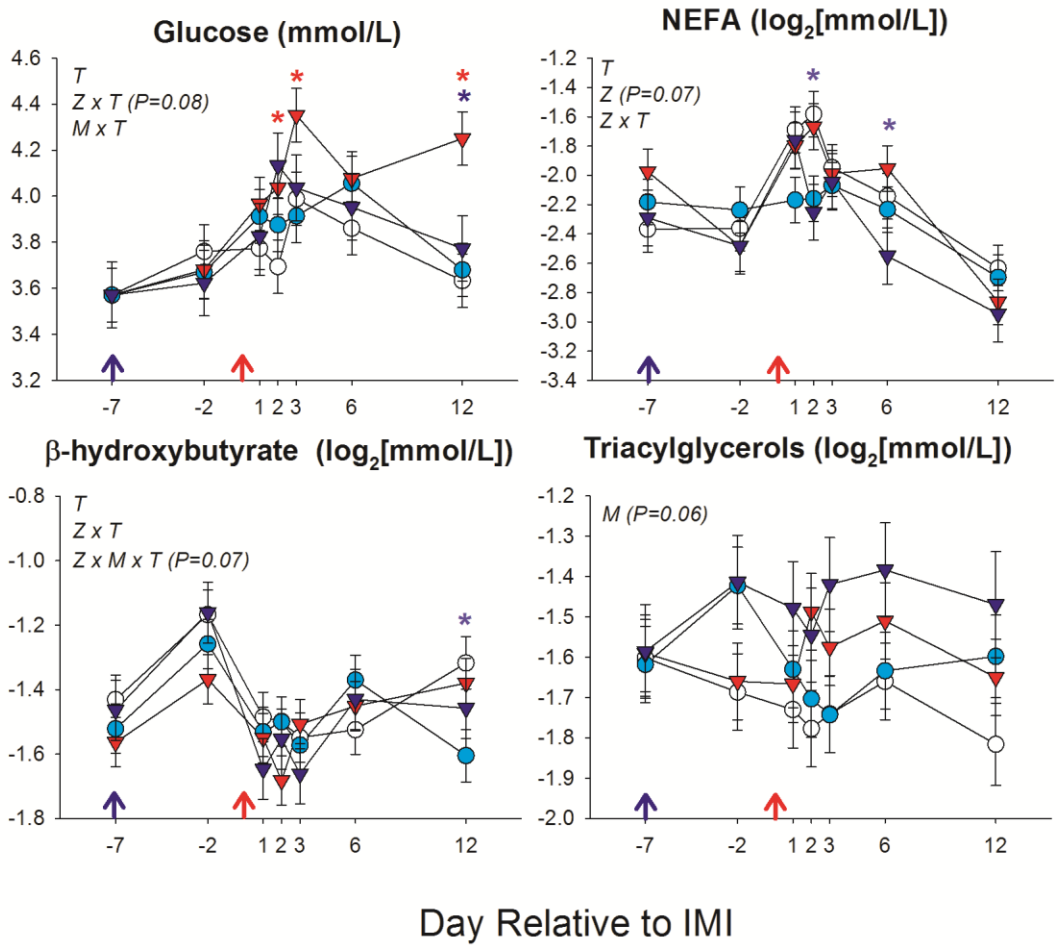

Figure 15. Plasma concentration of blood metabolic parameters from -7 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) and TZD x time (Z x T) are denoted with * and *, respectively.

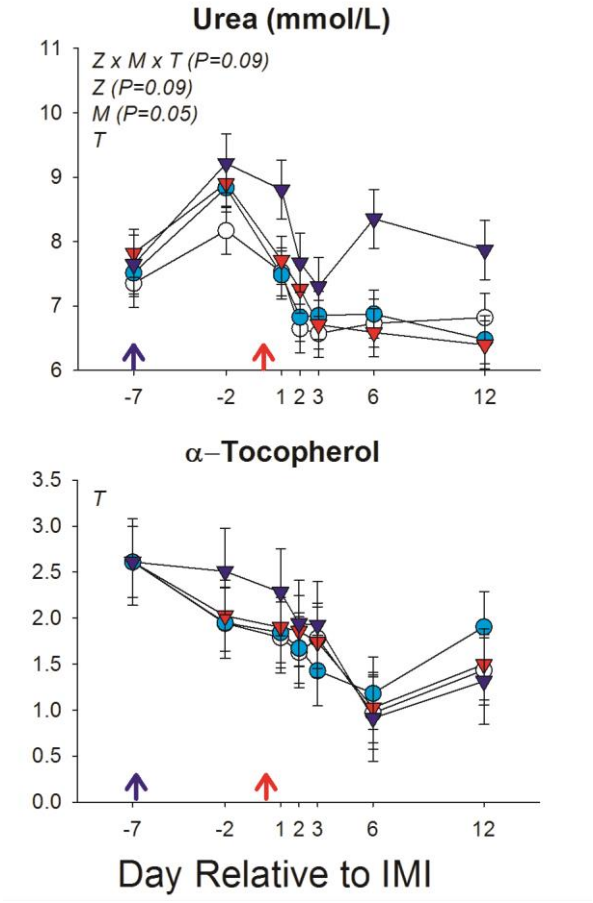

Figure 16. Plasma concentration of urea and a-tocopherol from -7 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) and TZD x time (Z x T) are denoted with * and *, respectively.

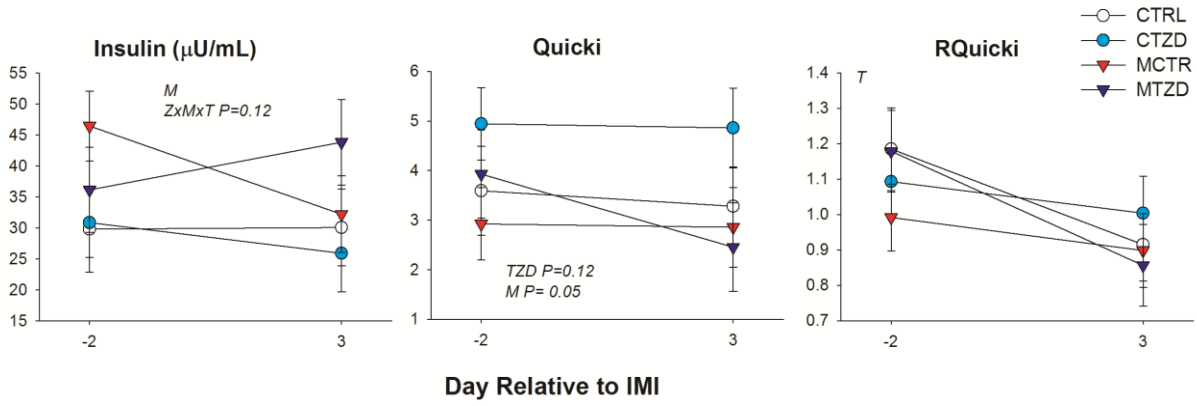

Figure 17. Plasma concentration of insulin plus Quantitative Insulin Sensitivity Check Index (QUICKI) and the Revised QUICKI (RQUICKI) before (-2 d) and after (3 d) intramammary infusion (IMI) of *Streptococcus Uberis* (d 0) during TZD administration in lactating dairy goats.

3.4 Blood minerals

The Ca^{+2} was overall increased in TZD-treated goats prior to IMI and tended to remain higher compared to the non TZD-treated goats after IMI mainly due to higher Ca in MTZD goats (Figure 18). The tendency for higher Ca in goats receiving *Strep. uberis* is likely driven by the higher Ca in MTZD goats (Figure 18). The concentration of Zn tended (P = 0.08) to be higher in TZD-treated compared to non TZD-treated goats and was overall higher in the former compared to the latter after IMI (Figure 18). Zn concentration in blood did not change during the first 3 days post-IMI and increased thereafter in all animals. Zn concentration was higher in MTZD compared to the other groups at d -2 and 6 relative to IMI but the Zn was higher in CTZD animals at 12 d post-IMI. As for the Ca, the observed pattern of Zn in blood was mostly driven by MTZD group. Mg^{+2} level decreased after IMI but not differences between groups were detected (Figure 18).

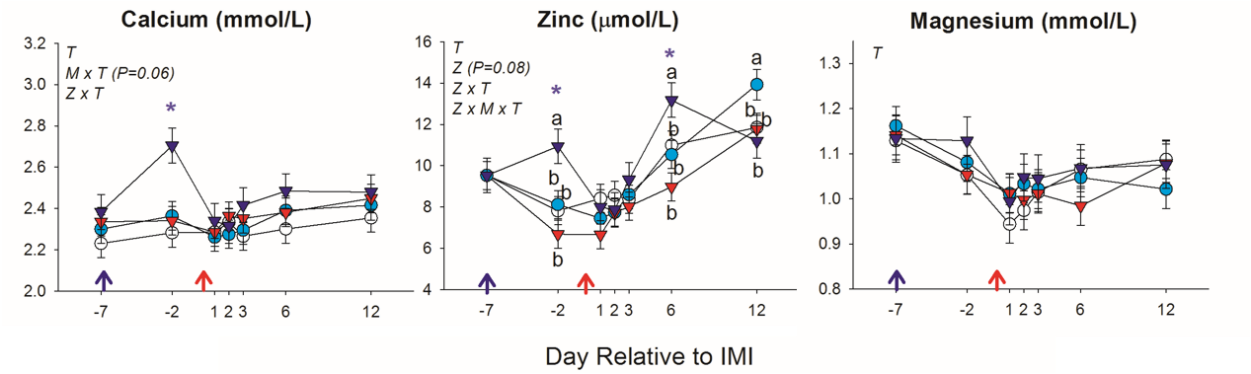

Figure 18. Plasma concentration of blood minerals from -7 to 12 d relative to intramammary infusion of *Streptococcus Uberis* (IMI, d 0). At d -7 started daily 2,4-thiazolidinedione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) and TZD x time (Z x T) are denoted with * and *, respectively.

3.5 Inflammation markers and liver stress

The level of the positive acute phase protein (+APP) haptoglobin was 0.50±0.47 g/L (mean±SD) prior IMI with a significantly lower overall level in TZD treated vs. control animals after 1 week of TZD treatment (Figure 19). Haptoglobin level rose rapidly after IMI in all

animals, reaching values >2 g/L with an overall larger persistence of high levels in *Strep. uberis* treated animals, mostly due to MCTR group (Figure 19).

The inflammatory marker and marker of neutrophils killing capacity myeloperoxidase was overall lower in TZD-treated compared to control goats after 1 week of TZD treatment but increased significantly in TZD treated goats after IMI and remained higher compared to control groups until the end of the experiment (Figure 19). The CTZD had an overall larger myeloperoxidase compared to the other groups. The liver stress marker γGT was not different between groups prior IMI but was overall higher in TZD-treated vs. control animals after IMI. This was mostly due to an increase of the parameter in CTZD 6 d post-IMI (Figure 19).

Among the negative acute phase protein (-APP) only albumin was affected by time while total cholesterol decreased after IMI in non-TZD treated groups with significantly lower values compared to TZD-treated goats at 6 d post-IMI (Figure 19). Retinol tended to be affected by M×T and Z×T interactions mostly due to a tendency for higher values in MTZD at -2d post-IMI and lower value in MCTR at 6 d post-IMI compared to the other groups (Figure 19). In contrast, the activity of the negative acute phase protein and antioxidant paraoxonase and the total bilirubin, as an index of liver clearance capability, decreased in all groups after IMI without being affected by TZD treatment (Figure 20).

The other measured +APP, ceruloplasmin, was increased over time after IMI in all groups without being significantly affected by TZD or IMI (Figure 20).

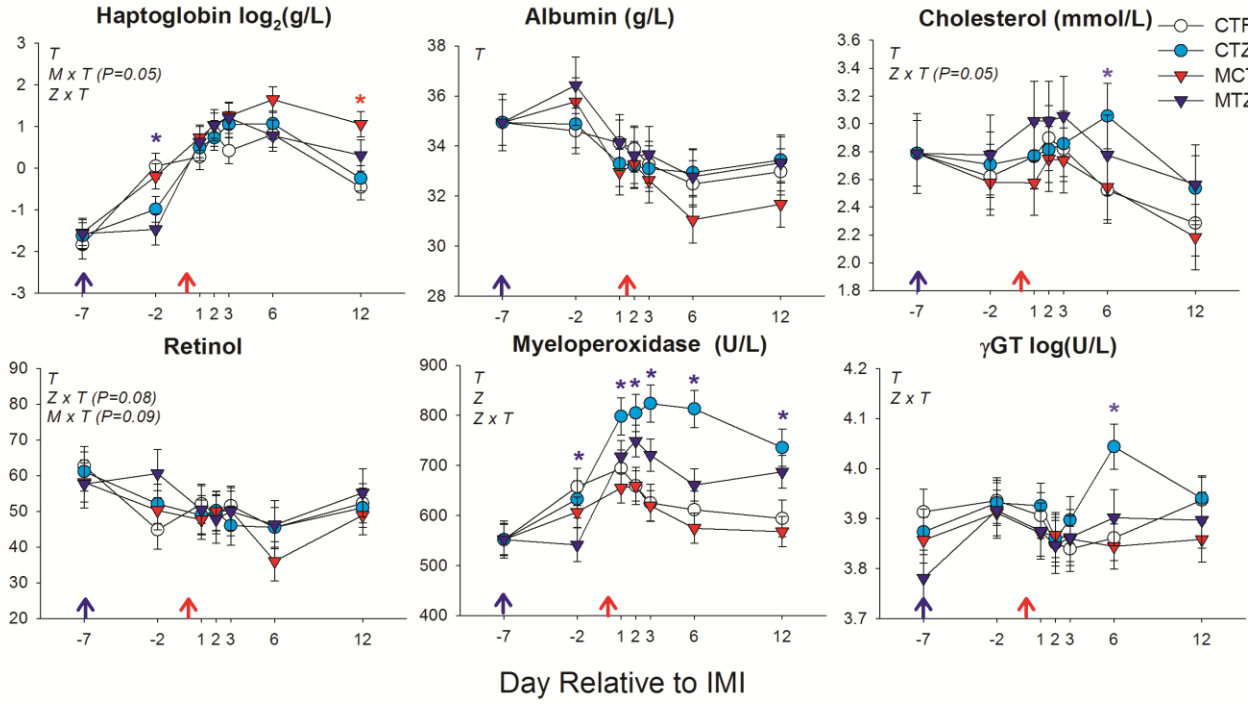

Figure 19. Plasma concentration of blood inflammatory markers, including the positive acute phase protein haptoglobin, the negative acute phase protein albumin and the negative acute phase protein index total cholesterol and plasma concentration of neutrophils killing capacity marker myeloperoxidase and phagocytosis % by neutrophils from -7 to 12 d relative to intramammary infusion (IMI) of *Streptococcus Uberis* (d 0).At d -7 started daily 2,4-thiazolodinidenione (TZD) or saline i.v. injections. Significant differences between mastitis × time (M x T) and TZD x time (Z x T) are denoted with * and *, respectively.

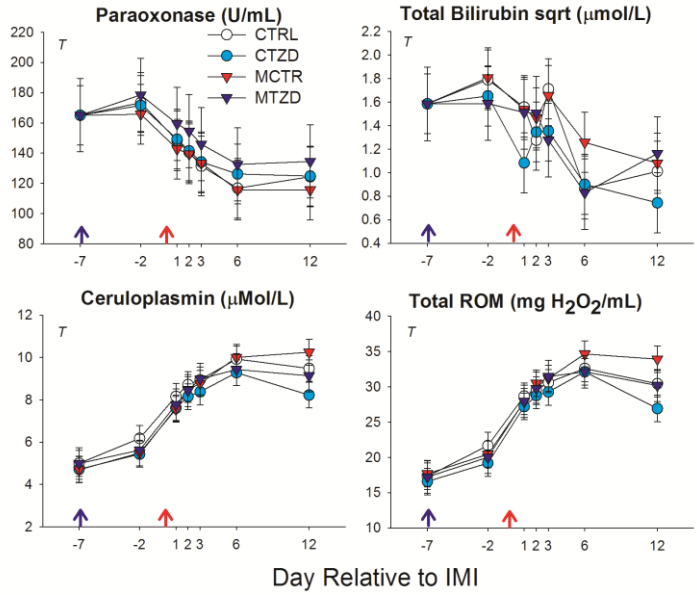

Figure 20. Plasma concentration of blood inflammatory markers -7 to 12 d relative to intramammary infusion (IMI) of *Streptococcus Uberis* (d 0).At d -7 started daily 2,4-thiazolodinidenione (TZD) or saline i.v. injections.

3.6 % PMN and phagocytosis in blood

The % PMN was 62.5±1.7 (mean±SEM) before IMI but was not different between groups. After IMI the % PMN was overall decreased in all groups (Figure 21). The goats treated with *Strep. uberis* had a larger decrease in proportion of granulocytes compared to the goats receiving intramammary saline (Figure 21). The % phagocytosis of all leukocytes and of PMN was only affected by time but was not affected by TZD or mastitis while monocytes phagocytosis was reduced by mastitis (Figure 21).

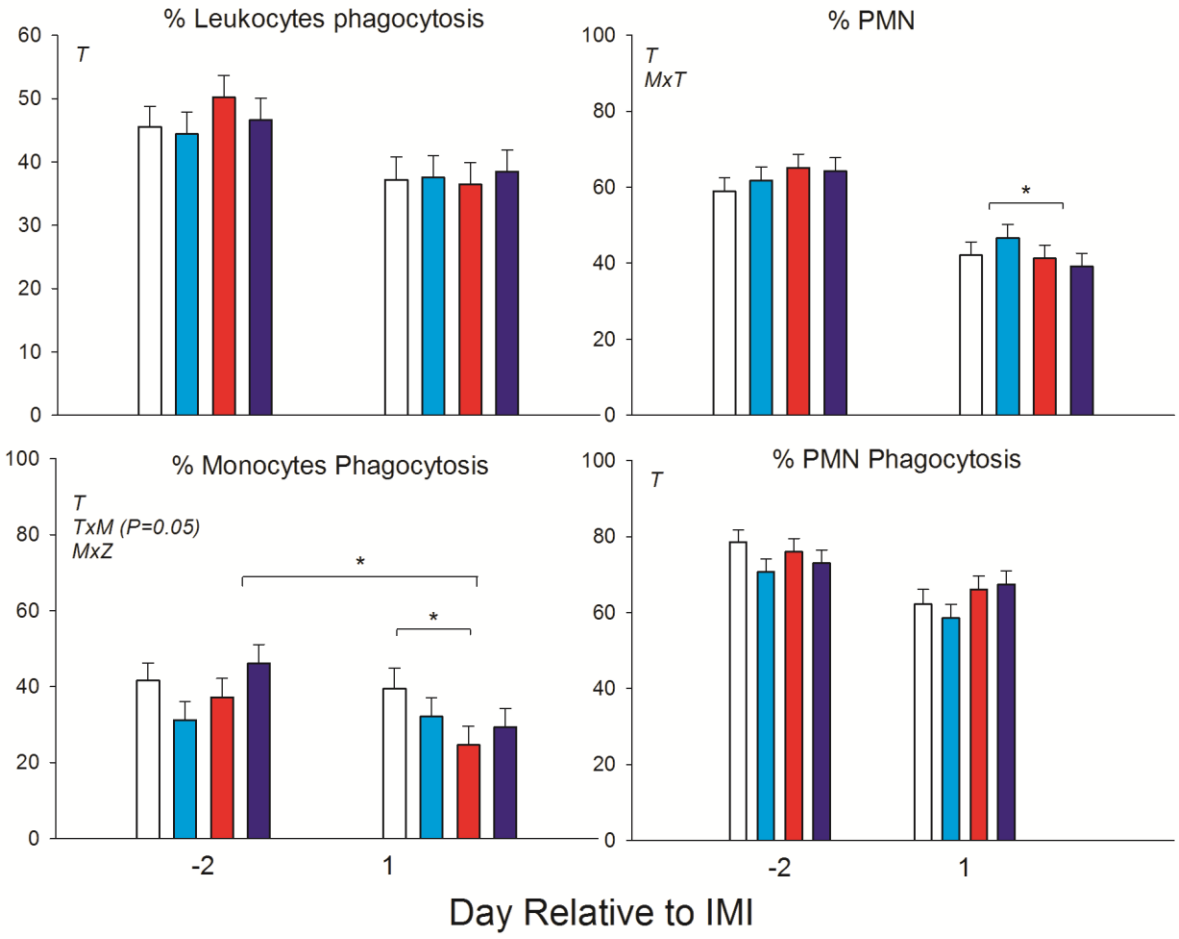

Figure 21. Percentage phagocytosis in 100 µL whole blood obtained from one lactating goat using the recommended (1x reagent) or half (0.5x reagents) of amount of reagents using the Phagotest kit (Glycotope, Germany).

3.7 Gene Expression

A negative correlation between Ct (or Cq) values and RIN has been reported in several publications (Becker et al., 2010, Fleige and Pfaffl, 2006). This is due to the decrease in abundance of integer mRNA (i.e., higher Ct values) in samples with higher degradation (i.e., lower RIN). A RIN≥5 was suggested as adequate for RTqPCR analysis (Fleige and Pfaffl, 2006). Due to the large proportion of samples with a RIN<5 in both adipose tissue (60%) and MEC (50%), most of our samples would have been inadequate for RTqPCR. The reason for such a large proportion of samples with a low RIN is unclear, because samples were snap frozen in dry ice, kept at -80°C during storage, and in ice during analyses. Despite the low RIN, we tested if the degradation observed with the RIN affected the RTqPCR data. We performed correlation analysis between Cq and RIN. Contrary to the data reported in previous publications above cited, there was not an overall significant inverse correlation between Cq values and RIN in all measured genes (Figure S10). Only the Cq values of *FASN* were negatively correlated with the RIN ($r=-0.23$; $P<0.05$). This negative correlation was however balanced by the significant ($P<0.05$) positive correlation between Cq and RIN in *GAPDH* (Figure S10). Therefore, the lack of correlation is indicative of absence of effect of RNA degradation determined by RIN on RTqPCR results in our experiment. These results can be partly explained by the very short sequence amplified by our primers (amplicon size around 100 bp) which might have avoided the effect of RNA degradation. Due to the lack of correlation between RIN and Cq, we deemed our RTqPCR results reliable.

Subcutaneous adipose tissue: Results for mRNA expression in adipose tissue are reported in Figure 22. The *TNFA* gene was undetectable in most of the samples so results are not shown. No effects were observed on expression of measured genes by mastitis or TZD treatment with

exception of a tendency for a Z×M×T for *SREBF1* due to a larger overall increase in expression of the gene in MTZD compared to the other groups after IMI.

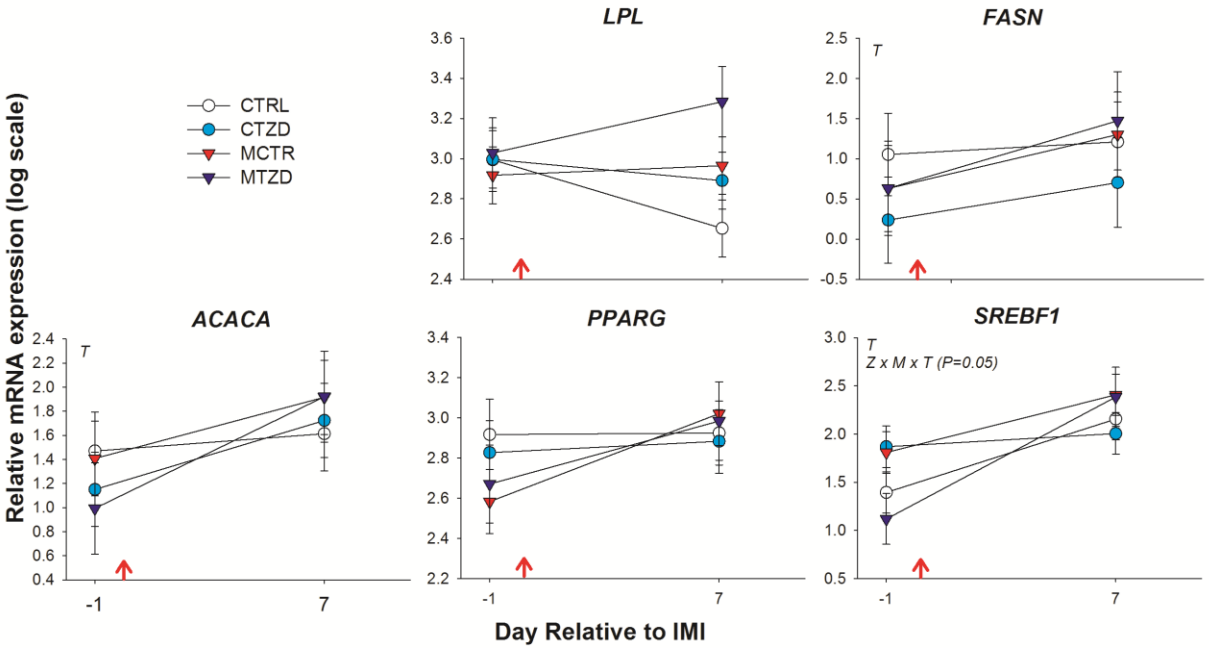

Figure 22. Expression of selected genes in subcutaneous adipose tissue from -1 to 7 d relative to intramammary infusion of *Streptococcus Uberis* (d 0), during administration of 2,4-thiazolodinidenione (TZD) or saline i.v. injections in lactating dairy goats.

Mammary epithelial cells (MEC): Results for the expression of genes related to mammary epithelial marker (*MUC1*), chemoattractant (*IL8* and *CCL2*), and lipid metabolism/PPARγ target genes in MEC are reported in Figure 23. Expression of *MUC1* was significantly affected by mastitis treatment, with a lower expression in mastitis-treated goats at 3 d post-IMI, and a larger expression in TZD-treated vs. control goats at 8 d post-IMI. Mastitis induction significantly increased the expression of *CCL2* post-IMI. Expression of *IL8* overall decreased over time and was higher in goats treated with *Strep. uberis*, especially the day after IMI (Figure 23). The expression of PPARγ and its target genes all increased or tended to increase through time (Figure 23). TZD-treated goats tended (P = 0.08) to have an overall higher

expression of *PPARG* especially at 3 d post-IMI (Figure 23). The expression of *SCD1* tended to be higher in TZD-treated goats after IMI (Figure 23). The expression of *FASN* was negatively affected by mastitis, while TZD treatment prevented a decrease in expression of *FASN* but delayed the increase in expression of *LPL* in mastitis-treated goats after IMI (Figure 23).

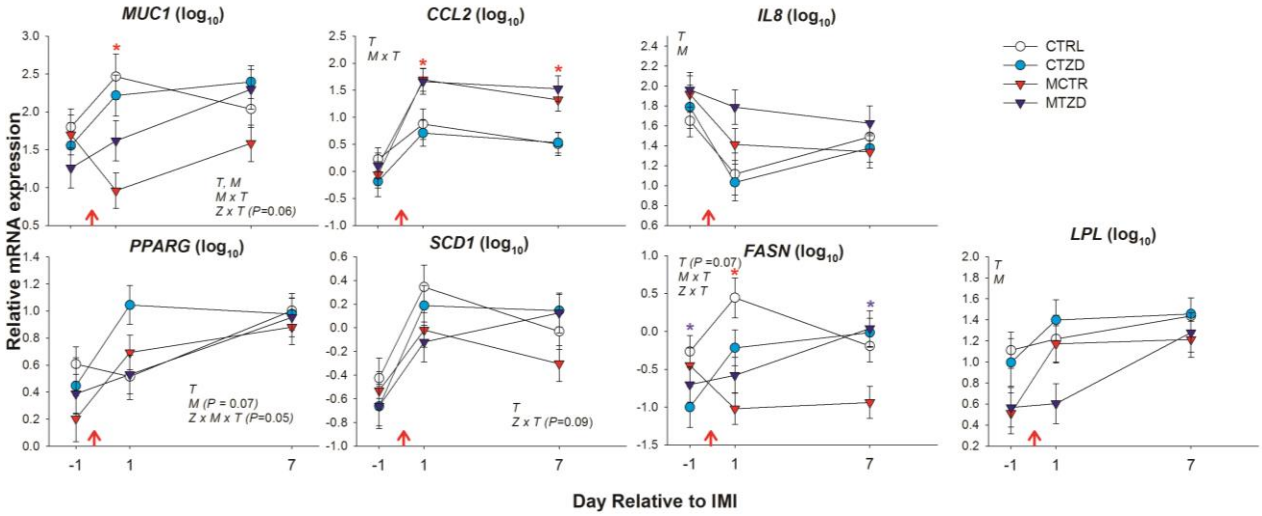

Figure 23. Expression of selected genes in mammary epithelial cells (MEC) from -1 to 7 d relative to intramammary infusion of *Streptococcus Uberis* (d 0), during administration of 2,4-thiazolodinidenione (TZD) or saline i.v. injections in lactating dairy goats. Significant differences between mastitis × time (M x T) and TZD x time (Z x T) are denoted with * and *, respectively.

3.8 Size of adipocytes

The average area of adipocytes was significantly increased over time and was overall affected by Z×T×M because the CTZD group had a larger increase after IMI compared to any other group (Figure 24). The adipocytes with medium areas (3,000 to 5,000 μm^2) were overall smaller in TZD vs. control goats at 1 day prior IMI (i.e., 6 days of TZD treatment) and small adipocytes (1,000 to 3,000 μm^2) were less abundant in TZD treated goats at 7 days post-IMI (Figure 24).

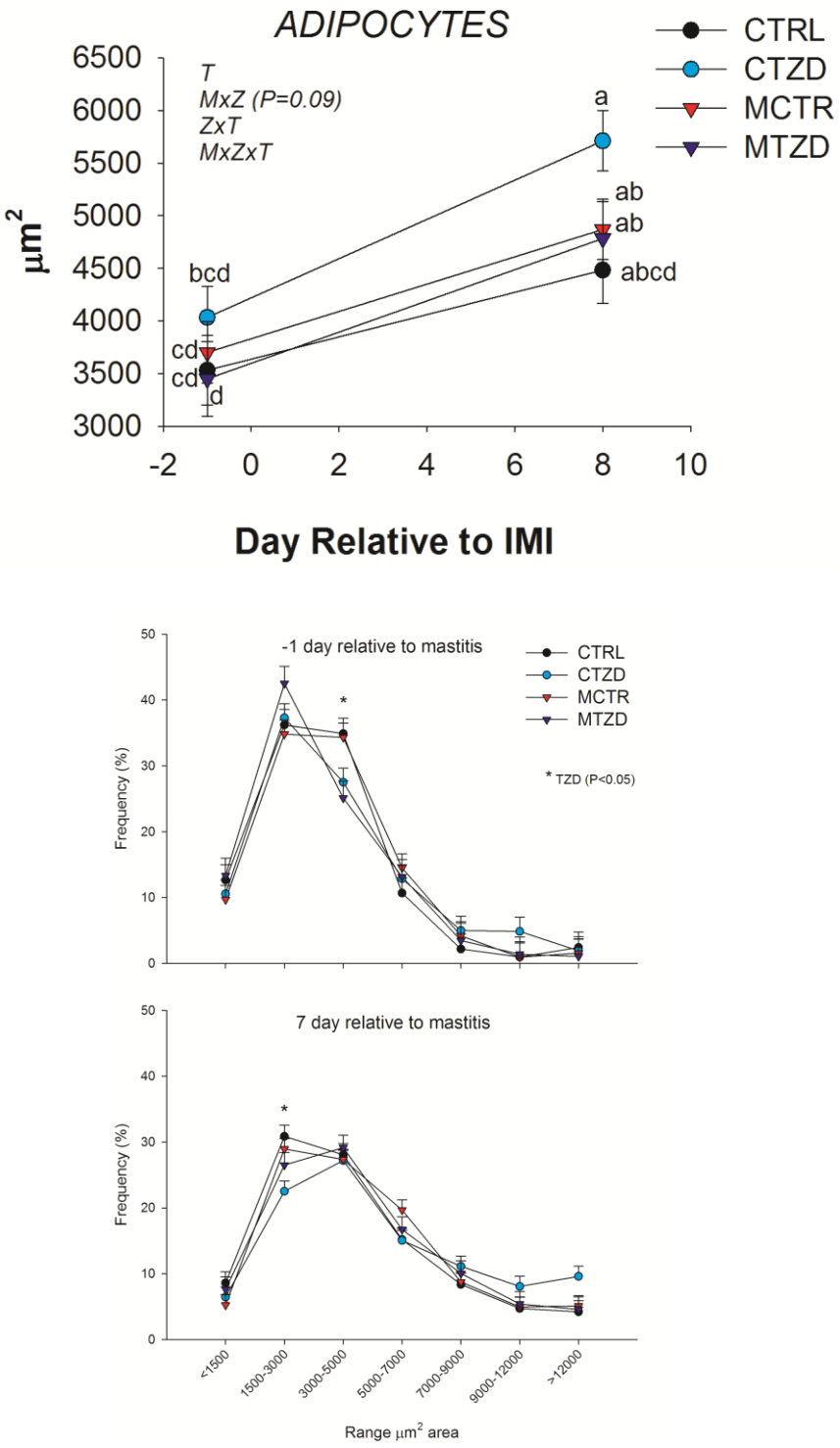

Figure 24. Measurement of the of the area of adipocytes and of the frequency of adipocytes by size before (-1 d) and after (7 d) intramammary infusion (IMI) of *Streptococcus Uberis* (d 0), during TZD administration in lactating dairy goats.

4 Discussion

In the present study all the animals receiving intramammary infusion with *Strep. uberis* developed subclinical mastitis. This was evidenced by the large increase in SCC 24h post-IMI, the significant (although modest) increase in rectal temperature between 12 and 36h post-IMI, decrease in milk yield, absence of any visible abnormality of the milk, increased expression of *CCL2,* a chemoattractant for neutrophils and macrophages (Kiku et al., 2016) and crucial for the MEC-macrophages crosstalk (detected to be upregulated in cows receiving IMI with *Strep. uberis* (Moyes et al., 2009a), and a larger decrease in % PMN in blood because of migration into the mammary. Our data confirmed the reliability of the model developed by (Lasagno et al., 2012) that used the same dose of *Strep. uberis* to induce subclinical mastitis in lactating goats.

Goats receiving intramammary *Strep. uberis* had an increase in plasma glucose, NEFA and decrease in BHBA concentrations after IMI. The pattern of these parameters is consistent with the data reported in a study where induction of mastitis was obtained using the gram negative bacteria *E. coli* in primiparous Holstein dairy cows (Moyes et al., 2014). Our data are somewhat consistent with the increase in NEFA and glucose in plasma in fed-restricted multiparous dairy cows receiving intramammary *Strep. uberis*, although the increase in NEFA was observed by 12h but not at 36h post-IMI in that study (Moyes et al., 2009b). In our study the increase in NEFA was also observed in CTRL group, which did not receive intramammary *Strep. uberis* but had an increase in rectal temperature at 12h and 44h post-IMI. The same group appeared to have had an increase in SCC prior IMI and had a slightly larger proinflammatory situation compared to the other groups prior IMI, as suggested by the numerically higher value of haptoglobin and lower value of albumin prior to IMI compared to the TZD-treated group. The CTRL group also appeared to have had a similar pattern as MCTR group for several

inflammatory-related parameters, such as albumin, cholesterol, myeloperoxidase, and NEFA, despite not having a significant increase in SCC. On examination of the inflammatory-related parameters, it is apparent that all the animals had an inflammatory response as indicated by a general increase in haptoglobin, ceruloplasmin, total ROM, and myeloperoxidase and a general decrease in magnesium, albumin, cholesterol, and paraoxonase, all markers of inflammatory-like conditions (Bertoni et al., 2008, Bionaz et al., 2007). The change in inflammatory markers was however not accompanied by a significant increase in SCC in goats receiving saline in the mammary. The increase in inflammation in goats not receiving *Strep. uberis* could have been partly due to the injection of saline in both halves of the mammary. However physiological saline is known to be relatively innocuous when injected into bovine mammary (Pyorala, 1987).

In the study by Moyes and collaborators (Moyes et al., 2009b), the cows fed-restricted had an increase in TG post-IMI, similar to the tendency we observed in our study. The increase in TG after IMI is not explained by the decrease in uptake of fat by the mammary gland, because a decrease in milk fat synthesis, although only numerically, was observed only for MCTR group, nor can be it explained by a very unlikely increase in feed intake or larger NEFA, which was also present in CTRL group that did not experience increase in TG post-IMI. An increase in TG in blood can be caused by insulin resistance (Ginsberg et al., 2005); however, the insulin sensitivity indexes did not indicate a significantly greater insulin resistance in goats receiving *Strep. uberis*. Recently, it was argued and somewhat demonstrated that the insulin indexes are probably not a good indicator of real insulin sensitivity in dairy cows (Mathews et al., 2016) and a glucose clamp should have been performed to measure insulin sensitivity. Also, pro-inflammatory cytokines decrease insulin sensitivity (Dandona et al., 2004, Kushibiki et al., 2001). Therefore, it

remains possible that, despites the lack of any indication of insulin resistance by the insulin sensitivity indexes, goats with IMI experienced some decrease of insulin sensitivity.

Contrary to our findings, there was in the study from Moyes et al. (Moyes et al., 2009b) no a decrease in BHBA but our data were more similar to the data reported by Graugnard et al., (Graugnard et al., 2013), in which IMI with LPS was performed in dairy cows. In our study the decrease in BHBA after IMI was observed in all animals, which might be indicative of either an overall response to inflammation (see above) or a metabolic change in all animals unrelated to IMI. The increase in glucose in our study can be associated with the decrease in milk yield and, especially, lactose yield, as demonstrated by Moyes and collaborators (Moyes et al., 2014). All the above observations are indicative of a similar metabolic response among cows and goats to IMI using *Strep. uberis*.

The increase in glucose and NEFA after IMI observed in our and other studies can be also associated with the inflammatory situation. The pro-inflammatory cytokine TNFα is known to induce insulin resistance in monogastrics (Hotamisligil, 1999). The work from Kushibiki and collaborators (Kushibiki et al., 2001) demonstrated that this effect is conserved in ruminants; however, a more recent study by Yuan and collaborators (Yuan et al., 2013) did not demonstrate any effect of TNFα injection on glucose, NEFA, or insulin in early lactating dairy cows. In our case, the increase in glucose and NEFA was not associated with an increase in insulin resistance, as indicated by the lack of any effect on insulinaemia and the calculated insulin sensitivity indexes. Data from Moyes and collaborators (Moyes et al., 2009b) did not find any effect on insulin level in blood after IMI in fed-restricted animals while a large increase in blood insulin post-IMI was observed in cows having a positive energy balance. The prevention of insulin resistance in heifers treated with TNF was achieved by treatment with TZD by Kushibiki and

collaborators (Kushibiki et al., 2001). We did not observed any significant effect of TZD on insulin sensitivity, with only a numerically greater sensitivity prior to IMI. Lack of effect on insulin sensitivity is in accordance with data from Schoenberg and Overton (Schoenberg and Overton, 2011) where the TZD was used in pre-partum dairy cows and Yousefi and collaborators (Yousefi et al., 2016) that treated dairy cows with pioglitazone, another synthetic PPARγ ligand during the entire transition period.

As stated above, the insulin sensitivity indexes used may not be applicable to ruminants. Regarding the level of TG discussed above, another indirect suggestion of a change of insulin sensitivity is the larger increase in adipocyte size accompanied by a lower NEFA in CTZD animals after IMI. An increase in adipose size is a consequence of TG accumulation. Adipose tissue accumulate TG under influence of insulin; thus, the data appear to indicate that adipose tissue in CTZD group had greater insulin sensitivity compared to the other groups. However, we are unable without an insulin or glucose clamp, to provide a definitive conclusion about insulin sensitivity in this study.

The observed increase in NEFA after IMI could be a response to lower feed intake, as high plasma NEFA and BHBA concentrations are indicative of negative energy balance (Goff and Horst, 1997). We did not measure the feed intake, but none of the other data, including body weight, are indicative of a significant and/or prolonged reduction of feed intake. In addition, the goats were producing a relatively small amount of milk (approx. 1 Kg/d) and they were in late lactation; thus, a negative energy balance is unlikely. However, as discussed above, the response to mastitis in the present study is somewhat similar to dairy cows in negative energy balance in the study of Moyes et al. (Moyes et al., 2009b) and the body condition score of the goats in our study was 1.6±0.5 (mean±SD; scale 1-5), which is in the low range compared to the values

reported in literature for this breed of goats and stage of lactation (Atasever et al., 2015). In our experiment, the ration was not optimized for lactating goats. This was done with the purpose to be consistent with the diet originally provided by the commercial farm where the goats were purchased. The diet may have not been adequate for the goats, as indicated by several of the above discussed parameters and by the overall relatively poor body condition. Taking together our findings support the reliability of the subclinical mastitis model used in the present experiment. Data also indicated a response to mastitis more similar to animals in negative energy balance, indicating a possible nutritional deficiency.

Regarding response to treatment, 2,4-thiazolidinedione is the basic molecular compound for the chemical synthesis of all thiazolidinedione molecules known to be specific and potent PPARγ agonists, including rosiglitazone and pioglitazone (Jain et al., 2013, Nazreen et al., 2015). Because of this, the 2,4-thiazolidinedione is assumed to be a PPARγ agonist; however, evidence are lacking in this regards. Indirect support for this compound being a PPARγ agonist is coming from prior studies in ruminants. In heifers and dairy cows it was demonstrated that 2,4-thiazolidinedione injection counteracted the insulin resistance after TNF injection (Kushibiki et al., 2001) and significantly decreased NEFA post-partum in dairy cows (Smith et al., 2009), respectively. Based on the above evidence, we assumed in the present study, that 2,4-thiazolidinedione is a PPARγ agonist.

Milk yield was not affected by TZD in normal goats but TZD treatment prevented the decrease of milk yield after IMI with *Strep. uberis*. Administration of TZD during the present trial did not significantly affect milk composition but, as with milk yield, milk fat and lactose were not decreased in MTZD as much as with MCTR. A direct effect of TZD on prevention of milk fat synthesis decline can be inferred by the higher expression in MEC of *FASN* in MTZD

vs. MCTR at 7 d post-IMI and a numerical higher expression of *SCD* in the same time point. The higher expression of *FASN* and the lower expression of *LPL* in MTZD vs. MCTR may indicate a different effect of TZD on *de novo* vs. preformed fatty acids in milk after IMI (Bionaz and Loor, 2008a). Interestingly, milk fat depression is characterized by a larger decrease in expression of *FASN* compared to *LPL* (Harvatine et al., 2009). It has been previously observed *in vitro* that expression of *FASN*, but not *LPL*, is increased upon activation of PPARγ by rosiglitazone (Kadegowda et al., 2009). The response of *FASN* to rosiglitazone appears to be consistent among ruminants, as recently reviewed (Bionaz et al., 2013). Overall, those data might indicate a slight activation of PPARγ in MEC by 2,4-thiazolidinedione.

During inflammatory episodes such as mastitis, plasma concentration of acute phase proteins like haptoglobin and ceruloplasmin are likely to increase. Indeed, in the present study all groups had an increase in plasma haptoglobin, indicating that all animals were experiencing inflammatory conditions. The mean±SD level of haptoglobin before IMI was 0.28±0.21, higher than what observed in healthy cattle (Bertoni et al., 2008, Bionaz et al., 2007, Trevisi et al., 2012) and higher than what was previously observed in healthy goats (Gonzalez et al., 2008). A level of haptoglobin that is considered high (or animals having a significant acute phase reaction) is when >0.3 g/L; thus, our animals had some basal level of inflammation. In our experiment haptoglobin was higher than 0.3 g/L after IMI in all groups and the concentration in plasma of negative acute phase protein such as albumin, paraoxonase, total cholesterol and retinol was decreased, indicating a significant inflammatory status after IMI (Bertoni et al., 2008). The larger increase of haptoglobin in non-TZD vs. TZD treated goats before IMI and after IMI, particularly in MCTR goats, and the higher level of total plasma cholesterol in TZD vs. control goats is suggestive of improved liver activity by TZD (Bertoni, 2013 #227). Taking these findings

together, there is an indication that the animals treated with TZD had a lower inflammatory status before IMI but, after IMI, the same animals had a faster recovery of the inflammatory status due to a better response by the liver. Thus, the TZD treatment appears to have improved the response to mastitis.

Recruitment and activation of neutrophils is known to be at principal defense mechanisms of innate immunity. In addition, during inflammation, neutrophils are the major cell type observed in the mammary gland (Rainard, 2006 #56); thus, their ability to migrate to the site of infection plays a critical role in the inflammatory response. In the present study, the decrease in the % of blood PMN phagocytosis in plasma 1 d after the IMI, suggests a chemotactic process by neutrophils in all groups but more robust in goats receiving *Strep. uberis*. A large neutrophil migration in goats receiving *Strep. uberis* is also suggested by higher *IL8* expression in MEC, although not increased compared to baseline. Although not difference in phagocytosis of blood PMN was detected between groups, the larger increase in MPO is indicative of a larger microbial killing capacity in TZD-treated vs. control goats (Klebanoff, 2013). Neutrophil granulocytes have a high abundance of the enzymes MPO, constituting the majority of azurophilic granules. The MPO produced the antimicrobial compound hypohalous acids, which can be released into circulation but it is also pivotal for bacteria killing after phagocytosis (Kato, 2016; Klebanoff, 2013). The increase in blood MPO can be due to an increase in neutrophils count (Mayyas, 2014) and/or by an increase of MPO expression by neutrophils. It has been demonstrated that PPARγ controls MPO expression in certain activated macrophages (Kumar, 2004) but rosiglitazone decreases MPO content in neutrophils in rabbit (Liu, 2009) and pioglitazone has been shown to decrease both neutrophils and MPO in several species (Croasdell, 2015). It is not possible to determine the reason for the increase of MPO detected in the present study because we did not

performed a whole leukocytes count; however, the lack of difference of % PMN between groups can indicate an increase in release and/or expression of MPO by neutrophils in TZD group. Higher MPO has been also associated with greater inflammation (Loria, 2008). Inflammatory markers did not indicate higher inflammation, but rather, reduced inflammation, in TZD-treated vs. control goats; thus, the increased MPO in our study in TZD-treated animals remains unexplained. Overall, our data indicate that, despite TZD not having an effect on PMN phagocytosis, the bacteria killing capacity of the neutrophils was augmented.

Data also indicated an effect of TZD on adipose tissue by decreasing the release of NEFA and slightly changing the adipocyte size profile. The histological data indicated that TZD treated goats had a significant increase in small adipocytes after 1 week of TZD treatment. An increase of small adipocytes is indicative of active adipogenesis. The formation of new adipocytes generally precede the formation of large adipocytes (Moreno-Navarrete and Fernández-Real, 2012). Our data are consistent with this general observation because the proportion of large adipocytes was larger in TZD-treated animals in later stages. PPARγ is a well-established master regulator of adipogenesis (Tontonoz and Spiegelman, 2008b). Therefore, the use of TZD appeared to have had a biological effect on adipose tissue. Surprisingly, no TZD effect was observed in the expression of target genes in adipose tissue. Lack of effect on expression of genes in adipose tissue is consistent with a study conducted in pregnant dry dairy cows (Schoenberg and Overton, 2011). However, in another study where non-pregnant dry cows were used, the injection of TZD affected the expression of several genes, including PPARγ target genes, but the effect was only temporary and, despite a continuous injection, it disappeared (Hosseini et al., 2015).

Despites the absence of a significant effect on expression of several of the main lipogenic genes in adipose tissue the presence of lower NEFA and larger adipocytes, especially in CTZD group, is indicative of lipid accumulation. An accumulation of triglycerides in adipose tissue can compete for lipid precursors with the mammary gland. This cannot be fully proved in the present work but, if present, we should have detected a decrease in milk fat production. We did not observed significant changes in milk fat. We observed however a tendency for higher expression in MEC of several lipogenic genes and targets of PPARγ (Bionaz et al., 2013), such as *FASN*, *SCD1*, and *PPARG*. Based on this we can speculate that 2,4-thiazolidinedione in our experiment helped the mammary tissue to maintain milk fat synthesis despites the completion with the adipose tissue for fatty acids.

The lack of effect of TZD on expression of PPARγ target genes in adipose tissue and the very small effect in expression of the same genes in MEC is indicative of 2,4-thiazolidinedione being a weak activator of PPARγ. However, recent *in vitro* works performed in bovine mammary cells indicated that in order for 2,4-thiazolidinedione to activate PPARγ a large amount of the vitamin A metabolite 9-*cis*-retinoic acid is required (Bionaz et al., 2015).The 9-*cis*-retinoic acid is a specific activator of Retinoic-X-Receptor, the obligate heterodimer of PPAR (Bionaz et al., 2013). As discussed above, the goats in the present experiment appeared to have a response to *Strep. uberis* similar to cows in negative energy balance. Our goats were thin and they came from a farm were they received only hay and a small amount of supplement. This feeding regime probably provided an insufficient amount of retinol. It is therefore possible that our goats had some deficiency in vitamin A and, thus, a lower level of 9-*cis*-retinoic acid reducing the response to PPARγ activator. We did not measure level of 9-*cis*-retinoic acid in plasma in the preset experiment but, despite measuring a normal level of retinol in plasma, the β-carotene was

undetectable (results not shown). Therefore, it is possible that the limiting effect on expression of genes was caused by an insufficient supplement/storage of vitamin A.

5 Conclusions

The sub-clinical mastitis model used in the present experiment was previously established. Here we have confirmed the validity of this model through the measurement of inflammatory parameters and gene expression. Thus, the model was sufficient and can be used for future studies.

Administration of TZD to lactating dairy goats undergoing subclinical mastitis had a relatively modest effect on milk yield and components and on overall metabolism with no effect on insulin concentration or insulin sensitivity indicies; however, the combination of several parameters, including NEFA, TG, and adipose histological observations, appear to indicate an improve insulin sensitivity in TZD-treated animals. The TZD injection had a strong effect on the inflammatory response and SCC. Especially noteworthy was the effect on the liver, with a better overall response to the inflammatory situation post-IMI. Despite not having any effect on leukocyte phagocytosis, TZD increased the killing capacity of neutrophils (i.e., myeloperoxidase).

Although we detected effects of TZD injection on inflammatory response, we did not observed a large effect on expression of PPARγ target genes in adipose and MEC. This was somewhat unexpected and might suggest TZD being a weak activator of PPARγ. It is also possible that, in the present study, the lack of a nutrigenomic role of TZD was due to the low BCS. This latter point deserves further investigations. Overall, we can conclude that TZD injection improved the overall response to mastitis with no or minimal effect on milk synthesis.

CHAPTER IV

EXPERIMENT 2: ROLE OF PEROXISOME PROLIFERATOR ACTIVATOR RECEPTOR GAMMA IN RESPONSE TO INDUCED SUBCLINICAL MASTITIS IN LACTATING GOATS

1 Introduction

Mastitis is a multifactorial disease that affects dairy animals resulting from an inflammation of the mammary gland, mostly caused by pathogens (Rinaldi et al., 2010). It is the most costly disease in the US dairy industry worldwide (e.g., 11% of total U.S. milk production) with negative consequences to animal well-being and milk quantity and quality (Jones, 2009). Mastitis can be treated using antibiotics, but the possibility of antibiotic resistance makes this practice dangerous and consumers are requesting to avoid or minimize use of antibiotics (Erskine et al., 2002). Therefore, alternatives to antibiotics are in high demand and means to prevent mastitis is a priority for the dairy industry.

Practices to aid in preventing mastitis include the maintenance of animals in a clean environment and pre- and post-dip procedures performed during milking. Nutrition plays a role in mastitis prevention. Among nutrients, adequate level of lipophilic vitamins aids in decreasing mastitis prevalence (Heinrichs et al., 2009). Vitamin A and beta-carotene are important in mastitis prevention due to the stimulatory effects on immune cells (Erskine, 1993). A significant inverse association between risk of clinical mastitis and serum retinol (Vitamin A) were observed in Holstein cows, for which a 60% relative reduction in risk per 100 ng/mL increase in serum retinol was reported (LeBlanc et al., 2004). In addition, beta-carotene can acts as an antioxidant, reducing the damage by superoxide produced during phagocytosis (Sordillo et al., 1997).

Besides the role on the immune cells, nutrition can prevent mastitis by a nutrient-gene interaction, also known as nutrigenomics, by which dietary compounds can affect the animal's metabolism and performance by affecting the transcription of genes (Bionaz et al., 2015). Compounds in a diet interact with the expression of genes through transcription factors. Clear nutrigenomic roles have been established for ligand-dependent nuclear receptors, especially the peroxisome proliferator-activated receptors (PPAR). PPARs are known to be activated by synthetic ligands, such as thiazolidinedione and fenofibrate, and natural ligands, such as fatty acids (Bionaz et al., 2013).

Among PPAR, the isotype PPARγ has been well-studied in monogastrics. PPARγ is known to play important anti-inflammatory roles (Yessoufou and Wahli, 2010). In Experiment 1, the use of the putative PPARγ agonist 2,4-thiazolidinedione (TZD) in dairy goats receiving intramammary infection to induce sub-clinical mastitis improved overall liver function and increased the level of myeloperoxidase in blood. The latter is an indicator of an improved killing capacity of neutrophils. Despite TZD being a putative activator of PPARγ, we failed to detect a substantial effect on expression of target genes in mammary epithelial cells and adipose tissue, raising the question about the potential activation of PPARγ by TZD. In Experiment 1, goats only received hay without adequate vitamins, including vitamin A. Moreover, work from our lab demonstrated that 9-*cis*-retinoic acid (a metabolite of vitamin A) is essential for the *in vitro* activation of PPAR by TZD in bovine mammary alveolar cells (Bionaz et al., 2015) and in primary mammary goat cells (unpublished data). Therefore, it is plausible that in Experiment 1, TZD failed to fully activate PPARγ because of an insufficient amount of vitamin A and, as consequence, its metabolite 9-*cis*-retinoic acid.

In the present work we hypothesized that continuous activation of PPARγ by TZD in lactating dairy goats supplemented with adequate amount of vitamin A can improve the response to subclinical mastitis. The objectives of this study were to test whether TZD-induced PPARγ activation in dairy goats receiving adequate amount of vitamins 1) improves the metabolic and inflammatory response to induced subclinical mastitis and 2) has a nutrigenomic effect on liver, mammary cells, and macrophages.

2 Material and Methods

2.1 *Animal management*

The Institutional Animal Care and Use Committee (IACUC) of Oregon State University approved all procedures for this study (protocol#4448)

Twelve lactating *Saanen* goats (mean±SD; 53.6±16.2 days in milk, 69.2±7.1 kg of body weight [BW], 2.6±0.6 body condition score [1-5 scale]) negative to milk bacterial analysis were housed in individual pens at the Hogg Metabolism Barn at Oregon State University. The goats were randomized by BW, milk yield, and components into two treatments (6 goats/group). Ten days of adaptation to the new environment was allowed prior starting the experiment. Animals were fed twice a day at 7AM and 7PM. The ration was calculated individually for each goats (using individual BW and milk yield) according to the NRC (2001) for small ruminants in mid-lactation.

Dry matter (DM) of individual feed ingredients was determined prior to starting the experiment. DM of grain was analyzed by standard microwave method, while alfalfa and orchard hay samples were submitted to analysis of ingredients composition (Table 6) by standard wet chemistry techniques at a commercial laboratory (Dairy One Forage Testing Laboratory, Ithaca, NY).

Table 6. Nutrient Composition of the forages used in the diet fed to *Saanen* lactating goats during the experimental period.

Chemical analysis		Alfalfa hay	Orchard Grass hay
Dry Matter (DM)	%	89.7	91.4
NE_L Mcal/Lb	% DM	0.69	0.53
Crude Protein (CP)	% DM	23.2	12.2
Adjusted crude protein	% DM	22.6	12.2
Soluble Protein	% CP	46	26
Degradable Protein	% CP	77	-
NDICP	% DM	4.1	-
ADF	% DM	25.7	37
aNDF	% DM	33.2	60
Lignin	% DM	7.1	-
NFC	% DM	27.3	18.6
Starch	% DM	0.8	-
WSC	% DM	10.1	-
ESC	% DM	8.5	-
Crude Fat	% DM	2.9	-
Ash	% DM	13.35	-
TDN	% DM	65	59
Calcium	% DM	0.97	0.31
Phosphorus	% DM	0.24	0.2
Magnesium	% DM	0.51	0.24
Potassium	% DM	2.64	2.28
Sodium	% DM	0.389	0.055
Sulfur	% DM	0.34	0.2
Chloride	% DM	0.58	-
Iron	ppm	1,460	223
Zinc	ppm	20	18
Copper	ppm	8	8
Manganese	ppm	48	112
Molybdenum	ppm	5	0.4
Lysine	% DM	1.18	-
Methionine	% DM	0.36	-

Animals received twice a day orchard grass hay and alfalfa hay. The ration was supplemented in the AM feeding with an individually calculated amount of a commercial grain goat mix (Kountry Buffet, CHS Inc., Sioux Falls, SD) and a mineral mix (SWEETLIX® Caprine Magnum-Milk Mineral, SWEETLIX®, Mankato, MN).

Animals were also drenched once a day to reach the level of vitamins required. Vitamins requirements (IU/d) ranged from 10,700 to 16,000 for vitamin A, from 2700 to 4,000 for vitamin D, and from 330 to 500 for vitamin E, based on individual diet. The drench (i.e. 10 ml/goat) was a mixture of a commercial drench (Vitamins and Electrolytes, Durvet, MO, USA) plus vitamin D and E (generously provided by CHS, Tangent, OR, USA). The amount of each component of the drench is reported in Table 7. In Table 8 are reported the amounts of each compound of the diet provided to the animals.

Table 7. Composition of the drench of vitamins (10mL solution) fed to *Saanen* lactating goats during the experiment period.

Drench of vitamins	Daily Feed per goat (Kg as fed)
Vitamin D (CHS[1])	0.0002
Vitamin E (CHS[2])	0.0125
Vitamins and Electrolytes mix (Durvet[3])	0.0075

[1]Contained 12,000 IU/Kg (CHS, MN, USA)
[2]Contained 227,000 IU/Kg (CHS, MN, USA)
[3]Contained a maximum of 37% of sodium, and a minimum of 3% of potassium, 5,000,000 IU/lb of Vitamin A, 2,000,000 IU/lb of Vitamin D3, 2,000 IU/lb of Vitamin E, 5mg/lb of Vitamin B12, 2,000mg/lb of menadione, 1,500mg/lb of riboflavin, 2,500mg/lb of d-pantothenic acid, and 500mg/lb of thiamine hydrochloride, 5,000mg/lb of niacin, 7,500mg/lb of ascorbic acid, 500mg/lb of pyridoxine hydrochloride and 130mg/lb of folic acid (Durvet, MO, USA).

Two kids per goat were left to nurse until 4 days prior intramammary infection. Afterwards, the goats were milked twice a day at 6AM and 6PM in a stanchion using a portable milking machine. Teats of the goats were pre-dipped before milking and post-dipped after milking using 0.5% iodine solution.

Table 8. Ration fed to *Saanen* lactating goats during the experiment period.

Components	Daily Feed per goat (Kg as fed)			
BW (kg)	60	70	80	90
Milk yield (kg/d)	2.0-3.0	3.5-4.8	3.0-3.5	2-3.2
Alfalfa Hay (kg AF/d)	0.6	1.7	1.5	0.8
Orchardgrass Hay (kg AF/d)	2.0	1.0	2.7	2.8
Kountry Buffet (kg AF/d)	0.35	0.32	0.32	0.32
SweetLix Minerals (g AF/d)	15.0	15.0	15.0	15.0

2.2 Experimental design

The overall experimental design is reported in Figure 25. The experiment included two objectives. The first objective was to assess the effect of 2,4-thiazolidinedione (TZD) on milk fat synthesis and the second objective to assess the effect of TZD on mammary infection. Thus, the experiment can be divided in two sections: the first section (from start of the experiment until mastitis induction) was performed to fulfill the first objective whereas the second session (from the mastitis induction until the end of the trial) was performed to fulfill the second objective. Therefore, in the present paper only materials and methods and results pertinent to the second part of the trial (i.e., mastitis induction) are reported. Some part of Materials and Methods describes the entire trial.

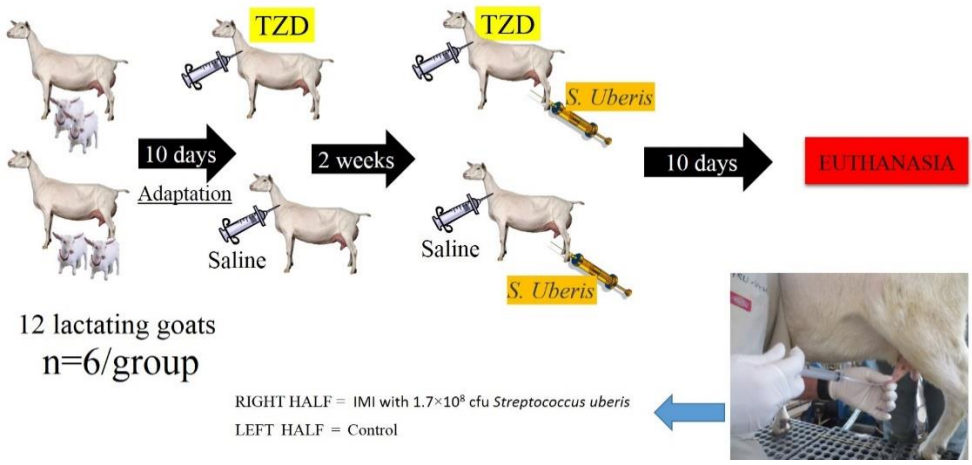

Figure 25. Experimental design.

2.3 2,4-thiazolidinedione injection

After the adaptation period, the goats started to receive daily injections of 8 mg/kg of BW of 2,4-thiazolidinedione (n=6; TZD; Cat# SC-216281, Santa Cruz Biotechnology, Dallas, TX) or 10 mL of sterile physiological saline (n=6; CTR; Cat# 002479, Henry Schein, Dublin, OH)

throughout the whole study (25 days). The daily injection was performed at 10AM. The dose of TZD was as for Experiment 1.

2.4 Intramammary infusion of Streptococcus uberis

In order to induce subclinical mastitis, after two weeks of repeated daily TZD or saline i.v. injections all goats received an intramammary infusion (IMI) of $1.7x10^8$ *Streptococcus uberis* (*Strep. uberis*) in 10 mL sterile physiological saline (Cat# 002479, Henry Schein, Dublin, OH) in the right half of the mammary gland, keeping the left half as a control. The pre-dosed aliquot of *Strep. uberis* (strain 0140J) in 1.5 mL sterile vials was provided by the laboratory of Peggy Dearing, College of Veterinary Medicine, Oregon State University. Prior to IMI, the teat ends were carefully cleaned with individual moistened towels and disinfected with swabs containing 70% ethanol. The infusion was performed with the aid of a disposable sterile urinary catheter (TomCat, USA). After IMI, each half mammary was thoroughly massaged upward into the gland cistern for 30 s to distribute the inoculation dose.

2.5 Measurements and sample collection

Milk was collected aseptically from both halves of the mammary gland for bacteria analysis 3 days prior IMI, just before IMI, and 24 h post-IMI. Before sampling, the teat was treated with teat dipping solution and cleaned using disposable paper towels. The orifice of the teat was disinfected with swabs containing 70% ethanol. Approx. 1 mL of milk was collected in a sterile 1.5 mL tubes. The samples were immediately put in ice and shipped overnight to the Ag Health Laboratories, Inc (Sunnyside, WA) on ice packs for a milk bacterial culture on blood agar plate. All the samples were negative to any pathogen prior IMI. To ascertain status of the mammary gland a California Mastitis Test was performed at the last milking just prior IMI.

Milk yield was recorded daily throughout the experiment. Goats were milked using a portable milking machines with two claws. In order to avoid cross-contamination, before IMI each goat was milked using one claw; after IMI both claws were used to milk one goat with one claw exclusively used to milk the right half (i.e., the infected half) and the other claw was used to milk the healthy half (Figure 26). Milk yield was measured at each milking (i.e., 6AM and 6PM). Milk samples were collected for components analysis just before IMI, 12 hours and 1, 2, 5, 6 and 7 days post-IMI. Samples were shipped with a preservative (Bronopol) to the Willamette National Dairy Herd Information Association (Salem, OR) to measure somatic cell count (SCC), lactose, fat and protein percentage.

Figure 26. Use of a portable milking machines with two claws before and after the IMI.

Rectal temperature (RT) was checked using a rectal thermometer prior starting IMI, 12 hours post-IMI and subsequently twice a day (AM and PM milking) until the end of the experiment. BW and body condition score (BCS) were recorded weekly throughout the study.

Blood samples were collected prior to the morning milking from the jugular vein using a 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ). Samples were collected prior IMI, 12 hours post-IMI, every day during the first 3 days post-IMI (i.e., 24h post-

IMI, 2d, and 3d post-IMI), and at 6 and 10 d post-IMI. Samples were collected into two evacuated tubes (10mL, BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ), one containing serum clot activator and the other sodium heparin. After blood collection, tubes containing sodium heparin were placed on ice (4 °C), while the tubes with clot activator were kept at room temperature (~30min) until centrifugation. Serum and plasma were obtained by centrifugation at 1,200 × *g* for 15 minutes. Aliquots of serum and plasma were frozen (-80°C) until further analysis.

2.6 *Blood Metabolites and inflammatory markers*

Aliquots of plasma and serum were shipped in dry ice to the Istituto di Zootecnica, Universitá Cattolica del Sacro Cuore, Piacenza, Italy, for metabolic and inflammatory profiling.

Plasma samples were analyzed for 19 parameters. These includes metabolic parameters glucose, cholesterol, urea, creatinine, non-esterified fatty acids (NEFA), triacylglycerol (TAG) and β-hydroxybutyric acid (BHBA); inflammatory-related parameters albumin, haptoglobin (Hp), ceruloplasmin, paraoxonase (PON), myeloperoxidase (MPO), total bilirubin, total reactive oxygen metabolites (ROMt), ferric reducing antioxidant power (FRAP) and zinc (Zn); liver status biomarkers gamma-glutamyl transferase (γGT) and aspartate aminotransferase (AST/GOT) plus total protein. In addition, the parameter globulin was obtained by subtracting albumin to total proteins. The analyses were performed following the procedures described previously (Bionaz et al., 2007; Trevisi et al., 2012) using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA) for all parameters.

2.7 *Phagocytosis and % polymorphonuclear leukocytes (PMN)*

Phagocytosis and % PMN were performed before starting the experiment (i.e. baseline), prior to IMI and 6 d post-IMI. The phagocytic capacity of PMN and monocytes isolated from

100 μL heparinized whole blood was determined using the Phagotest kit (Glycotope Biotechnology, Heidelberg, Germany) following the manufacturer's instructions but using half of the amount of each reagent as described for Experiment 1. The % of PMN (>80% of PMN are neutrophils in goat, Jones et al, 2007) was determined by gating nucleated cells using the forward (cell size) and side scatter (cell complexity) in samples used as control (i.e., samples were preserved at 4°C instead of 37°C after addition of bacteria to avoid induction of phagocytosis; see Figure 27).

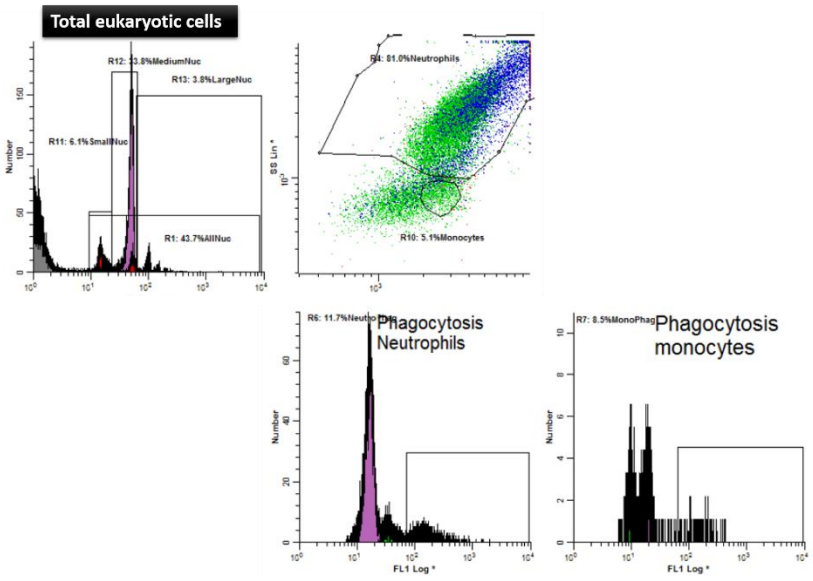

Figure 27. Schematic visualization of the steps to determine % phagocytosis in PMN and monocytes and % of PMN among nucleated cells in whole blood of goat using flow cytometer following the instructions of the Phagotest kit (Glycotope, Germany).

2.8 *Mammary epithelial cells and macrophages isolation*

The mammary epithelial cells (MEC) and macrophages were isolated from 250 mL of milk using magnetic sorting. MEC samples were isolated 2 d post–IMI and macrophages were isolated at 5 d post-IMI. Milk samples were collected just after milking in 250 mL DNase/RNase free sterile tubes (Cat# 430776, VWR, USA) and immediately kept on ice (4 °C) until isolation (~1 hr).

The magnetic isolation was performed as follow. Tubes were centrifuged at 1,500 × g for 10 min to pellet the cells. Cells were washed twice with 12 mL of sterile phosphate buffered saline – PBS [calcium and magnesium chloride free] (Cat# PBL06-500ML, VWR, USA) and centrifuged at 1,000× g for 10 min. Before the last wash the cells went through a cell strainer (BD facol cell strainer – 70μm nylon, Cat#732-2758, VWR, USA). The final pellet was resuspended in 1 mL of PBS solution plus 0.1% bovine serum albumin (BSA) (Cat# AK8917-0100, Akron, Boca Raton, FL) and transferred in a 1.5 mL tube pre-wetted with the PBS+0.1% BSA solution. The cell isolation was performed using the KingFisher Duo (Cat# 5400100, Thermo Scientific, USA) following the manufacturer's instructions for Thermo Scientific Dynabeads isolation with some modifications. In brief, 1 mL solution of cells were transferred to a KingFisher Duo - 96 well plate (Cat# 97003500, Thermo Scientific, USA), plus 2 μL/sample of an antibody against the epithelial-specific marker cytokeratin 8 (Cat# MA1-19037, Thermo Fisher, Rockford, IL) for MEC and a monoclonal antibody CD14 for macrophages isolation (CAM 36A, Cat# CT-BOV2027, Monoclonal antibody center, Pullman, WA), respectively. Twenty-five μL of Dynabeads® Pan Mouse IgG (Cat# 11041, Thermo Fisher, Rockford, IL) were added into the plate diluted with PBS+0.1% BSA. The isolation steps consisted in the conjugation of the antibody with the Dynabeads® by incubation for 30 minutes at 6°C, following by 20 minutes incubation with the cells and two washing steps.

The cells positively separated using the magnetic beads were counted using an automated cell counter (Moxi-Z, Cat#MXZ001, ORFLO®, ID, USA). The isolation of MEC provided 40.5 x 10^6±69.3 x 10^6 cells (mean±SD) from 250 mL of milk with a cell diameter of approx. 11 μm while from the same amount of milk the isolated macrophages were 103.6 x 10^6±145.8 x 10^6 cells with a cell diameter of around 8 μm (Figure 28). Cells positively isolated by the magnetic

beads (i.e., positive) and cells remaining after isolation (i.e., negative) were transferred to 1.5 ml tubes. Centrifugation at 1,000 × *g* at 4 °C for 5 min was performed to pellet the cells. Then, 1mL of cold TRIzol® (Cat# 15596018, Ambion, Carlsbad, CA) was added to the cell pellet and mixed by pipetting to dissolve the cells content. The samples were stored at -80°C until RNA extraction. The evaluation of the enrichment of MEC was performed on 12 random samples measuring the expression of cytokeratin 8 (*KRT8*), kappa casein (*CSN3*) and lactalbumin (*LALBA*), while on macrophages cells the cluster of differentiation 14 (*CD14*) expression was measured on positive cells mean±SD 292.9±42.7 and on negative cells mean±SD 85.5±42.7.

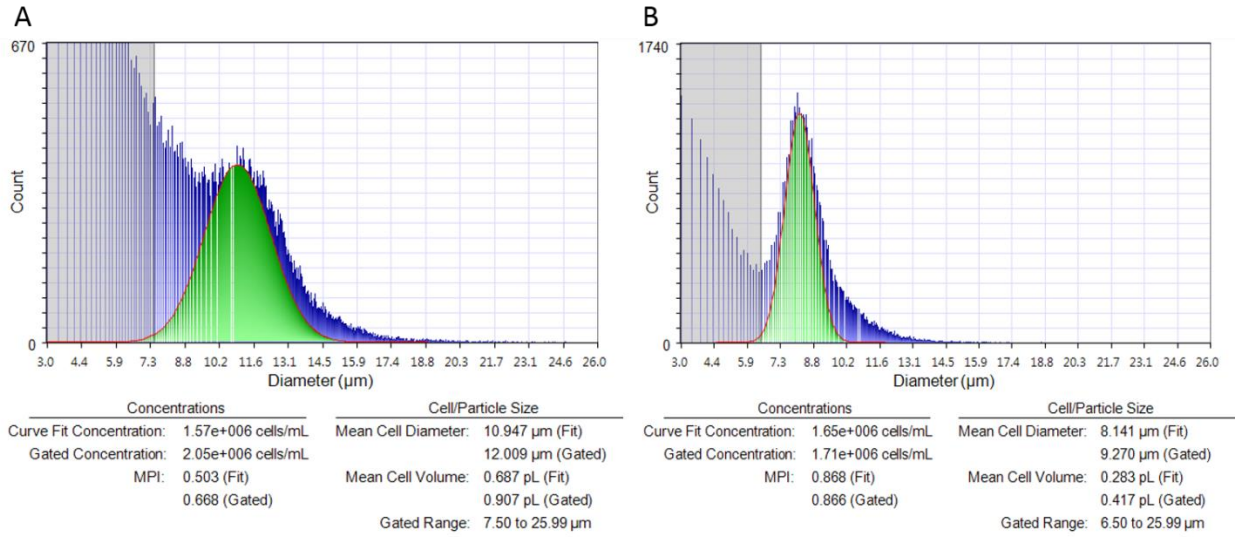

Figure 28. Representation of Moxi-Z chromatogram for the measurement of A) mammary epithelial cell and B) macrophages after cell isolation using magnetic sorting.

2.9 *Euthanasia and Tissue collection*

At the end of the trial goats were euthanized by rapid intravenous injection of barbiturate (Beuthanasia D®) in order to harvest various tissues including liver, mammary, and adipose tissue for gene expression analysis. All tissues were harvested from 9 goats (6 in TZD group and 3 in control group) using sterile scalpel and forceps, cleaned with RNase decontamination solution (RNaseZap®, Cat# AM9780, Ambion). Tissue was quickly blotted with sterile gauze to

remove residual blood and snap-frozen in liquid nitrogen and subsequently store at -80ºC until RNA extraction.

2.10 RNA isolation and quality assessment and quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Prior to RNA isolation, frozen liver samples were weighed (~100 mg) and immediately placed into 1 mL ice-cold TRIzol® in a 2 ml RNase/DNase Free tubes (Metal Bead beating tubes, Cat# 13117-50, Qiagen, USA). MEC and macrophages samples were already immersed in 1mL of TRIzol®. In all samples immersed in TRIzol®, 1 bead (Cat# 69989, 5mm, Qiagen) per tube was added using the QIAGEN bead dispenser. Tubes were loaded into a Bullet Blender Next Advance (Laboratory Instruments, USA) and samples were homogenized twice for 1 min at maximum speed with 1 min resting on ice. Once the tissue or cells were completely homogenized, RNA was extracted following a previously published protocol (Bionaz and Loor, 2007) and RNA cleaning was performed using RNeasy Plus Mini Kit (Cat# 74134, Qiagen) following the manufacturer's instructions without DNA digestion. The RNA concentration was measured using SpectraMax® Plus 384 (Molecular Devices, Sunnyvale, CA, USA). The purity of RNA was assessed by ratio of optical density OD260/280, which was above mean±SD 1.5±0.5 for all samples. The RNA integrity number (RIN) was assessed by electrophoretic analysis using a 2100 Bioanalyzer Instruments (Agilent, USA). RIN values had a mean±SD of 7.4± 0.7 for liver samples, 6.9±1.2 for MEC samples, and 7.0±1.2 for macrophages samples.

.The cDNA synthesis was performed according to (Bionaz and Loor, 2007) with some modifications. A portion of the hepatic RNA was diluted to 100 ng/µL with DNase/RNase free water (Cat# 10977-015, Life Technologies, Grand Island, NY) prior cDNA synthesis. cDNA was synthetized using 100 ng of RNA for macrophages and liver and 90 ng of RNA for MEC, 1 µL Random Primers (Cat# 48190-011, Invitrogen, Carlsbad, CA, USA), and 9 µL of DNase/RNase-

free water. The mixture was incubated at 65°C for 5 min and kept on ice for 4 min. A total of 9 µL of Master Mix composed of 1 µg dT18 (Cat#A15612, Invitrogen, Carlsbad, CA, USA), 2 µL 10 mM dNTP mix (Cat# 18427-013, Invitrogen, Carlsbad, CA, USA), 4 µL 5×Reaction Buffer (Cat# EP0441, Thermofisher), 0.25 µL RevertAid Transcriptase (Cat# EP0441, 200 U/µL; Thermofisher), 0.125 µL Rnase Inhibitor (Cat# EO0382, 40U/µL, Thermofisher), and 1.625 µL DNase/RNase-free water was added. The second step of the cDNA synthesis reaction was performed as follow: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. The synthesized cDNA was diluted 1:4 with DNase/RNase-free water (1 part of cDNA in 4 parts of water, i.e., 20% dilution). The following procedures up to completion of RT-qPCR were done as described for the liver samples, with three modifications: 10 µL of DNase/RNase-free water was added into the first step, the master mix contained 1µL of 10 mM dNTP mix and 0.25 µL of Maxima Reverse Transcriptase (Cat#200 U/µL; Thermofisher; Cat. No. EP0742) were used. A total of 4 cDNA (20 µL × 4 = 80 µL solution) were synthesized in a single 0.2 mL PCR tube for each sample. This provided sufficient cDNA to perform PCR in triplicate for >30 genes.

The target genes selected to be evaluated in liver RNA samples were PPARγ (*PPARG*), PPARβ/δ target gene pyruvate dehydrogenase kinase 4 (*PDK4*), PPARα target gene carnitine palmitoyltransferase 1A (*CPT1A*), PPARγ target gene apolipoprotein E (*APOE*), the acute phase protein haptoglobin (*HP*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). For MEC we measured transcription of inflammatory-related genes interleukin 8 (*IL8*), chemokine (C-C Motif) ligand 2 (*CCL2*), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*), and tumor necrosis factor alpha (*TNFA*). Also, the milk fat synthesis related gene stearoyl-CoA desaturase (delta-9-desaturase) (*SCD1*) was measured in MEC. For macrophages the genes evaluated were related to the inflammatory response (*IL8, CCL2, TNFA, NFKB1,*

transforming growth factor beta 1 [*TGFB1*], interleukin 4 [*IL4*], and interleukin 10 [*IL10*]. In addition, nitric oxide synthase 2 (*NOS2*), small ubiquitin-like modifier 1 (*SUMO1*), cluster of differentiation 14 (*CD14*), *PPARG* and *GAPDH* were measured in macrophages.

2.11 Primer design and evaluation

Primers were designed as previously described by Bionaz and Loor (2008) with modifications. Briefly, *Capra hircus* specific sequences were searched in National Center for Biotechnology Information (NCBI at https://www.ncbi.nlm.nih.gov/) and blasted against the sheep genome in the University of California Santa Cruz Genome Browser (https://genome.ucsc.edu/) in order to determine the exon-exon junctions using Primer Express (V3.0.2, Applied Biosystems). Primer-pairs were designed with the following features: minimum amplicon size of 90 bp (most ranged between 100 and 120 bp) and limited 3' G + C percentage. Primer pair sequences were aligned against NCBI database through BLASTN to determine uniqueness and similarity with annotated sequences.

Primer pairs were verified through a 20 µL PCR reaction, composed of 1 µL each of forward and reverse primers, 10 µL of 1×SYBR Green master mix (Cat# 4367659, Applied Biosystems, Woolston Warrington, UK) and 8 µL of cDNA (a pool cDNA from all RNA samples - i.e., liver, macrophages and MEC), using the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15s at 95°C followed by 1 min at 60°C. Five microliters of each PCR product was run in 2% agarose gel stained with EZ-vision® (Cat# N391-15MLDRP, Amresco, Solon, OH), and the remaining 15 µL were cleaned with DNA clean & Concentrator Kit (Cat# 11-302C, Zymo Research, USA) and sequenced at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. The details of primer pairs and relative amplicon sequences are shown in Tables 9 and 10.

Table 9. Gene symbol, GenBank accession number, sequence and amplicon size of primers used to analyze gene expression by quantitative PCR.

Gene	Accession #	Primers[1]	Primers (5'-3')[2]	bp[3]	Source
Nuclear receptor					
PPARG	NM_001285658.1	F. 156	TCCGCTCCGCACTACGA	100	Goat Exp. 1
		R. 255	TGATTGCACTTTGGTACTCTTGGA		
PPAR target genes					
APOE	XM_018062563.1	F. 347	GGCCGCCTCTGGGATTAC	98	This manuscript
		R. 444	AGCACCGTCAGTTCCTGAATG		
CPT1A	XM_018043311.1	F. 274	CATCAGATTCAAGAATGGCATCA	100	This manuscript
		R. 373	CTTGGCGTACATGGTCGACAT		
PDK4	XM_005678949.3	F. 1041	CTGATGAACCAGCACATCCTTATATT	92	This manuscript
		R. 1132	GCCGCCACATCACAGTTTG		
Inflammation					
HP	XM_005692202.3	F. 522	GAATGTGAGGCAGTGTGTGGAA	143	This manuscript
		R. 664	AGTGTGGCTCCCGAGATGAG		
IL8	XM_005681749	F. 282	CAACGGAAAAGAGGTGTGCTTA	90	Goat Exp. 1
		R. 371	GGATCTTGCTTCTCAGCTCTCTTC		
CCL2	XM_005693218	F. 164	AAATTCAATAAGAAGATCCCCATACAG	100	Goat Exp. 1
		R. 264	CCTAGGATGGTCTTGAAAATCACA		
NFKB1	XM_005681365.2	F. 528	GGTGGTCGGCTTTGCAAA	91	This manuscript
		R. 618	TACACAGGCGTCTGTCATTCG		
TNFA	NM_001286442	F. 178	CTCTTCTGCCTGCTGCACTTC	100	Goat Exp. 1
		R. 283	AAGATGACCTGAGTGTCTGAACCA100		
IL4	NM_001285681.1	F. 229	CTGCCCCAAAGAATGCAACT	90	This manuscript
		R. 318	TGTTCAAGCACATGTGGTTCCT		
TGFB1	NM_001314142.1	F. 850	TACTGCTTCAGCTCCACAGAAAAG	100	This manuscript
		R. 949	ACCCCTTGGGTTCGTGAATC		
IL10	XM_005690416.2	F. 731	CGGCGCTGTCATCGTTTT	100	This manuscript
		R. 830	CTTTGTAGACACCCCTCTCTTGGA		
CD14	NM_001285583.1	F. 141	TAGCGCCGTTCAGTGTATGG	102	This manuscript
		R. 242	TACTGCTTCGGGTCGGTGTT		
NOS2	XM_013971952.1	F. 492	CTTCAAAGAGGCAAAAATAGAGGAA	120	This manuscript
		R. 611	GCCTGCTTGGTGGCAAAG		
Post-transcriptional modifiers					
SUMO1	XM_018060803.1	F. 202	GCCTTACTCTGCAGGAAGCAA	100	This manuscript
		R. 301	TCACTGCTATCCTGTCCAATGACT		
Lipid Metabolism					
SCD	NM_001285619	F. 432	GGCGTTCCAGAATGACGTTT	100	Goat Exp. 1
		R. 531	AAAGCCACGTCGGGAATTG		
Internal control genes					
RPS9	XM_005709411	F. 312	AGGTCTGGAGGGTCAAATTCAC	100	Goat Exp. 1
		R. 418	CAGGGCATTACCTTCGAACAG		
UXT	XM_005700842	F. 158	AGCGGGACTTGCAAAAGGT	100	Goat Exp. 1
		R. 258	AGCTTCCTGGAGTCGCTCAA		
YWHAZ	XM_005689196.1	F. 475	TGGTGATGACAAGAAAGGGATTG	100	Goat Exp. 1
		R. 574	TCTGATAGGATGTGTTGGTTGCA		
GAPDH	XM_005680968	F. 305	CCATCTTCCAGGAGCGAGATC	100	Goat Exp. 1
		R. 407	CCAGCCTTCTCCATGGTAGTG		

[1]Primer direction (F – forward; R – reverse) and hybridization position on the sequence.
[2]Primer sequence. Exon-exon junction are underlined
[3]Amplicon size in base pair (bp)

The sequencing product was confirmed through BLASTN (megablast) at the NCBI database. The accuracy primer pairs were evaluated by the presence of a unique peak during the dissociation step at the end of qPCR.

Table 10. Sequencing results of PCR amplicon obtained from the primer-pairs used for this experiment.

Gene	Sequence
APOE	CGACTCTCTGACAGGTGCAGGAGGAGCTGCTCAACACCCAGGTCATTCAGGAACTGACGGTAGGTC
CPT1A	GGGTCCAGCGAGTCCCTCCAGTTGGCTCATCGTGGTTGTGGGCGTGATGTCGACCATGTACGCGCAAG
PDK4	CCTAGAGGAACCCAAGCCTCATTGGAAGCATTGATCCAAACTGTGATGTGGCGGC
HP	CACGTGGACAGACGCAGAGGACATAGGTGGGTCGCTGGATGCCAAAGGCAGCTTCCCCTGGCAGGCCAAGATGGTC TCCCACCATAACCTCATCTCGGGAGCCACACTA
NFKB1	GCAATCGTGCAGAAAGTATTTGAACACTGGAAGCACAGAATGACAGACGCCTGTGTAA
IL4	GAGGAGAGGCTGGATTGAGCTTAGGCGTATCTACAGGAACCACATGTGCTTGAACAA
TGFB1	CAAGGTCGTCGCTCTACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCACGAACCCAAGGGGTA
IL10	GCAGAGCAGCGGTGGAGCAGGTGAAGAGAGTCTTCAATATGCTCCAAGAGAGGGGTGTCTACAAAGA
CD14	CGTCGTGGCGGCGGCACAGCCTGGACCAGTTTCTCAAGGGAGCCAACACCGACCCGAAGCAGTA
NOS2	GCGGGAGCGGTACAAGGAGATAGAAACAACAGGAACCTACCAGCTGACGGGAGATGAGCTCATCTTTGCCACCAA GCAGGCA
SUMO1	ACAGTGGGGGAGAAGGAGGAGAATATATTAAACTCAAAGTCATTGGACAGGATAGCAGTGANAA

2.12 Quantitative PCR (qPCR)

qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Cat# 4309849 , Applied Biosystems, Grand Island, NY). Within each well, 4 µL of diluted cDNA combined with 6 µL of mixture composed of 5 µL Power SYBR® Green PCR Master Mix (Cat# 4368706, Thermo Fisher Scientific, Waltham, MA USA), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of DNase/RNase-free water. Three replicates were run for each sample and a non-template control (NTC) was run for each gene analyzed. qPCR was conducted in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) located in Emily Ho's laboratory at Oregon State University using the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15s at 95°C followed by 1 min at 60°C. A dissociation curve was performed (gradient from 95C to 60°C to 95°C) to check for presence of amplified product(s) and melting temperature of amplicons. The PCR efficiency and quantification cycle values were obtained for

each sample using LinRegPCR (Ramakers et al., 2003). The genes ribosomal protein 9 (*RPS9*), ubiquitously-expressed transcript (*UXT*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) were used as internal control genes (ICG) for the liver and macrophages qPCR, while *RPS9*, *YWHAZ* and *GAPDH* were used as ICG for the MEC samples. The reliability of the selected ICG and the stability of the normalization factor through the calculation of the pairwise variation V (the lower the better; with an acceptable V value of 0.2) were assessed using geNorm software (Vandesompele et al., 2002). The V-value was 0.044 for liver samples, 0.13 for macrophages samples, and 0.19 for MEC samples. Data were then normalized with the geometric mean of the three best ICG as previously described (Vandesompele et al., 2002).

2.13 Statistical analysis

Prior to statistical analysis all data except % phagocytosis and gene expression were arithmetically transformed to have the same mean at time 0 relative to IMI (i.e., baseline) for TZD and CTR group. This was achieved by subtracting from each data point the difference between TZD and CTR at time 0 relative to IMI. For milk data the correction was done relative to the left half of the CTR group using the same approach as above. Data were checked for normal distribution of the residuals and outliers using PROC REG of SAS. Data with a studentized t >3.0 were removed. Normal distribution of residuals was assessed using PROC UNIVARIATE of SAS. Data with a significant ($P<0.01$) Shapiro-Wilk test were considered not normally distributed and log or square root transformed prior statistical analysis. Data were analyzed with the PROC GLIMMIX procedure of SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). Fixed effects in the model were treatment (i.e., TZD or CTR; Z), time relative to IMI (T) and all interactions as fixed effect. For milk yield and milk composition, SCC and MEC and

macrophages gene expression, mastitis (M) was also included in the main effect (including interactions). Goat was considered as random effect in all analysis. The covariance structure SP (POW) for repeated measures with unequal spatial time was used for several parameters including blood and milk composition, while the autoregressive (1) covariance structure for repeated measures was used for equally spaced parameters, such as milk yield and DMI analysis. Gene expression data had no time effect in the model. Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.11$, respectively.

3 Results

3.1 Dry matter intake, rectal temperature and body weight

Dry matter intake and body weight decreased after IMI while rectal temperature increased within 12h post-IMI and tended to remain higher than pre-IMI for the first 5 days post-IMI (Figure 29).

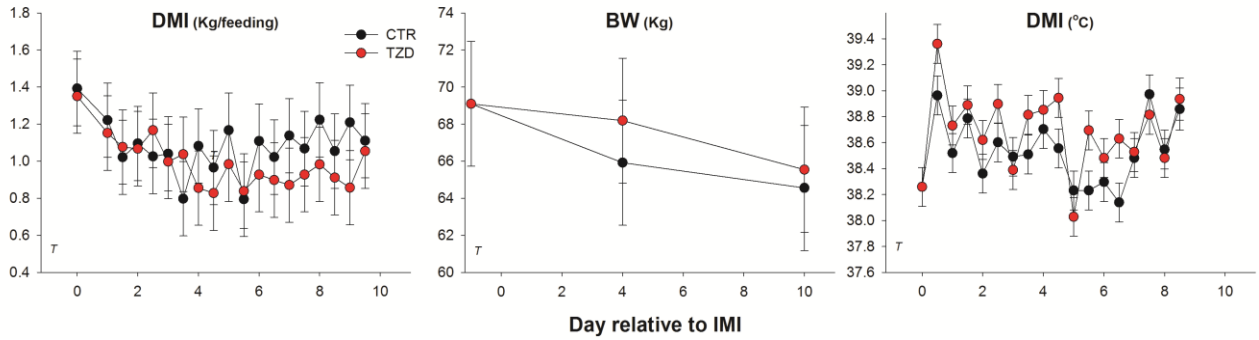

Figure 29. Performance parameters (DMI = dry matter intake; BW = body weight; RT = rectal temperature) in *Saanen* goats treated with 2,4-thiazolidinedione (TZD) or saline (CTR) after intramammary infusion of *Strep. uberis* (IMI; time = 0). Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect. Interaction of these effects is denoted by "x"

3.2 Milk yield, SCC and milk composition

Results for milk yield, SCC and milk components are shown in Figure 30. Milk yield was affected by time and tended to be lower in mammary halves receiving *Strep. uberis*. This was however mostly due to a higher milk yield in TZD-control compared with TZD-mastitis. SCC where increased by *Strep uberis* infusion with differences compared with control observed from 12h post-IMI until the end of the trial. However, SCC was not affected by TZD TRT.

Milk fat percentage was affected by TRT×Time due to an overall higher milk fat% in TZD-treated groups compared to control. Lactose and protein percentages were affected by the interaction Time×Mastitis where mammary halves receiving IMI had a decrease of lactose % just

after IMI that remained lower until the end of the trial and an increase of protein % at 12 and 24 hours post-IMI. Lactose and protein % were not affected by TZD TRT. A tendency for a TRT×Mastitis interaction was observed for the % of solid nonfat (SNF) due to numerically higher SNF in mastitis halves 1 day post-IMI (Figure 30).

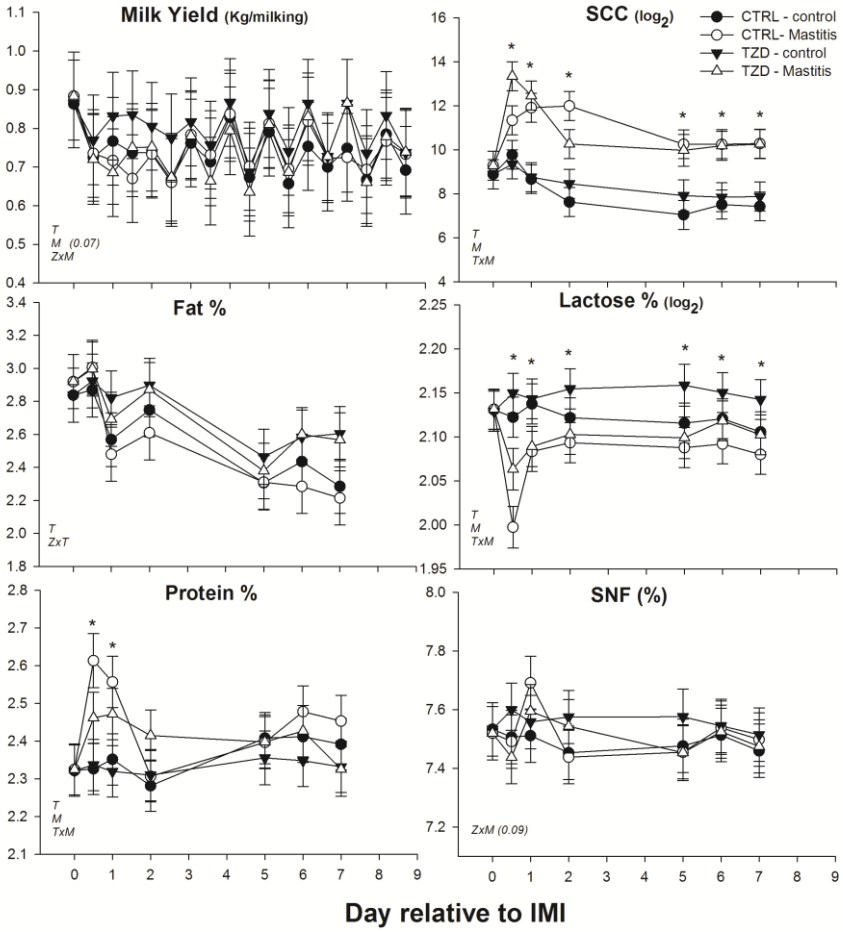

Figure 30. **.** Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with *Strep. uberis* (Mastitis) in *Saanen* dairy goat on milk yield, SCC and milk components. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect. Interaction of these effects is denoted by "x".

Results for the yield of milk components are reported in Figure 31. Lactose, protein, and fat yield were all decreased by time. Lactose yield tended to be lower in mastitis halves (*P = 0.08*) within 2 d post-IMI. Lactose and protein yield were not affected by TZD. Milk fat yield

was decreased by mastitis with a tendency for the TZD-treated animals towards greater fat yield than control groups over time (P = 0.11).

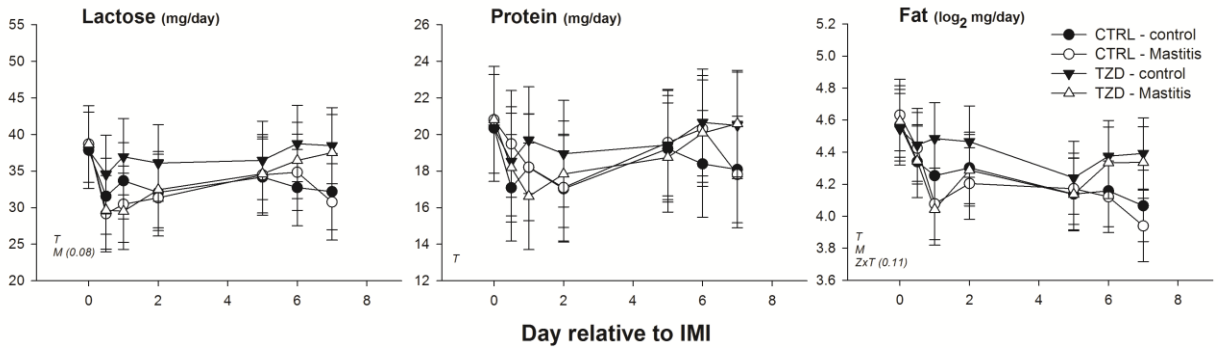

Figure 31. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with *Strep. uberis* (Mastitis) in *Saanen* dairy goat on yield of milk components. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect. Interaction of these effects is denoted by "x".

3.3 *Blood biomarkers*

Metabolism: results for glucose, NEFA, TAG, BHBA, creatinine, and urea are available in Figure 32. Glucose, NEFA, TAG, creatinine, and urea were affected by time but no effect of TZD was detected. Glucose had a peak 12h post-IMI, NEFA had increased while TAG decreased 12d post-IMI. An increase through time was also observed for creatinine and urea. BHBA plasma concentration was not affected by TZD or time.

Inflammation and minerals: main effects of Time, TZD, and their interactions on inflammatory biomarkers and Zn are presented in Figure 33. The blood level of the positive acute phase protein (+APP) haptoglobin was affected by time and a tendency for an effect of TZD through time (P = 0.07) was observed, where the TZD group had a greater increase after IMI (P = 0.006) compared with the CTRL group, remaining higher than CTRL up to 2 days post-IMI (P = 0.03) and returning to the level of the CTRL group within 3 d post-IMI. The other +APP ceruloplasmin was affected by time. Although, TZD group had numerically greater ceruloplasmin concentration over time when compared with the CTRL group, no TRT effect was

detected. The inflammatory marker and marker of neutrophils killing capacity myeloperoxidase (MPO) was not affected by TRT or Time, but a numerically larger MPO in TZD group was detected when compared with the CTRL group over time. The concentration of Zn was affected by time, where it decreased 12 h post-IMI in both groups.

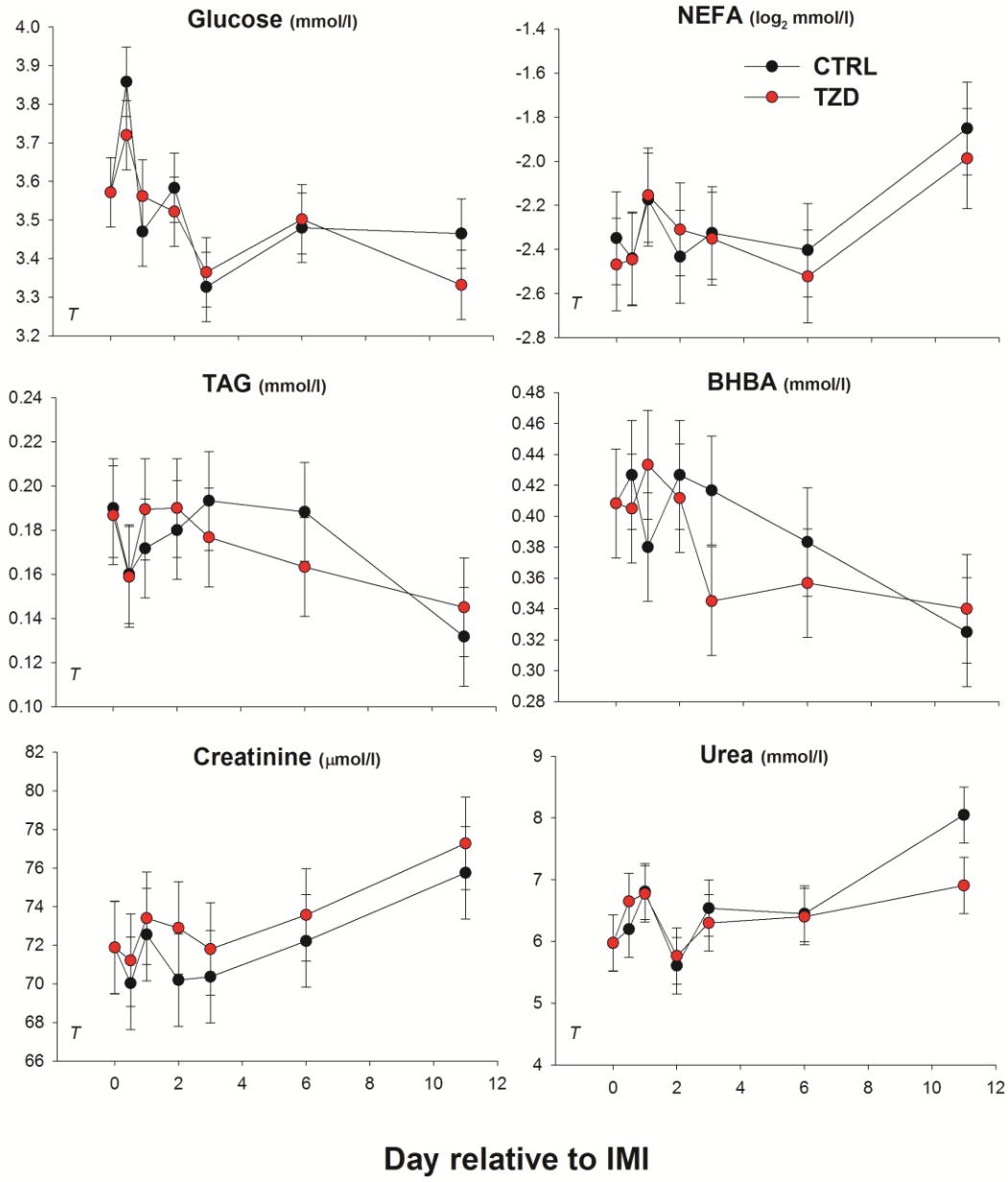

Figure 32. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with Strep. uberis (Mastitis) in *Saanen* dairy goat on metabolic parameters in blood. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect. Interaction of these effects is denoted by "x"

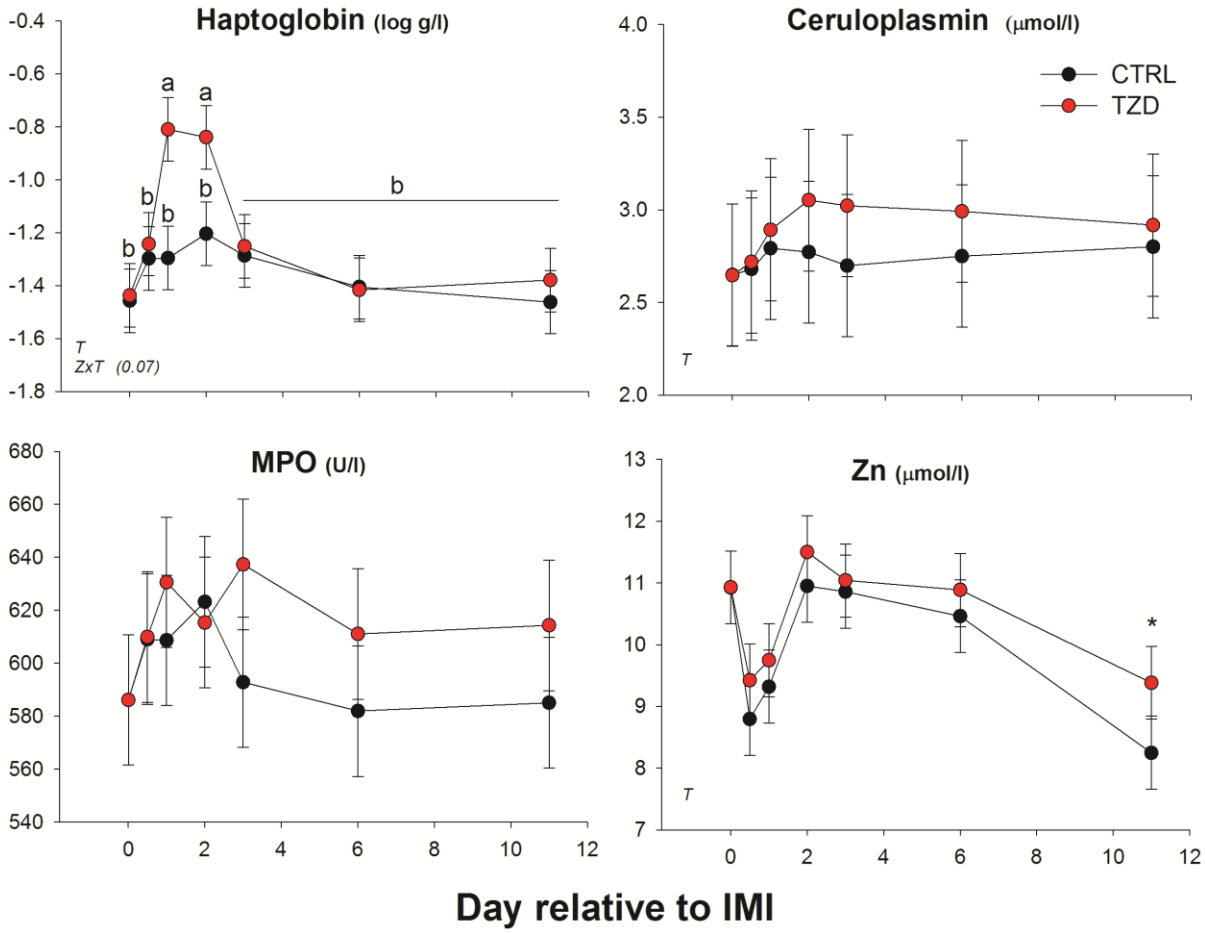

Figure 33. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with *Strep. uberis* (Mastitis) in *Saanen* dairy goat on inflammatory response markers (haptoglobin (log_2), ceruloplasmin and myeloperoxidase [MPO]) and Zinc level in blood. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect. Interaction of these effects is denoted by "x".

Liver function: main effects of Time on liver function biomarkers is presented in Figure 34. Plasma bilirubin was affected by time (T; P = 0.01) and an interaction effect of TRT×Time (TRT*T; P = 0.02) was detected. The amount of globulin was affected by Time (T; P = 0.01) and tended to be greater in the TZD group when compared with the control over time (TRT x T; P = 0.09). The plasma concentration of total protein, albumin, AST/GOT, cholesterol and PON was only affected by time (T; P ≤ 0.02), however they were not affected by TRT. GGT blood level was not affected by TRT or Time.

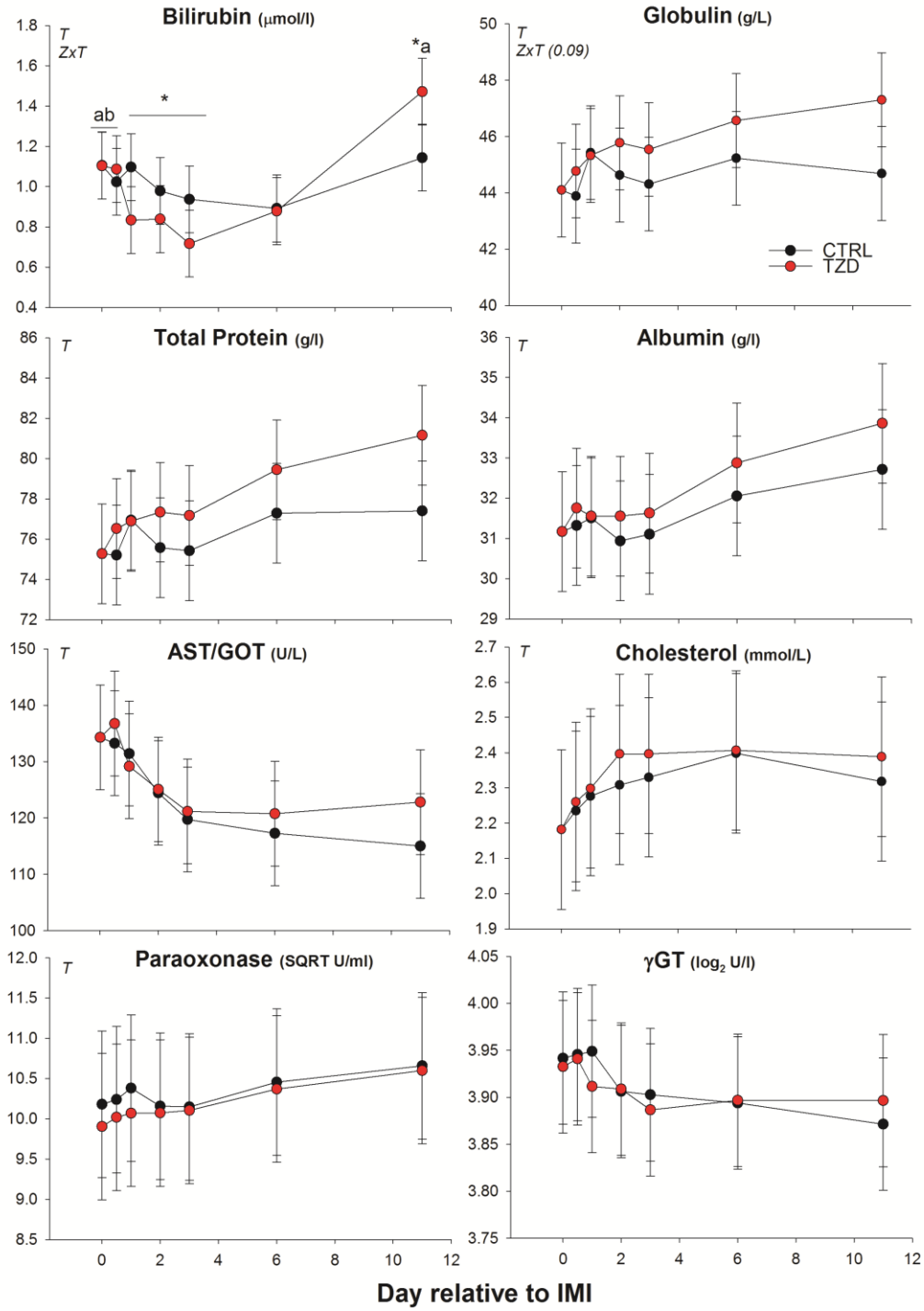

Figure 34. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with Strep. uberis (Mastitis) in Saanen dairy goat on liver function markers measured in blood [AST/GOT = aspartate aminotransferase, γGT = gamma-glutamyl transferase]. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect.

Oxidative stress: main effects on oxidative stress parameters are presented in Figure 35. Total reactive oxygen metabolites increased through Time and tended (P = 0.06) to be higher in TZD-treated goats. Ferric reducing antioxidant power was not affected by TZD or Time.

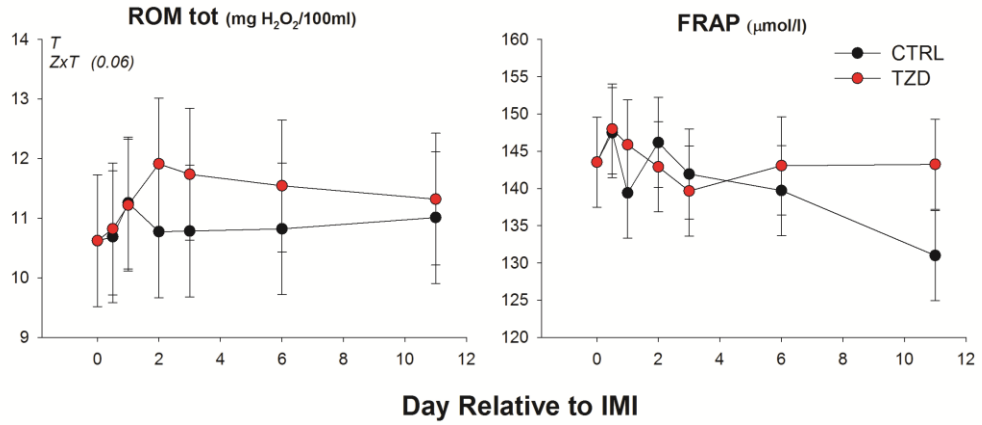

Figure 35. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with *Strep. uberis* (Mastitis) in *Saanen* dairy goat on oxidative stress [total reactive oxygen metabolites [ROM tot] and ferric reducing antioxidant power [FRAP]. Overall statistical significance is indicated in the plot as follow: T = time effect; M = mastitis effect; Z = TZD effect.

3.4 % PMN and Phagocytosis

Main effects on % PMN and phagocytosis are reported in Figure 36.

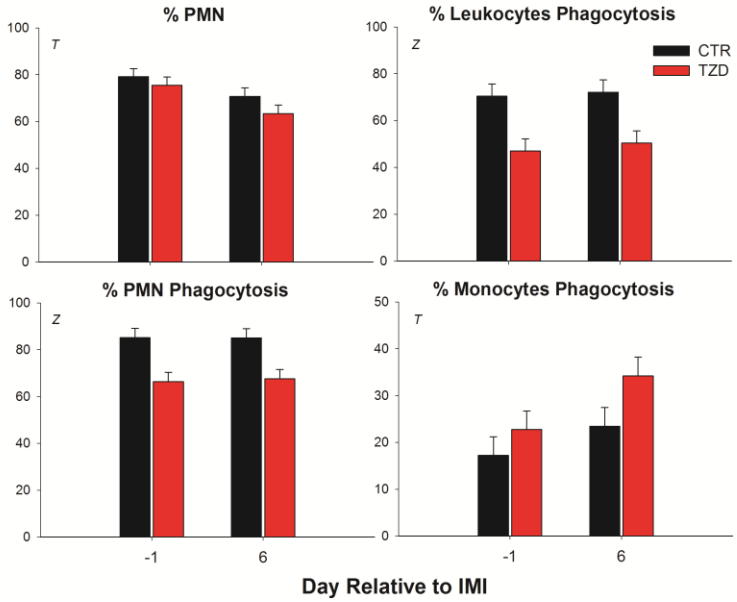

Figure 36. Effect of 2,4-thiazolidinedione (TZD) daily injection and intramammary infusion (IMI) with *Strep. uberis* (Mastitis) in *Saanen* dairy goat on % poymorphonucleated cells (PMN) and phagocytosis in all leukocytes, PMN, and monocytes. Overall statistical significance is indicated in the plot as follow: T = time effect; Z = TZD effect.

The % PMN was overall decreased from pre- to post-IMI in both treatments but not effect of TZD was detected. TZD treatment detected % of phagocytosis in all leukocyte, mainly driven by a decrease in PMN phagocytosis. The phagocytosis in monocytes was increased after IMI but was not significantly affected by TZD treatment.

3.5 Gene Expression

No effect of TZD in expression of measured genes was detected in liver (Figure 37).

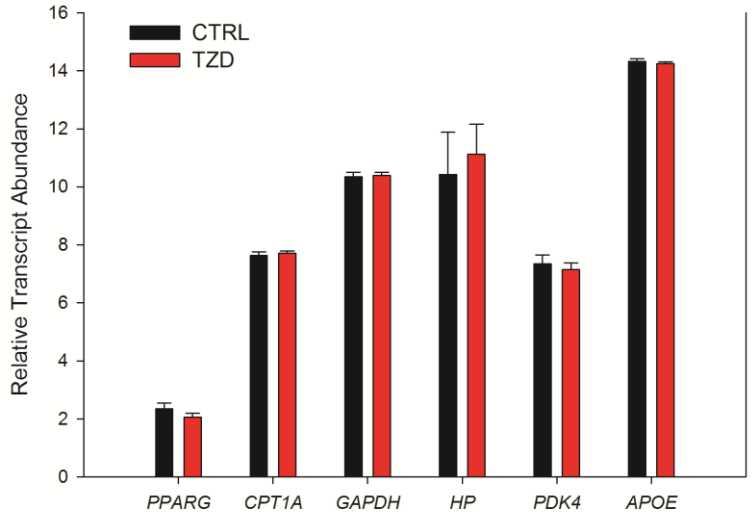

Figure 37. mRNA expression of the peroxisome proliferator-activated receptor gamma (*PPARG*), PPAR target genes (*APOE*, *CPT1A* and *PDK4*), haptoglobin gene (*HP*) and *GAPDH* in liver tissue of lactating goats treated with saline (CTRL) and 2,4-thiazolodinidenione (TZD).

In MEC pro-inflammatory related genes were all up-regulated by mastitis but none were affected by TZD treatment (Figure 38). The lipogenic *SCD1* gene was decreased by mastitis in all groups but was also overall lower in TZD-treated animals. (Figure 38).

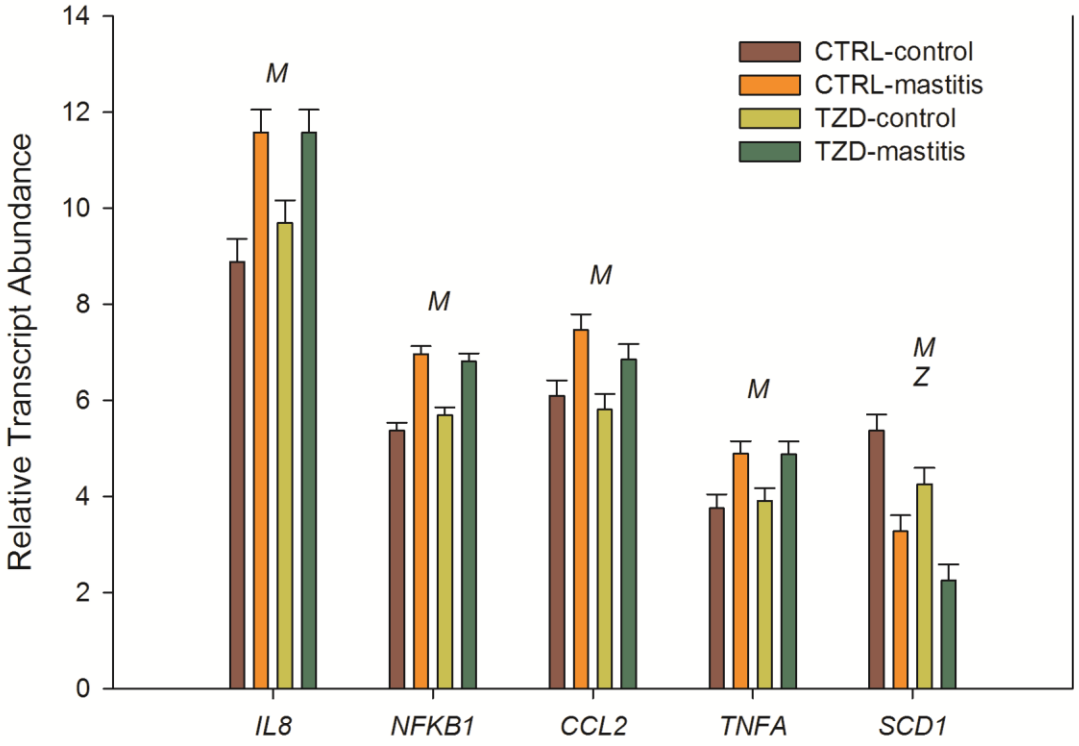

Figure 38. Effect on eexpression of inflammatory- and milk fat synthesis- (*SCD1*) related genes in mammary epithelial cells (MEC) after administration of 2,4-thiazolidinedione (TZD) or saline (control) in combination with intramammary infusion of *Strep uberis* to induce subclinical mastitis. Cells were isolated 5 days post-IMI. Letters in the plot denote overall statistical ($P \leq 0.05$) effect for mastitis (M) or TZD (Z).

Results for mRNA expression of genes related to inflammatory response in macrophages isolated from milk are reported in Figure 39. No effects were observed on expression of measured genes by TZD treatment with the exception of a tendency ($P = 0.09$) for a lower *IL4* expression. However, the genes related to the classical macrophage activation (i.e., *NFKB1, IL8* and *CCL2*) were affected by mastitis but no effect was detected for *TNFA*. Among the markers for the alternative macrophages activation *IL10* was not affected by TZD or mastitis whereas expression of *TGFB1* tended to be increased by mastitis induction ($P = 0.06$). The macrophage cell marker *CD14* was increased by mastitis induction. Expression of *NOS2* tended to be

increased by mastitis induction (P = 0.07). *PPARG* and *SUMO1* mRNA expression was not affected by TZD or mastitis (Figure 39).

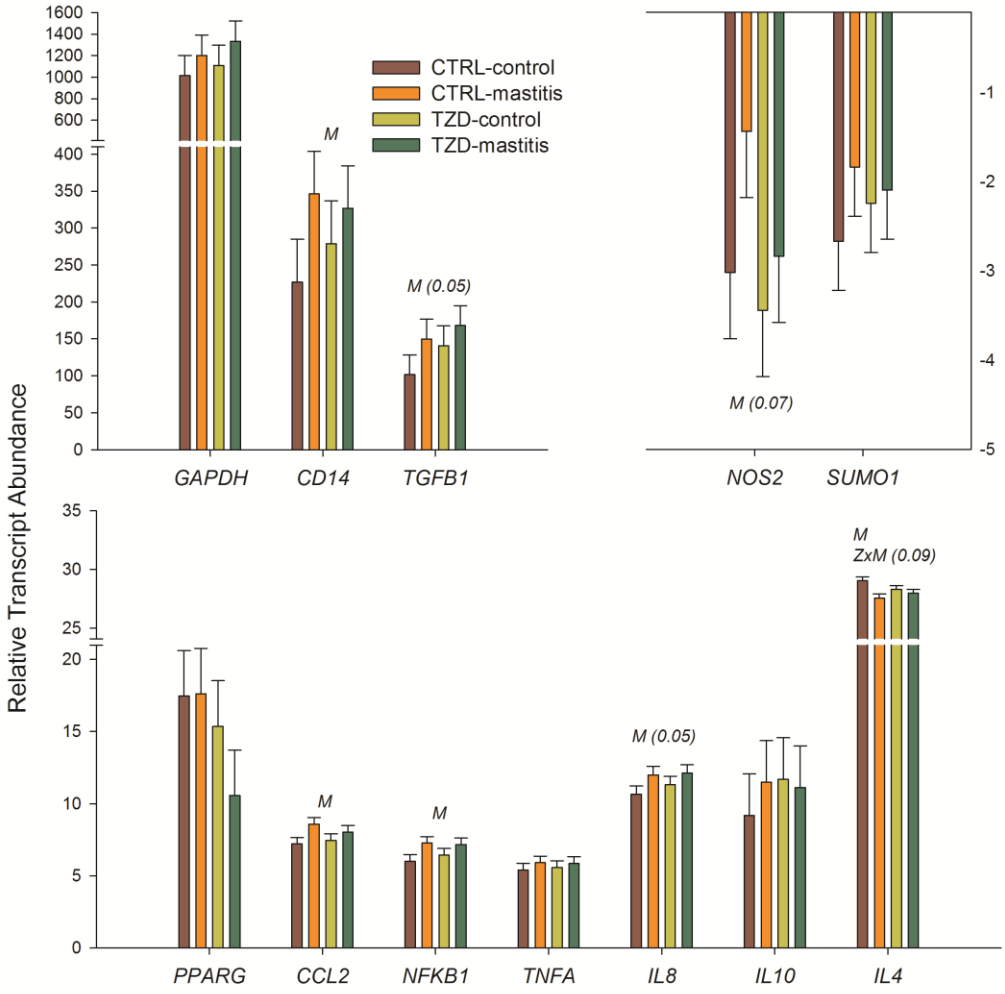

Figure 39. Effect on expression of monocytes/macrophages marker *CD14*, *GAPDH*, *PPARG*, and inflammatory-related genes in macrophages by administration of 2,4-thiazolidinedione (TZD) or saline (control) in combination with intramammary infusion of *Strep. uberis* in lactating dairy goats. Cells were isolated 5 days post-IMI. Letters in the plot denote overall statistical ($P \leq 0.05$) effect for mastitis (M), TZD (Z), or interaction (MxZ).

3 Discussion

Experiment 2 differs from Experiment 1 in several instances: a) goat were in early-mid vs. late lactation; b) goats grazed for 2 years prior the trial and were fed adequate amount of supplements vs. fed only hay and relatively low amount of supplements which results in goats having a good vs. poor body condition at the beginning of the study; c) mastitis was induced in one half vs. both halves and the control was nothing vs. saline intramammary infusion; d) TZD was injected for 2 and vs. 1 week and at prior IMI and at 10AM vs. 12 PM; e) goats were milked twice a day (approx.. 3.6 kg/d; higher milk yield) vs. 1 milking per day (approx. 1 kg/d; low milk yield); f) goats were individually fed vs. group fed; g) presence of only 2 vs. 4 groups.

Despite the above differences it is important to compare the two studies. Similar to what observed in Experiment 1, IMI induced a consistent increase in SCC that remained higher then control halves until the end of the trial. In the present study we did not observe lower SCC in TZD-treated vs. control animals as detected in Experiment 1. Different than Experiment 1 was the larger decrease in milk yield in the half receiving *Strep. uberis* after IMI in TZD vs. control goats. In Experiment 1 we had to remove two goats from the TZD group because they ceased milk production. This could have affected the final results by maintaining goats that did not substantially decrease milk yield. More evident in Experiment 2 compared to Experiment 1 was the maintenance of milk fat after IMI in TZD vs. control goats. This observation appears to partly support the proposed role of PPARγ in controlling milk fat synthesis (Bionaz et al., 2013, 2015); however, *SCD1*, the only genes related to milk fat synthesis measured in MEC in the present experiment, and a putative PPARγ target gene (Bionaz et al., 2013), was down-regulated 5 days post-IMI, indicating that either this gene is not a PPARγ target gene or that PPARγ was

not activated by TZD. Furthermore, *SCD1* is consistently down-regulated during milk fat depression (Harvatine et al., 2009). Expression of *SCD1* was also down-regulated by IMI in a similar manner between TZD and control goats which appears to be consistent with the large decrease in milk fat production in these halves. Decrease of milk fat synthesis after IMI is very often observed (Swanson et al., 2009 and Gunther et al., 2009). The fact that *SCD1* was lower expressed in TZD despite a higher milk fat synthesis in this vs. control goats is indicative of other genes and/or factors responsible for the maintenance of milk fat synthesis in TZD-treated animals.

The effect on milk fat synthesis cannot be ascribed in this case to NEFA concentrations (Palmquist, 2006), considering that this parameters increased after IMI despite the decrease in milk fat in all halves with the exception of the control half of TZD-treated goats. The same can be concluded looking at BHBA and TG values. None of them was significant different between groups. Interesting is also the fact that the half mammary from the control goats not receiving IMI responded similarly to the half mammary receiving IMI; however, this was not the case for the TZD-treated goats. It is unclear the reason for such "uncoupling", but it appears that the TZD treatment prevented any negative consequence to the other half of the mammary during IMI.

As for Experiment 1, our data clearly supports the effectiveness of the subclinical mastitis model used. The SCC were high with no clinical sign of mastitis, milk yield and components changed, the change in metabolic parameters such as the spike in glycaemia and momentarily decrease of Zn (Erskine et al., 1993), and the increase in haptoglobin and other inflammatory parameters, all indicate a successful induction of subclinical mastitis. Furthermore, we detected a robust increase in expression of most of the inflammatory-related genes in MEC and macrophages, indicating a robust inflammatory response. The above effects were however less

pronounced compared to Experiment 1. This can be partly due to the IMI in only one half in Experiment 2 compared to both halves in Experiment 1, at the least for the blood parameters. Different than Experiment 1 was the lack of significant increase in MPO in TZD vs. control animals. In Experiment 2 the MPO was only numerically higher in TZD vs. control goats. Interestingly, TZD-treated goats had a very large reduction of % leukocytes phagocytosis. It is unclear the reason for such an effect; however, these data in combination with MPO data might indicate that TZD had increased the total number of leukocytes in TZD-treated goats, Unfortunately a complete blood count was not performed to asses if this is true.

The haptoglobin level after IMI was different between the two experiments. In Experiment 1 the change was more gradual and haptoglobin remained high until the end of the trial with somewhat similar pattern in all animals; in Experiment 2 the change in haptoglobin was relatively modest and with a quick spike only in TZD-treated animals. None of the negative acute phase proteins was different between the two groups in Experiment 2 while differences were observed in Experiment 1. The modest effect on the above parameters can also be due to the difference in body condition and diet between the two experiments. In Experiment 1 data were indicative of a response to mastitis in our goats similar to cows experiencing negative energy balance (NEB). In our case the response to mastitis induction was different than cows in NEB. The modest metabolic and inflammatory response detected in Experiment 2 are indicative of a better, more robust, faster, and more effective response to mastitis in all animals despite TZD treatment likely due to a better nutrition and body condition. It can be also possible that the animals were already receiving more than adequate amount of any component which already maximizes the activation of PPARγ, especially vitamin A (Bionaz et al., 2015), and the use of TZD had not additional effect, as indicated by expression of genes in MEC and macrophages.

Goats treated with TZD likely have increased the production of immunoglobulin, as measured by globulin. As immunoglobulins are the product of the adaptive immune system, the higher globulin observed are indicative of a better adaptive immune system in goats treated with TZD.

From an oxidative stress point of view, the goats treated with TZD had an overall greater reactive oxygen species production (RMO tot). It is unclear the reason for such an increase. Considering the % phagocytosis and the absence of differences in MPO between the two groups it is plausible that the higher ROM tot was caused by a larger catabolic state in TZD vs. control animals. The capacity of TZD-treated animals to be protected from oxidative stress was not lower than controls as indicated by several antioxidant parameters, such as paraoxonase and FRAP.

As mammary epithelial cells and resident macrophages in mammary gland are the first line of defense against IMI, the inflammation related genes measured in MEC, as expected were all affected by mastitis. These findings are in accordance with other studies that demonstrated up-regulation of genes that stimulate the immune response, i.e., *IL8* and the migration stimulatory chemokines i.e., *CCL2*, upon intramammary infection with *Escherichia coli* and *Streptococcus uberis* in bovine mammary gland tissue (Buitenhuis et al., 2011; Moyes et al., 2009). However, no effect of TZD was observed for any of inflammation-related genes.

PPARγ is expressed in human macrophages (Ricote et al., 1998; Jiang et al., 1998). Other studies have observed a regulatory role of PPARγ on macrophage with a consequent decrease in inflammation (Ricote et al., 1998). In goats, *PPARG* is expressed in macrophages but was not affected by TZD or mastitis. Macrophages can be activated through two major pathways and by different cytokines. TNFα and NOS2 are markers of the classical activation of macrophages and

IL10 and TGF-β are markers of the alternative activation (Chawla, 2010). Some studies done in human macrophages suggested that PPARγ inhibits *TNFA* and *NOS2* expression (Meier et al., 2002 and Chung et al., 2000), which coupled with other studies indicate that PPARγ exerts anti-inflammatory effects by inducing the production of cytokines related to the alternative activation such as IL10 (Odegaard et al., 2007; Huang et al., 1999). However, the findings of our study are not consistent with what is reported in human macrophages, because the alternative activation markers measured *TGFB* and *IL10* were not affected by TZD and mastitis, except for a tendency of *TGFB* to be affected by mastitis. Moreover, expression of *TNFA* was not affected by TZD or mastitis, but the expression of *NFKB1, IL8* and *CCL2,* cytokines that trigger neutrophils chemotaxis, were increased by mastitis which is in accordance with Lewandowska et al. (2013) that demonstrated an up-regulation of *NFKB1* as well as *IL8* and *CCL2* in bovine macrophages infected with *Staphylococcus aureus.* A review published by Nagy et al. (2012) reported that PPARγ can maintain expression of *NOS2* repressed via Sumoylation of the specific nuclear receptor (Rigamonti et al., 2008). The expression of *SUMO1,* which codes for a protein of the post-translational modification complex (Hosseini et al., 2014), in goat macrophages was not affected by TZD or mastitis. Taking together these findings do not support the alternative activation of macrophages in goats by TZD.

The above blood parameters indicated that among the small effects observed in Experiment 2, the higher haptoglobin and lower bilirubin, indicated an overall better liver response in TZD vs. control goats. TZD had a strong effect on liver. Based on this consistent effect on the liver in both experiment 1 and 2. Based on this consistent effect on the liver we wanted to test if TZD had a nutrigenomic effect in the liver. Considering the abundance in expression of PPARα and PPARβ/δ compared to PPARγ in liver (Bionaz et al., 2013), it is

possible that TZD activated all PPAR isotypes and not only PPARγ. However, the data on PPAR-isotypes specific target genes in liver suggested that TZD did not activate any PPAR isotype. The lack of effect on haptoglobin expression in liver is in accordance with the level of haptoglobin in liver at two weeks post-IMI.

5 Conclusions

In conclusion, treatment of goats with TZD as a PPARγ activator had a modest effect on the immune system as well as on the liver function when goats were adequately fed and received a NRC recommended level of vitamins, including vitamin A. The lack of a large response could be due to adequate feeding because goats in Experiment 1 had a relatively larger response to inflammation compared to Experiment 2. Considering that animals in Experiment 2 were in better body condition and better fed that in Experiment 1, these findings suggest that nutrigenomic intervention maybe beneficial only during state of deficiency; thus, our data indicate that nutrigenomic interventions maybe be more effective only in the presence of dietary deficiencies. The lack of effects by TZD on expression of genes in liver, MEC, and macrophages cast doubts on the activation of PPARγ by TZD. Overall, we failed to demonstrate our hypothesis of a better response to mammary infections in goats fed adequate amounts of vitamin A. TZD treatment had some minor beneficial effect on the response to mastitis; thus, it does not appear to be particular beneficial. The most interesting findings are the large reduction of phagocytosis and the preservation of the milk fat synthesis in the untouched half of the mammary in TZD-treated goats. This might suggest a detrimental effect on phagocytosis and a beneficial effect on milk fat synthesis by TZD.

CHAPTER V

CONCLUSIONS

The original hypothesis of this study was that activation of PPARγ improves the response to mammary infections. To activate PPARγ we used in lactating dairy goats the putative PPARγ agonist 2,4-thiazolidinedione (TZD), and we induced subclinical mastitis by intramammary infusion of *Strep. uberis*. We verified that the subclinical mastitis model used in this study was an effective means to test our hypothesis. The use of TZD improved the response to a mammary infection in both studies with differences in magnitude and modes. In both studies, we observed an improved liver response to the mammary infection. The two studies differed, in the diet provided to the goats (Experiment 1 the diet can be considered at the low end of current recommendations and in Experiment 2 at the high end of current recommendation) and on the body condition score of the animals at the beginning of the study (thin in Experiment 1 and normal in Experiment 2). The effect of TZD on inflammatory response was larger in Experiment 1 vs. Experiment 2, indicating that the dietary level and/or the body condition of the animals strongly impacted the effect of the treatment. The immune system also was affected differently between the two studies. In Experiment 1 TZD improved the bacteria killing capacity of neutrophils without affecting phagocytosis while in Experiment 2 the killing capacity of the neutrophils was only increased numerically but a large decrease in phagocytosis was observed. Our data also suggested an improvement of the adaptive immune system in Experiment 2 by which globulin level in blood increased. We do not have an explanation for the decrease in phagocytosis in Experiment 2. It can concluded, that diet and/or body condition may have impacted the effect of TZD on the immune system. Commonality between the two experiments was the lack of effect of TZD on expression of inflammatory-related genes with a minor effect

on milk fat synthesis related genes (slightly increased in Experiment 1 but decreased in Experiment 2), indicating that TZD had no or only a minor nutrigenomic effect on PPARγ gene targets. In the present study we used TZD as agonist of PPARγ; data from the two experiments suggest that TZD failed to effectively activate PPARγ (and any of the PPAR isotypes) to a detectable level at least in the liver. Thus, the effects observed *in vivo* can be unrelated to the activation of PPARγ or are the results of a weak, although consistent and undetectable, activation of the nuclear receptor by TZD. In support of this are i*n vitro* data (see Appendix) where it was demonstrated that TZD is a not or only a very weak activator of PPARγ at physiological levels of 9-*cis*-retinoic acid.

Taking together the findings from both *in vivo* experiments, we can conclude that TZD (and, maybe, a weak activation of PPARγ by TZD) can be an effective means to improve the animal response to a mammary infection, but the effect can be considered important only when animals are not receiving an adequate, NRC recommended diet. In that case, the only large benefit observed was on milk fat synthesis. Thus, the original hypothesis can be considered partly demonstrated. This study revealed that reality is more complex that hypotheses and we remain with more questions than answers. After this study it remains to be determined: 1) if 2,4-thiazolidinedione is an effective agonist of PPARγ (and other PPAR isotypes) *in vivo* and, if so, we need to determine the magnitude of PPARγ activation by TZD; 2) the reason for the beneficial effect of TZD on the liver; 3) the effect of TZD on myeloperoxidase and neutrophils in general and the contrasting effect on phagocytosis based on the dietary level and/or body condition; and 4) the effect of TZD and the role of PPARγ in controlling milk fat synthesis.

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APPENDIX I

Streptococcus uberis 0140J complete genome. Sequence ID: ref\|NC_012004.1\|Length: 1852352N

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1038 bits(562)	0.0	759/855(89%)	9/855(1%)	Plus/Minus

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Query 1   TCATTTGTCCCACCTTCGACGGCTAGCTCCAAAT-GGTTACTCCACCGGCTTCGGGTGTT 59
          |||| | ||||||||| | ||||| ||||| ||| |||||| ||||| ||||||||||
Sbjct 19494 TCATCTATCCCACCTTAGGCGGCTGGCTCCTAATAGGTTACCTCACCGACTTCGGGTGTT 19435

Query 60   ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGC 119
          |||||||||||||||||||||||||||||||||| |||||||||||||||||| ||
Sbjct 19434 ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC 19375

Query 120  ATGCTGATCTACGATTACTAGCGATTCCAGCTTCATATAGTCGAGTTGCAGACTACAATC 179
           |||||||| |||||||||||||||| |||||| ||| |||||||||| ||||||||
Sbjct 19374 GTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATC 19315

Query 180  CGAACTGAGAACAACTTTATGGGATTTGCTTGACCTC-GCGGTTTCGCTGCCCTTTGTAT 238
          ||||||||||  ||||| | |||| ||||| | || ||| || || | | |||||
Sbjct 19314 CGAACTGAGATTGGCTTTAAGAGATTAGCTTGCCGTCACCGGCTT-GCGACTCGTTGTAC 19256

Query 239  TGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCC 298
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 19255 CAACCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC 19196

Query 299  CCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAA 358
          |||||||||||||||| | ||||||||||  ||||||||||||||||||||||||||
Sbjct 19195 CCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAGTGCCCAACTTAATGATGGCAA 19136

Query 359  CTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA 418
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APPENDIX II

Activation of PPAR by2,4-thiazolidinedione: dose-effect of 9-cis-retinoic acid and PPAR-isotype specific activation

Introduction

The dairy industry is in high need of new and relatively inexpensive approaches to prevent mastitis due to the high cost associated with this disease. The demonstration of a role of PPARγ in preventing mastitis can help to satisfy that need, especially considering that it can be activated by nutritional compounds that can be manipulated in the ration of animals (Bionaz et al., 2015).

Despite being 2,4-thiazolidinedione (**TZD**) a putative PPARγ agonist (Jain et al., 2013), in the Experiments 1 and 2 involving *in vivo* study on goats we did not observe any large effect on expression of PPARγ target genes in adipose tissue (Experiment 1) or mammary epithelial cells (Experiments 1 and 2). This was somewhat unexpected although consistent with data in bovine, especially for adipose tissue (Schoenberg and Overton, 2011). The data cast doubts on the capacity of TZD to effectively activate PPARγ *in vivo*. Prior data from our laboratory (Bionaz et al., 2015) indicated that TZD is a potent activator of PPAR in the presence of 9-*cis*-retinoic (**RA**) acid; however, in the experiments performed to obtained these data, 10 μM (approx. 3,000 ng/mL) of RA were used. The level of RA in plasma of dairy animals is in the order of 0.5 to 10 ng/mL; thus, at the least 300-fold lower than what used for the *in vitro* experiments. Furthermore, effects observed in Experiments 1 and 2 indicated that TZD affected liver function. PPARγ is very low expressed in liver (Bionaz et al., 2013); therefore, it can be the case that TZD activate other PPAR isotypes besides PPARγ.

The hypothesis of the present study is that TZD is a weak PPARγ activator and can activate also other PPAR isotypes. The objective of the present study were 1) to assess the activation of PPAR by TZD with incremental doses of RA starting from doses similar to what observed in plasma of dairy animals, and 2) to determine if TZD is a specific PPARγ activator by combining to the treatment with TZD a combination of PPAR isotype-specific inhibitors. We used a gene reporter assay in combination with primary goat mammary cells isolated from lactating goats to accomplish our objectives.

Material and Methods

Isolation of primary mammary goat cells

Primary mammary goat cells (PMG) were isolated from the mammary tissue collected at the euthanasia from three control goats from Experiment 2. Mammary tissues were collected in 50 mL tubes containing 25 mL of media (DMEM; Caisson Labs, cat# DML 10-500ML). Before isolation 50 mL of fetal bovine serum, 5 mL Penicillin Streptomycin (Pen/Strep; Amresco, cat#K952-100mL), and 1.5 mL of fungizone (Gibco, cat#15290-018) were added to the DMEM. After briefly (30 s) dipping the tissue in 70% ethanol to reduce superficial contamination, tissue pieces from the center of the mammary gland were washed by passing the tissue in 3 sterile and fresh sterile PBS with 10,000 U/mL of Pen/Strep. Afterwards, tissue was finely minced with a disposable sterile surgical blade (Miltex, cat#4-310) and samples was transferred to a 50 mL conical flask with cold collagenase solution [Worthington, cat#CLS 1; 75 mg/mL in HBSS (Gibco, cat#14025-092)] and softly shacked for 15 min (50 ml of collagenase solution per 1.5 g of tissue) at 37°C. The cell suspension was then filtered through 70 μm cell strainer (BD Falcon, cat#352350). The filtered cells were washed twice using sterile PBS and by centrifuging at 150 x

g for 2 min. Cell were washed twice. . Cells were resuspended in DMEM and counted using Moxi-Z (Orflo). Cells were immediately plated in 75 cm^2 flasks with DMEM and cultivated at 37°C with 5% CO_2 in a humidified incubator. When cells reached approx. 80-90% confluence were detached using 0.25% Trypsin and frozen in 2-4 cryovials containing Hyclone Adavencestem Cryo Med (GE Healthcare Hyclone) and immediately stored at -80°C overnight. The day after cells were transferred in liquid N for long-term storage.

Cell culture

PMG cells were cultivated in a high-glucose Dulbecco modified Eagle's medium - Nutrient Mixture F-12 (DMEM/F-12), which is a media formulated for mammalian cell culture (Cat# 11039-021, Thermo Fisher). The DMEM/F-12 was supplemented with 10% fetal bovine serum (Cat# 1500-500; Seradigm, Radnor, PA), penicillin/streptomycin (10 mL/L, Cat# 97063-708; Amresco, Solon, OH), and Fungizone® Antimycotic (3 ul/mL, Cat# 15290-018; Life Technologies, Grand Island, NY). When cells reached ~ 90% confluence were passed and seeded at 10,000 cells/well in 96-well plate 24 h prior plasmid transfection. Transfection of plasmids and treatments were applied using a reduced serum medium Opti-MEM® (Cat# 31985-070; Life Technologies, Grand Island, NY). Transfection of plasmids was performed using Lipofectamine® 3000 (Lipo3; Cat# L3000001; Life Technologies) at concentration of 0.3 µL/well. Cells were transfected with a luciferase 3X tandem PPRE (i.e., Peroxisome Proliferator-activated Receptor or PPAR response element) plasmid (Cat# 1015; Addgene, Cambridge, MA, USA). Cells were co-transfected with a renilla plasmid (Cat#16154; Life Technologies) at 50:1 ratio of luciferase/renilla plasmid in order to normalize the luciferase signal. The transfection efficiency was tracked by using an enhanced green fluorescent protein (EGFP) plasmid with a cytomegalovirus (CMV) promoter (generously provided by Siva Kolluri lab, Department of

Environmental and Molecular Toxicology, Oregon State University) at 50 ng/well. After 24 h of transfection cells were treated in biological and technical triplicates (see treatments in the following section). The luciferase and renilla signals were obtained 24 h post-treatment using a luciferase assay kit (Cat# PAE2920; Promega, Madison, WI) and read using a luminometer (Tropix 717, Applied Biosystems, Bedford, MA).

Treatments

Each treatment was performed in three biological replicates and technical triplicate or quadruplicate. Each experiment was performed in 3 separate batches at different days. In each experiment one of the treatment was to leave the transfected cells only with medium to use as control for each plate. The following chemical were used: PPARβ/σ antagonist GSK3787 (39-611-0, Tocris Biosciences, Bristol, United Kingdom), the synthetic PPAR□□ antagonist GW6471 (4618, Tocris Biosciences), the synthetic PPAR□ antagonist GW9662 (NC9970221, Cayman Chemical, Ann Arbor, Michigan, USA), the synthetic PPARγ agonist rosiglitazone (AGCR13570. AdipoGen International, San Diego, USA), 9-*cis*-retinoic acid (GR101, Enzo Life Sciences, Farmingdale, NY, USA), 2,4-thiazolidinedione (sc-216281, Santa Cruz Biotechnology, Dallas, TX, USA), DMSO (2166D, Research Organics, Cleveland, OH, USA), and ethanol for molecular biology (3916A, Decon Labs Inc., King of Prussia, PA, USA).

Experiment A: dose of 9-cis-retinoic acid

In order to test the dose of RA that aids TZD in activating PPAR we performed 3 experiments. In all 3 experiments we used 100 μM TZD. In the two of the experiments (experiments 1 and 3) we used DMSO as carried for the various compounds. The experiment 3 was performed using HP D300e Digital Dispenser, which uses only DMSO and not ethanol. In experiment 2 we used ethanol as carried for all compounds. In experiment 3 we normalized all

the wells with the same amount of DMSO. We did not normalized for experiment 1 and 2. The doses of RA tested were 0, 10, 100, 1000, and 10000 nM, corresponding to doses from 0.3 to 3,000 ng/mL.

Experiment B: TZD activation of PPAR isotypes

In order to test the activation of various PPAR isotypes by TZD we combined 100 μM of TZD with various combination of synthetic PPAR□□ PPARβ/σ, and PPAR□ antagonists at 10 μM concentration each.

Statistical analysis

Prior statistical analysis all data were checked for normal distribution of the residuals and outliers using PROC REG of SAS. Data with a studentized t >3.0 were removed. Normal distribution of residuals was assessed using PROC UNIVARIATE of SAS. Data with a significant (P<0.01) Shapiro-Wilk were considered not normally distributed and log transformed prior statistical analysis. Data were analyzed with the PROC GLIMMIX procedure of SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). Statistical significance and tendencies were declared at $P < 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

RESULTS

Activation of PPAR by TZD requires 9-cis-retinoic acid

Results of the 3 experiments to test the dose of 9-*cis*-retinoic acid necessary to aid in PPAR activation by TZD are reported in Figure 1. In experiment 1 we used up to 1000 nM of RA. Contrary to what expected and contrary to experiments 2 and 3, the data indicated that the combination of RA and TZD depressed activation of PPAR in PMG. Experiments 2 and 3 were

more consistent and indicated that dose of 10 μM is necessary for the activation of PPAR; however, the use of only RA without TZD had a same level of activation as the use of TZD+RA.

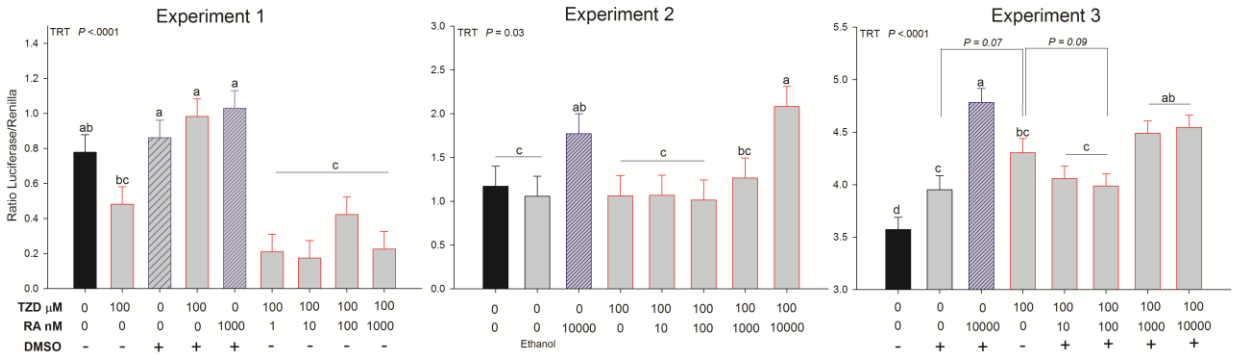

Figure 1. 2,4-thiazolidinedione (TZD) activates PPAR only in the presence of a large dose of 9-*cis*-retinoic acid (RA) in primary mammary goat cells transfected with 3xPPRE luciferase plasmid plus pTK-Green Renilla and subjected to the indicated treatments for 24h.

Activation of PPAR isotypes by 2,4-TZD

Experiments 1 and 3 were affected by treatment while a tendency of a treatment effect was observed in experiment 2 (P = 0.06). In experiments 1 and 2 the minimum activation of PPAR

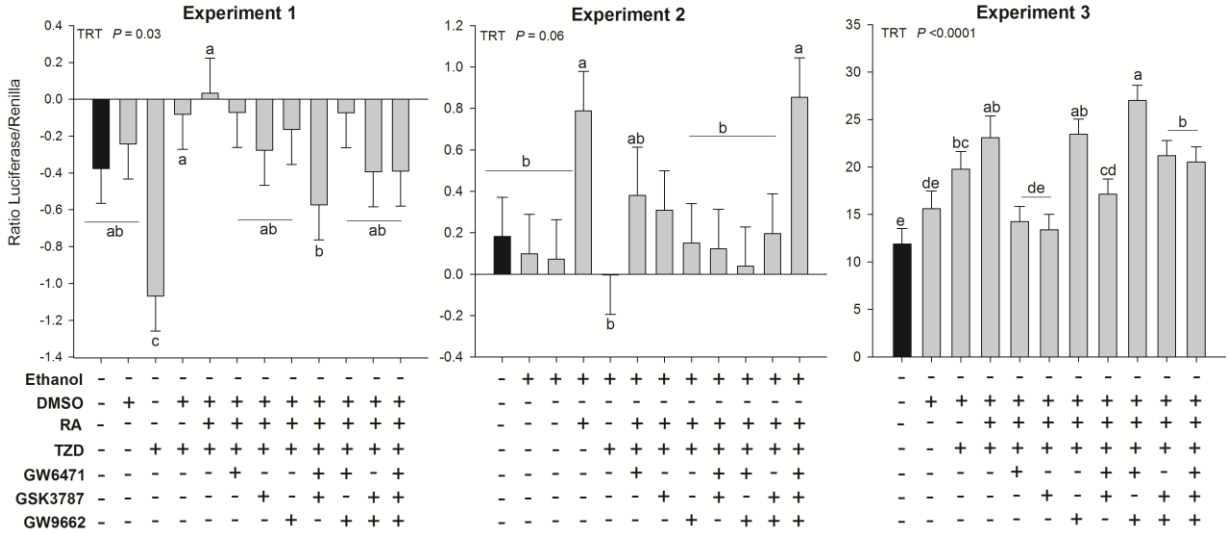

Figure 2. Primary mammary goat cells were treated for 24h with 100 μM of 2,4-thiazolidinedione (TZD) plus 9-cis-retinoic acid at a concentration of 10 μM, and with PPARβ/σ antagonist GSK3787, PPAR α antagonist GW-6471 and PPARγ antagonist GW9662.

was detected with the use of only TZD, which was even lower than lack of any treatment. The three experiments suggested that 2,4-TZD is a weak activator of PPARγ isotype while it seems to be a strong activator of PPARα and PPARβ/σ (Figure 2).

DISCUSSION

Bionaz et al. (2015) demonstrated that 2,4-TZD is a strong activator of PPAR but only in presence of 9-cis-retinoic acid a metabolite of vitamin A and a ligand for the Retinoic-X-Receptor, an obligate heterodimer for PPAR in bovine alveolar cells. The same pattern was observed in our in vitro experiment regarding to primary mammary goat cells by which to have a significant activation of PPAR by 2,4-TZD is needed 9-cis-retinoic acid at 10,000 nM.

Regarding to the potential activation of PPAR isotypes by 2-4,TZD the PPARα and PPARβ/σ were more affected than PPARγ.

CONCLUSIONS

All the above data are indicative of 2,4-TZD being a weak PPARγ activator.