Universitat Autònoma de Barcelona

# Strategies for the optimization of cow dry period

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MEMÒRIA PRESENTADA PER SÍLVIA PARÉS I RIERA

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# Resum

Les vaques lleteres necessiten un període no productiu entre lactacions per tal d'assolir alts nivells de producció en la següent lactació. Durant aquest període, especialment en animals altament productius, la glàndula mamària és més sensible a contraure infeccions que provocaran malestar a l'animal i pèrdues al productor. Per tal d'evitar-ho, actualment s'administren antibiòtics d'ampli espectre de forma preventiva, contribuint al problema de salut pública de les resistències bacterianes. Per tant, és interessant buscar estratègies per optimitzar l'eixugat i buscar alternatives a l'ús d'antibiòtic en la prevenció de les infeccions intramamàries durant l'eixugat.

En aquesta tesi hem treballat tres estratègies per millorar la immunitat i accelerar la involució de la glàndula mamària en l'eixugat. La primera estratègia es basa en la infusió de la proteïna de fase aguda M-SAA3, la qual s'ha descrit com a peça clau en la estimulació de la immunitat innata i com a proteïna activa en l'acceleració de la involució tissular mamària. En aquest cas, hem optimitzat la producció recombinant d'aquesta proteïna en un sistema lliure d'endotoxines per tal que es pugui administrar *in vivo*. La segona estratègia involucra l'administració de MMP-9 recombinant en un format nanoparticulat per tal que sigui més estable que la seva versió soluble i tingui un patró d'alliberament lent, per tal d'allargar la durada de l'efecte. La MMP-9 és la principal responsable de disgregar el teixit en l'eixugat, i per tant té un paper molt rellevant en la involució de la glàndula mamàra. Els resultats obtinguts demostren la seva funcionalitat *in vivo*. La tercera estratègia implica l'administració sistèmica de cabergolina, un bloquejador de prolactina, per tal d'accelerar l'aturada en la producció de la llet. S'ha estudiat el seu efecte en la involució i regeneració de la glàndula mamària per tal de comprovar que no es malmet la funcionalitat del teixit de cara a la següent lactació. Finalment, en l'últim estudi hem treballat en l'anàlisi dels fenòmens d'apoptosi, proliferació i senescència al voltant de l'eixugat, comparant MPC i PMC d'alta o baixa producció amb l'objectiu de trobar diferències que ens permetin trobar tractaments més efectius per cada grup.

## Summary

Dairy cows need a dry period between lactations in order to achieve high production levels on the next lactation. During the dry period, especially in high-productive animals, the mammary gland is more susceptible to contract infections that produce discomfort to the animal and economical losses to the farmer. In order to avoid intramammary infections, the common practice is to infuse antibiotics at dry-off as a preventive treatment, contributing to the emergence of bacterial resistances. Thus, it is interesting to find new strategies in order to optimize the dry period and avoid preventive antibiotic use.

In this thesis, we worked on three strategies to enhance immunity and accelerate involution of the mammary gland at dry-off. The first strategy is based on the infusion of the acute phase protein M-SAA3, which has been proven useful as an activator of the immune system and a stimulator of mammary involution. For this, we optimized the recombinant production of endotoxin-free M-SAA3 in order to allow its administration *in vivo*. The second strategy involves the administration of recombinant MMP-9 nanoparticles that are more stable than its soluble counterpart, and also show a slow-release pattern and thus a longer effect. MMP-9 is the main responsible protein for disaggregation of ECM and thus involution of the mammary gland. Our results proved its functionality *in vivo*. The third strategy involves the systemic administration of cabergoline, a prolactin blocker, in order to accelerate the cessation of milk production at dry-off; and also the analysis of tissular factors involved in involution and regeneration after cabergoline administration to assure that mammary involution and regeneration are not compromised for the next lactation. Finally, on the last study we evaluated the tissular differences between PMC and MPC producing high or low levels of milk on apoptosis and senescence factors among others, to find differences that would suggest new research lines in order to find more specific therapies for each group.

# List of abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| ALLIC | Asssociació lletera interprofessional de Catalunya |
| BAX | Bcl-2-associated X protein |
| BCIP/NBT | 5-bromo-4-chloro-3'indolyl-phospate / nitro blue tetrazolium |
| BCL-2 | B-cell lymphoma inhibitory factor |
| BHI | Brain-heart infusion |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium ion |
| CASP3 | Caspase 3 |
| cDNA | Complementary deoxyribonucleic acid |
| CK18 | Cytokeratin-18 |
| CMT | California mastitis test |
| CNS | Coagulase-negative *Staphilococcus* |
| DMEM | Dulbecco's Modified Eagle's medium |
| DTT | Dithiothreitol |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| EU | Endotoxic units |
| FBS | Fetal bovine serum |
| FESEM | Field emission scanning microscopy |
| GH | Growth hormone |
| HP | High producing |
| ICP-OES | Inductively coupled plasma optical emission spectroscopy |
| IGF-1 | Insulin-like growth factor-1 |
| IGFBP | Insulin-like growth factor binding proteins |
| IMI | Intramammary infection |
| $K^+$ | Potassium ion |
| LAL | Limulus amebocyte lisate |
| LAP | Lingual antimicrobial peptide |
| LIF | Cytokine leukemia inhibitory factor |
| LP | Low producing |

| | |
|---|---|
| LPS | Lipopolisaccharide |
| MALDI-TOF | Matrix-assisted laser desorption/ionization – time of flight |
| MMP-9 | Matrix metalloproteinase-9 |
| MPC | Multiparous cows |
| mRNA | Messenger ribonucleic acid |
| M-SAA3 | Mammary serum amyloid A3 |
| $Na^+$ | Sodium ion |
| NBF | Neutral buffered formaline solution |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PMC | Primiparous cows |
| PRL | Prolactin |
| PRLR | Prolactin receptor |
| rER | rough endoplasmic reticulum |
| RIA | Radioimmunoassay |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| RT | Room temperature |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| RU | Relative units |
| SCC | Somatic cell counts |
| SDS | Sodium dodecyl sulfate |
| SEC | Secretory epithelial cells |
| SIRT-1 | Sirtuin-1 |
| TBST | Tris buffered saline + Tween 20 |
| TEM | Transmission electron microscopy |
| TFA | Trifluoroacetic acid |
| TGF-$\alpha$ | Transforming growth factor-alpha |
| TGF-$\beta$ | Transforming growth factor-beta |
| TGF-$\beta$1 | Transforming growth factor-beta 1 |
| TIMP | Tissue inhibitor of metalloproteinase |
| TLR | Toll-like receptor |
| UAB | Universitat Autònoma de Barcelona |

# Table of contents

# CHAPTER I – General introduction

Dairy cows have been genetically selected since early domestication to produce high milk yields. To meet the growing global demand and to keep dairy farming profitable at the same time, average milk yield per cow, as well as average herd size, has increased in recent decades (De Vliegher et al, 2012). Nowadays, dairy cows are able to produce up to 50 kg of milk a day when they are on their peak production days, doubling the milk yield in the last 20 years. Despite the tremendous progress in genetics, nutrition, and management, today's milk production per human capita is 14% less than it was in 1960 (Gerosa and Skoet, 2012), and it is still necessary to improve the efficiency of milk production. Also, to sustainably contribute to food security of a growing and richer world population, livestock production systems are challenged to increase production levels while reducing environmental impact, being economically viable, and socially responsible (Van Wagenberg, 2017).

The process of selection has compromised other aspects of dairy production such as the animal's metabolism and health. Udder health and cow dry period are critical issues in dairy production and will be the focus of study of this thesis that seeks to contribute to the improvement of the productivity and welfare of dairy cows.

## Morphology and development of the mammary gland

### Anatomy of the mammary gland

The cow has four mammary glands grouped into a structure called an udder, which is located in the inguinal region of the cow's body.

The udder is divided in two halves that are separated by the median suspensory ligament, which is responsible for the attachment of the udder to the body.

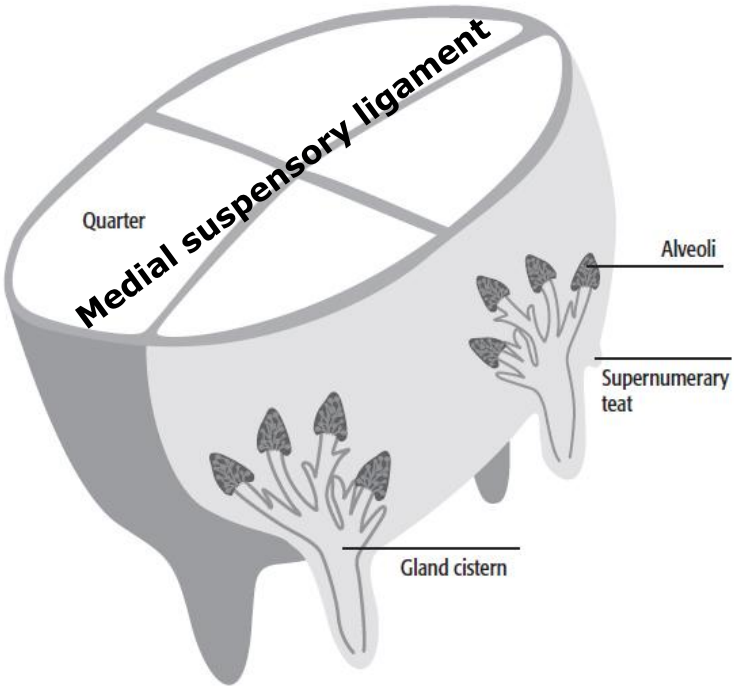


**Figure 3.** Scheme of cow's udder (adapted from heiferinyourtank.typepad.com)

Milk is secreted by alveoli, which are grouped into small clusters called lobules. The alveoli are lined with two layers of epithelial secretory cells, the functional unit of the mammary gland that synthesizes and secretes milk. Lobules are surrounded by connective tissue capsules (Schmidt, 1971; Turner, 1939,

1952), often referred to as stroma. Groups of lobules form larger structures called lobes, and are considered to be the parenchymal elements. The entire mammary gland is composed of lobules and lobes. The lobules are drained by small ducts. The smaller ducts or capillary milk ducts are lined with a single layer of epithelial cells and are capable of secretion. The ducts get progressively larger until they reach a cistern, which is connected to a teat by which milk is released upon suckling or machine milking (Schmidt, 1971; Turner, 1939, 1952; Gorewit, 1988).

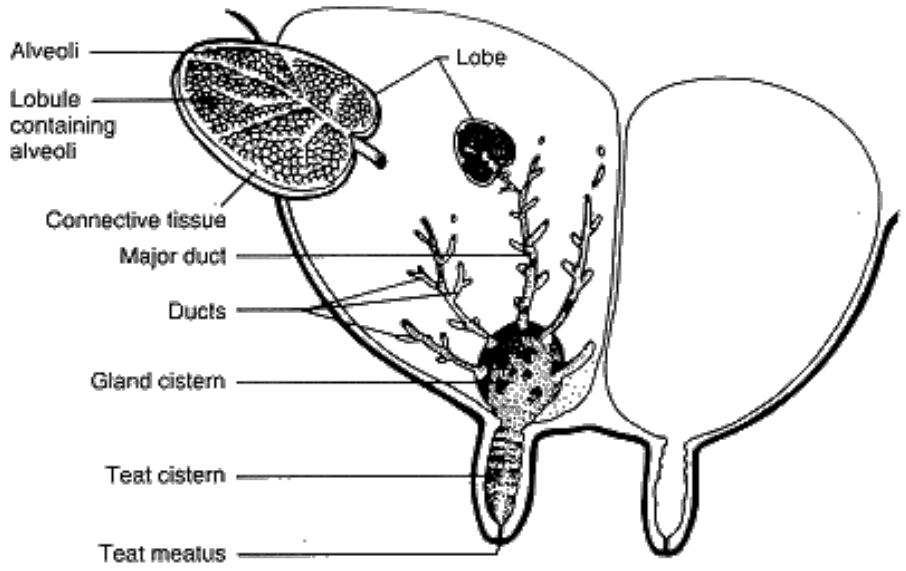


**Figure 4.** Inner structure of the mammary gland (Adapted from studyblue.com).

## Cell types in the mammary gland

There are three basic supporting tissues in the mammary gland, which are known as the mammary fat pad, stroma, and parenchyma. The mammary fat pad is adipose tissue infiltrated by vascular endothelial cells where the duct system grows within. The stroma is the connective tissue that surrounds the alveoli and provides structural support and anchorage to the mammary gland,

and it is generally composed of a number of different cell types and the extracellular matrix (ECM) (Borena et al., 2013). Resident tissue immune cells such as neutrophils and macrophages are present in the stromal component and can associate with the connective tissue structures and epithelial components. The parenchyma is the epithelial tissue, which contains a duct system and lobes.

Two main cell types comprise the mammary epithelium: basal and luminal. The basal epithelium consists of myoepithelial cells, which generate the outer layer of the gland, and a small population of stem cells, which supply the different cell types. The luminal epithelium forms ducts and secretory alveoli and contains populations of cells defined by their hormone receptor status. Together with the myoepithelium, the luminal epithelium generates a bi-layered, tubular structure that allows form to meet function during lactation when the outer myoepithelial cells contract to squeeze milk from the inner alveolar luminal milk producing cells. (Macias and Hinck, 2012).

Prior to parturition, percent area occupied by stroma, epithelium and lumen are at average 33% each. In contrast, in non-pregnant post pubertal heifers the numbers are 84%, 12% and 3% respectively (Sejrsen et al. 1982, Akers et al. 2006).

The cycle of pregnancy-associated mammary gland proliferation, differentiation, and involution occurs many times during the lifespan of diary cattle (Turner and Huynh, 1991). Long-lived populations of stem cells, which

have a near-infinite propensity to produce functional mammary gland cells, are responsible for this sequence of events (Holland and Holland, 2005).

## Secretory epithelial cells and milk production

The main functions of secretory epithelial cells (SEC) are to produce, assemble, modify, and secrete milk constituents into the luminal space within the alveolus. Hallmarks of a milk-producing SEC include abundant mitochondria, plentiful rough endoplasmic reticulum (rER), and a prominent supra-nuclear Golgi apparatus, indicative of the cell's high protein production and modification requirements. Several substances can pass the cell membrane from the blood stream (water, minerals, vitamins, immunoglobulins), whilst others need transporters and are produced in the secretory cells (proteins, fat, lactose).

Milk fatty acids are secreted following assembly by both the intra-cellular *de novo* synthesis of short-chain fatty acids, and the cleavage of pre-formed long-chain fatty acids that are derived from the blood supply. Milk proteins are generally classed in two fractions – casein (α-, β-, κ-, and γ-caseins) and whey (predominantly comprised of α-lactalbumin and β-lactoglobulin). The amino acid precursors are transported through specific transport channels. The lactose component of milk relies solely on glucose as the sugar precursor, and lactose is enzymatically formed in the Golgi apparatus prior to its secretion with proteins.

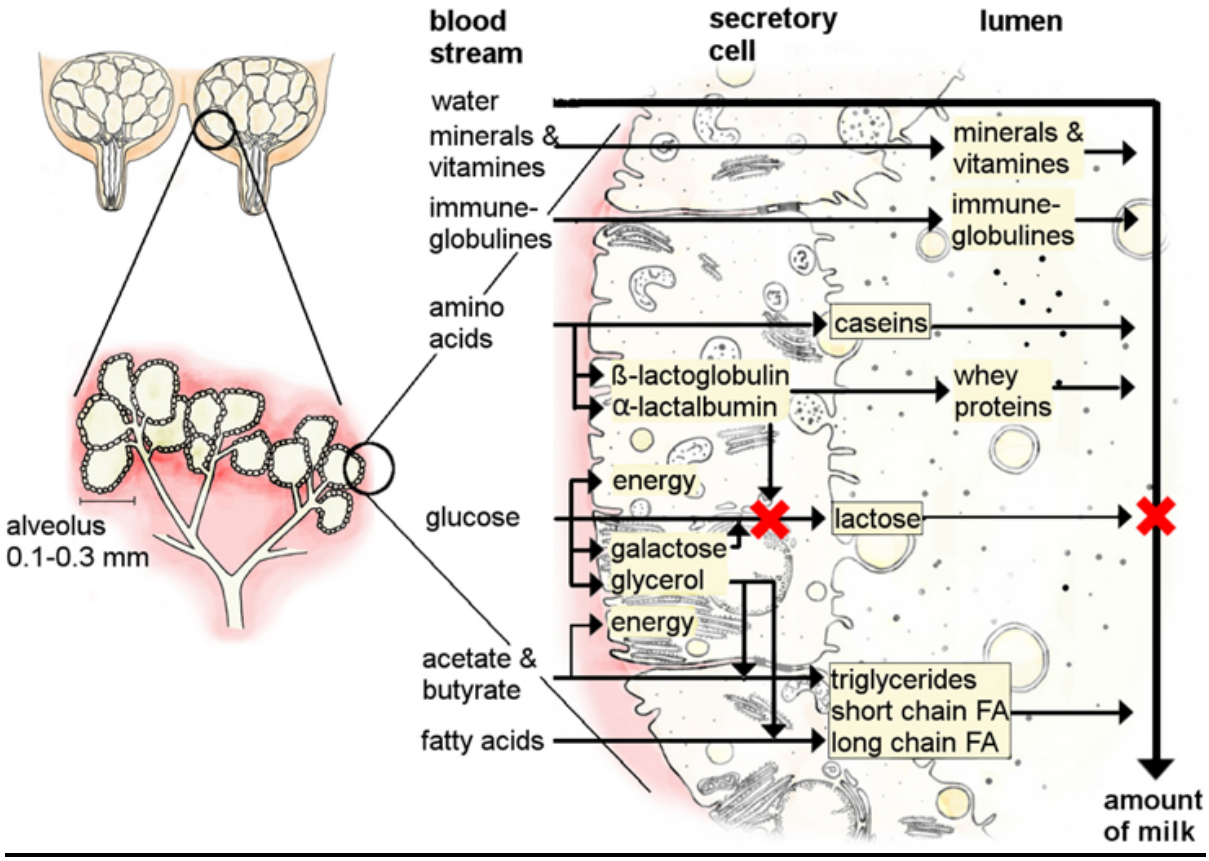


**Figure 5.** Structure of the alveoli and secretory tissue (image adapted from Strucken et al., 2015).

### Mammary stem cells and their progeny

The bovine mammary gland is a unique organ with regard to its frequently repeating cycles of growth and involution throughout the life of an animal. Over the last decades, evidence for the existence of mammary stem cells (MaSC) has been accumulated mainly in mice and humans (Borena et al., 2013). Some research groups such as Ellis and Capuco, 2002; and Holland et al., 2003 were able to isolate and describe this cell population from bovine mammary glands.

The two major categories of stem cells are embryonic stem cells and adult stem cells, which differ with regard to their tissues of origin and ability to

generate multiple cell lineages. Embryonic stem cells can be obtained from the embryo and are capable to differentiate into any cell type. On the other hand, adult stem cells are found in the tissues of adult organisms, and their regenerative potentials, under normal circumstances, are more limited than those of embryonic stem cells. These cells are multipotent because they are capable of forming the differentiated cell types of related lineage found within the tissue in which they reside (Capuco and Ellis, 2013).

Mammary stem cells (MaSC) are defined as cells that can generate the ductal and lobular components of the mammary epithelial tree, complete with all the cell types of the mammary epithelium, as well as having the ability to self-renew (Stingl 2009; Osiska et al., 2014).

Differentiation of mammary stem cells is crucial for the development of the mammary gland. The mammary stem cell lineage pathway begins with the adult stem cell and ends with the functionally active terminally differentiated cell. In the pathway between these 2 cells there are progenitor and intermediate cell types (Holland and Holland, 2005).

One of the main roles of adult stem cells is to proliferate, ensuring organ growth and maintaining tissue homeostasis of the resident organ. During proliferation, stem cells divide symmetrically and when maintaining tissue homeostasis, they divide asymmetrically. Symmetrical division of a stem cell involves mitotic division of the cell into two daughter stem cells or terminally differentiated cells. During asymmetrical division, the stem cell produces one daughter stem cell and one differentiated cell, both cells possessing dissimilar

phenotypes. Although adult stem cells have an unlimited proliferation capacity, they divide infrequently *in situ*. Progenitor cells, the progeny of stem cells, have a more limited proliferation capacity in comparison with stem cells, but divide more frequently. Lineage restricted progenitor cells have a tremendous proliferation capacity and are responsible for the generation of differentiated cells to ensure ductal growth, alveolar development and ultimately milk production (Choudhary, 2014).

## Development of the mammary gland

During its development, the mammary gland progresses through distinct stages: the embryonic and fetal period, when the mammary system develops; the neonatal and prepubertal periods of isometric growth; the peripubertal period when the gland grows allometrically and ducts elongate and branch; and sexual maturity, when branching continues and alveolar buds form (Hovey et al. 2002).

The mammary gland consists of a fat pad of mesodermal origin into which epithelial cells of ectodermal origin proliferate (Sheffield, 1988).

In all mammals, the mammary glands arise from a localized thickening of the ectoderm. The mammary bud is formed by the elevation of an epidermal "mammary crest" and a milk-line that forms on both sides of the midventral line in the embryo (Sheffield, 1988, Hovey et al. 2002). Ductal morphogenesis is primarily regulated by estrogen and growth hormone (GH), and the proliferative phase of alveolar morphogenesis requires progesterone and prolactin (PRL) (Neville et al. 2002).

Calves' mammary glands contain only rudimentary ducts with small club-like ends that regress within a short time after birth. They are composed of a teat, a primary duct, and several secondary ducts that end in modestly branched lobules positioned at the periphery of the mammary fat pad (Sheffield, 1988, Hovey et al. 2002). In heifers, the parenchyma initially grows isometrically relative to overall body development, but at about 3 months of age, prior to puberty, growth becomes allometric (Sinha and Tucker, 1969).

The mammary gland must undergo numerous changes to prepare for lactation. These changes require both gland maturation and alveologenesis and are primarily under the control of progesterone and PRL (Macias and Hinck, 2012).

**Lactogenesis**, also called mammary differentiation, starts at mid pregnancy with the progressive expression of many of the genes involved in the synthesis of milk components (Neville et al. 2002). When pregnancy begins, hormonal stimulus from PRL and progesterone induce a new developmental stage on the mammary gland, finally differentiating the tissue to the final functional stage. Around parturition, lactogenesis becomes complete and the gland undergoes a set of developmental processes that lead to the secretion of colostrum, and then milk. This last event is characterized molecularly by a further increase in expression of milk protein genes, closure of tight junctions between alveolar cells, and the movement of citoplasmatic lipid droplets and casein micelles into the alveolar lumina (Neville et al. 2002).

After termination of the suckling (or milking) stimulus, involution occurs and the secretory epithelium is remodeled to return almost to the pre-pregnant state (Neville *et al*. 2002).

## The lactation cycle

The standard lactation cycle in dairy cows typically lasts around one year. The cycle is split into four phases: early, mid, and late lactation, and the dry period. Lactation starts at calving and goes by for about 300 days.

Forty days after calving, cows are usually inseminated again, so that they can give birth and start a new cycle about one year after calving.

About sixty days before the expected calving date, cows are dried-off so that the mammary gland can involute and regenerate to achieve high production levels on the next lactation. This non-lactating period is called **the dry period**.

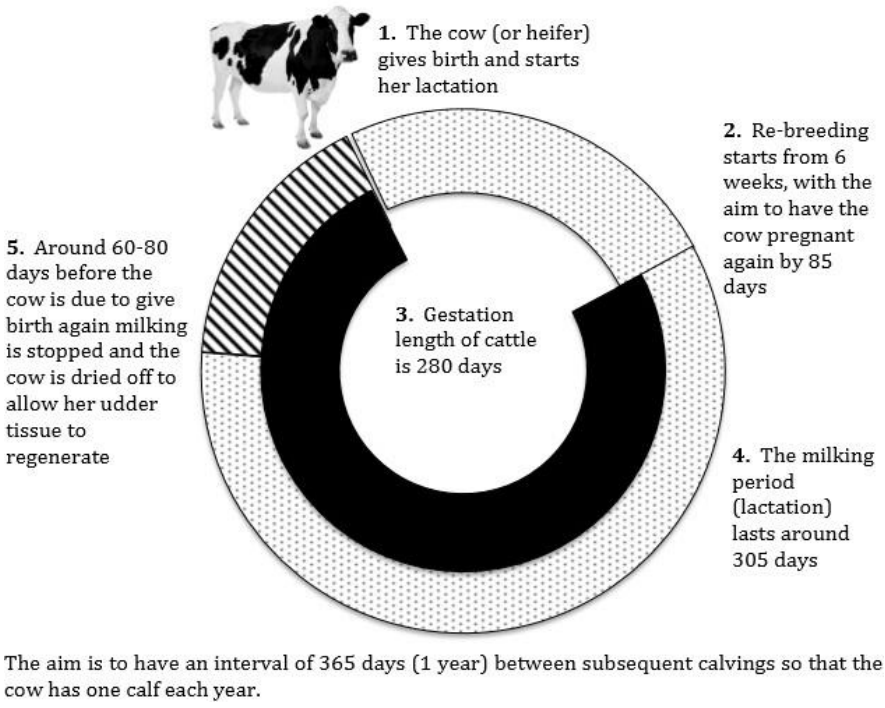


**Figure 1.** Scheme of the production cycle in dairy cows (Adapted from www.studyblue.com).

During the lactation cycle, the mammary gland and also the entire system of the animal undergo changes due to the metabolic demand of milk production, pregnancy and the hormonal changes involved.

Also, while lactation progresses, milk composition changes, and daily milk production decreases because some secretory cells enter a senescent state, being the dry period necessary to regenerate the mammary gland.

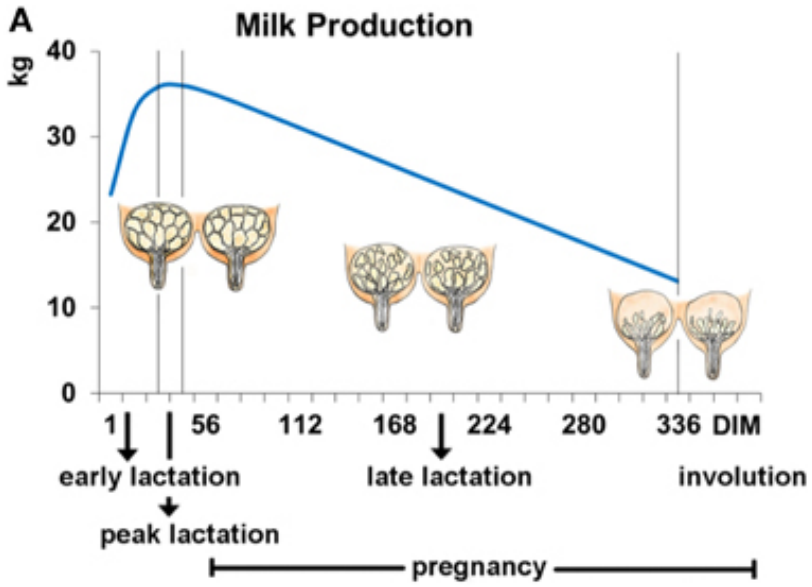


**Figure 2.** Changes in milk yield during the lactation cycle in dairy cows (Adapted from Strucken et al., 2015).

## Hormonal regulation of the lactation cycle

Galactopoietic hormones are those required for the maintenance of lactation, and include the reproductive hormones, oxytocin, PRL, and various metabolic hormones.

During pregnancy, progesterone increases until a few days prior to parturition, when it decreases. Proliferation of the mammary epithelium is dependent on

estrogen and progesterone (Hovey et al., 2002). However, estrogen also stimulates secretion of insulin-like growth factor-1 (IGF-1) from the stromal cells of the udder and thereby causes growth of epithelial cells. Progesterone helps mammary differentiation while inhibiting milk production. On the other hand, concentration of PRL, the main galactopoietic hormone, starts rising a few days prior to parturition, reaching the peak at parturition. In fact, no mammogenic activity occurs in the absence of GH and PRL (Hovey et al., 2002).

At the beginning of lactation, PRL has a predominant effect on the mammary tissue in order to differentiate the epithelial component to a functional stage. Also, the withdrawal of progesterone triggers the second phase of lactogenesis and the start of milk production (Neville et al., 2002).

Once lactation is started, suckling and removal of milk from the mammary gland maintain it. Oxytocin responds to suckling stimulus acting on the myoepithelial cells to produce milk ejection. Milk production is controlled by the lactogenic hormones PRL and GH during lactogenesis and galactopoiesis. Both PRL and GH are essential for the transition from a proliferative to a lactating mammary gland. Although GH dominates over PRL during galactopoiesis in ruminants, PRL is of fundamental importance in the development and differentiation of the mammary gland. While PRL seems to act directly on mammary tissue, the effects of GH are considered to be indirect, mediated through stimulation of IGF-1 production (Hovey et al., 2002). IGF-1 is involved in the regulation of somatic growth and cellular proliferation, but its action is controlled by a family of proteins, the insulin-like

growth factor binding proteins (IGFBP), that bind to IGF with different affinities. They have been shown to either inhibit or enhance biological effects of IGF-1 (Cohick, 1998). Whereas GH stimulates synthesis of IGF-1, PRL optimizes its action by suppressing the expression of IGFBP5, an inhibitor of IGF's action. An increasing list of local growth factors besides the IGFs has been shown to modulate survival and apoptosis in the mammary gland. PRL acts both directly and through mammary epithelial factors and activates several transcription factors. Binding of PRL to its receptor leads to the formation of receptor dimers, which mediate various cellular events. These include the activation of signal transduction pathways. Further effects include the modification of intra-cellular ion levels through the activation of potassium ($K^+$) and calcium ($Ca^{2+}$) ion channels.

The resulting downstream cellular responses lead to increased transcription of the milk-specific proteins $\alpha$-lactalbumin and caseins, maintenance of the differentiated secretory phenotype and promotion of cell survival. One of the key signaling molecules activated by PRL is Stat-5. Several of these proteins or polypeptides are also cytokines. The epidermal growth factors (EGFs) and members of the transforming growth factor family (TGF-$\beta$) are examples of such factors, which have been implicated in mammary function in ruminants. EGFs exert direct mitogenic effects and bind with varying affinity to the different EGF-binding proteins. There are low levels of TGF-$\alpha$ and TGF-$\beta$1 expression during lactogenesis and galactopoiesis, and higher levels during mammogenesis in heifers and during involution.

## The Dry Period

During lactation, the number of mammary epithelial cells gradually declines to approximately 50% of the number initially present at the onset of lactation. The number of cells must therefore be restored fully prior to the next lactation, so that the following lactation is not compromised (Capuco and Ellis, 2013).

Because of that, dairy cows need to undergo a dry period in order to reach maximum production levels on the following lactation. The dry period is critical to regenerate mammary epithelial cells (Choudhary, 2014) and allow mammary gland to involute and regenerate in order to be able carry on with the following lactation. It is the time to replace the senescent cells that have lost production ability, with new epithelial cells that can be used to produce milk during the next lactation (Choudhary, 2014).

The dry period is also associated to negative consequences due to milk accumulation in the mammary gland, also known as milk stasis. When there is too much pressure inside the gland, there is milk leakage, the teat canal plug can not be formed, and opportunist bacteria from the environment can enter the mammary gland and colonize it. As milk is an excellent culture broth for bacteria, and the temperature inside the gland is around 38ºC, this can easily evolve to an intramammary infection (IMI) (Halasa et al., 2007). Moreover, this situation is exacerbated because the dry period of dairy cows is highly affected by hormonal regulation due to the undergoing gestation. Sixty days prior to due date, hormonal levels of galactopoietic hormones are starting to rise, hampering the involution process of the mammary gland.

**The two phases of the dry period:**

There are two main phases characterized in the dry period of the dairy cow: an initial phase of **involution**, mainly due by apoptosis (wilde et al., 1997), followed by a **regeneration** stage in which the tissue is differentiated again for milk production. Even though we speak of two phases, both phenomena coexist at the same time, and the ratio between them is what matters (Stefanon et al., 2002).

Current management of dairy cows results in significant overlap of lactation and pregnancy, such that these animals are typically pregnant when milking is terminated during late lactation. Thus, when milk stasis occurs, the mammogenic and lactogenic stimulation of pregnancy opposes stimuli for mammary involution (Capuco and Akers, 1999).

Although extensive mammary cell loss does not occur during a typical bovine dry period, extensive tissue remodeling does occur, and this includes changes in cell populations, alveolar structure, and synthesis of ECM (Capuco and Ellis, 2013).

In mice, involution can be divided in two phases, a first reversible phase which can be reversed by reinitiating milk removal; and a second irreversible phase in which protease activity destroys the lobular-alveolar structure of the gland by degrading the ECM and basement membrane, as well as massive loss of alveolar cells. Despite that, concurrent pregnancy inhibits mammary involution in cows and may account for the maintenance of alveolar structure during the dry period. Consistent with the continued presence of intact alveoli, milk

production of non-pregnant beef cows has been reinitiated four weeks after weaning (Capuco and Akers, 1999).

Capuco and Akers hypothesized in 1999 that the dry period is critical for replacing progenitor cells, which have a limited life span and are responsible for expanding and maintaining the number of mammary secretory cells. Since then, several studies have been performed that support this hypothesis (Capuco and Ellis, 2013). If replacement of senescent cells is a critical event during the dry period, lack of a dry period of sufficient length to allow replacement may decrease the persistency of the ensuing lactation.

## Dry period length

The dry period has been vastly studied. In the review from 1999, Capuco and Akers suggested that it is necessary to apply 60-day dry periods in order to maximize milk production. Even though, other researchers have focused on trying to reduce the dry period length, in order to reduce economical losses and increase production per lactation.

Early retrospective reviews of milk production records suggested that dry periods of 30 d or less had negative effects on milk production in subsequent lactations of dairy cows (Klein and Woodward, 1942; Wilton et al., 1967). However, with the potential financial gains of shorter dry periods (Santschi et al., 2011b) being considered, research in this area has continued, collectively demonstrating the complexity of choosing the correct dry period length. For instance, 30-day dry periods were successfully implemented without affecting milk production in several controlled trials (Bachman, 2002; Gulay et al.,

2003), whereas others have shown the opposite effect (Bernier-Dodier et al., 2011; Steeneveld et al., 2013). Furthermore, it has been suggested that short dry periods (28 d: Pezeshki et al., 2007; 35 d: Santschi et al., 2011a) appear to only negatively affect the milk yield of primiparous cows, and if the dry period is extended slightly (40 to 45 d: Kuhn et al., 2006), these effects could be negated (Zobel et al., 2015). Shortened and skipped dry periods shift a portion of the milk production to the prepartum period and reduce milk production in the next lactation (Bachman and Schairer, 2003; Collier et al., 2004). In this context the research of new strategies to modulate factors regulating involution are of arising interest for the cow dry period optimization.

## Immune system role during the dry period and mastitis risk

The innate immune system is present in all multicellular organisms. It is the first line of defense against pathogens and works tightly with the adaptive immune system. It is characterized by the release of factors, mostly peptides and small proteins, and generally works by pattern recognition receptors. The mammary gland tissue, and particularly epithelial cells, participates actively in the inflammatory response induced both by bacterial infection and by the process of involution after cessation of milk removal (Loor et al., 2011).

During the dry period, the role of the immune system is to protect the gland from possible infections, remove remaining milk, and remove cell detritus. When the immune system is activated, several molecules from the innate immunity system, such as lactoferrin, play an important role inhibiting bacterial growth, although at the beginning of the dry period, the

concentration of citrate rises and competes with lactoferrin for iron binding. When citrate and iron bind, iron becomes available for bacteria to grow. When it binds to lactoferrin instead, it becomes chelated and unavailable for bacteria.

On the other hand, when the immune system is activated, there is a recruitment of immune cells such as macrophages, neutrophils and lymphocytes. Their main function is to phagocyte milk and cell debris and destroy pathogenic microorganisms. In high-productive (HP) animals, the huge amount of milk remaining in the gland hampers the effectivity of the immune system on the first days, because most of the phagocytic cells are engaged removing fat and protein debris from milk. Also, the immune system is not fully active against intramammary pathogens at dry-off because it is still downgraded by pregnancy hormones (Burvenich et al., 2007). Moreover, it seems that genetic selection for HP animals might have affected the innate immune system of the mammary gland (Loor et al., 2011). All this factors concur in a high risk of contracting mastitis during the dry period and to a slow involution and regeneration process that need long and non-productive dry periods.

## Bovine mastitis

Bovine mastitis is considered one of the most important causes of economical loss and welfare problems in dairy production in developed countries (Petrovski et al., 2006, Halasa et al. 2007). This disease is defined as the inflammation of the mammary gland parenchyma as a consequence of a microbial infection, injury, allergy or neoplasia (Kibeweb, 2017).

As mentioned previously, the dry period is one of the key points to take in account for mastitis prevention (Bradley and Green, 2004). The IMIs during the dry period usually turn into IMIs that will show up at the beginning of the following lactation and will cause welfare problems and economical losses to the farmer, associated to the decrease in milk production and treatment costs among other factors (Petrovski et al., 2006; Zobel et al., 2015).

Mastitis can curse on different ways and severities, from subclinical to acute, depending on the etiology of the pathogen causing the infection and the responsiveness of the animal's immune system to fight against it.

Usually, mastitis-causing pathogens are classified in two groups: **contagious** or **environmental**. Contagious pathogens are usually better adapted to the mammary gland's environment. They typically produce subclinical mastitis and spread from direct contact. On the other hand, environmental pathogens are opportunistic, and usually generate a rapid infection (Bradley, 2002). Because of the immense success of dry period antibiotics, environmental bacteria are now the most common causes of new IMIs during the dry period (Bradley and Green, 2004; Zobel et al., 2015).

## Common etiological causes

The etiological agents responsible for mastitis might vary from place to place depending on climate, animal management, and other factors (Deb et al. 2013).

According to most authors, the main contagious pathogens are considered to be *Staphilococcus aureus, Streptococcus dysgalactiae and Streptococcus agalactiae*, while the most common environmental pathogens causing mastitis belong to the *Enterobacteriaceae* family, more specifically *Escherichia coli, Streptococcus uberis* and *Klebsiella* spp (Gomes et al., 2016; Leitner et al., 2011; Oliveira et al., 2007; Tremblay et al., 2013).

### Staphylococcal mastitis

Within Saphylococcal mastitis, *S. aureus* and coagulase negative *Staphylococcus spp* (CNS) are the main mastitis-causing agents. The group of CNS comprises multiple staphylococcal species that are mainly environmental (Nam et al., 2010; De Vliegher et al., 2012), and has become one of the most important etiological causes in well-managed herds (Pitkala et al., 2004; Nam et al., 2010). *S. aureus* is a contagious pathogen that spreads easily from cow to cow and produces chronic subclinical mastitis and, occasionally, acute episodes (Swinkels et al., 2005).

### Streptococcal mastitis

Streptococcal mastitis is mainly caused by one of this three species: *S. dysgalactiae* and *S.* agalactiae (considered contagious), and *S. uberis* (considered environmental). Mastitis produced by *S. agalactiae* had high prevalence decades ago, but have been virtually eradicated from the dairy cow by appropriate use of penicillin-based antibiotics and management practices. Its presence is now usually related to poor management and missing biosecurity (Hillerton et al., 2005).

*Streptococcus agalactiae* is an obligated pathogen of the mammary gland transmitting directly among cows during milking. It infects the gland cistern and ducts of the mammary gland causing irritation, swelling and subclinical mastitis. The infected cow shows mere clinical signs without abnormalities drawn in milk. However, low production rates and high somatic cell counts (SCC) are usually registered (Kibebew 2017).

On the other hand, the environmental *S. uberis* is now considered one of the most common causes of clinical and subclinical mastitis.

### Coliform mastitis

The environmental pathogen *E. coli* is the main coliform producing mastitis. Coliforms induce a typical acute disease, short limited and mild, as they do not cause extensive damage to mammary tissue. They are also effectively controlled by natural antibiotics and phagocytes present in milk (Jain, 1979; Hovegeen et al., 2005).

## Prevention and control

Hygiene is a key point in mastitis prevention. Contagious pathogens can not live in the environment, so if the management practices are adequate, contagious mastitis are easy to prevent. Also, on-site diagnosis techniques such as California Mastitis Test (CMT) allow a quick and easy diagnosis, so that animals can be treated earlier. However, these practices are routinely applied during lactation but withdrawn during the dry period, increasing even more the risk to contract IMIs.

Hence, preventive administration of antibiotics and teat sealant are common strategies in dairy production. However European law policies are becoming stricter regarding the use of antibiotics, due to the public health problem of bacterial resistances, and it is likely that these practices are restricted in the near future.

For that, we need to find alternative strategies to antibiotic use for the prevention of mastitis in dairy production.

## Strategies to optimize the dry period

Several strategies have been considered in the past. From studying the effect of different dry-period lengths, to the administration of drugs, or vaccination, none of the strategies seems to be enough to maximize production efficiency and diminish the risk of mastitis.

In this context, there are some new lines of research that have been recently tested in order to stimulate the immunity of the mammary gland and accelerate the involution process. Different molecules have been tested, from natural products to chemically produced ones, and also probiotics and recombinant proteins or cytokines.

Table 1 summarizes some of the most important molecules tested with the aim of improving the dry period and their effects on immune system stimulation and involution markers.

In this thesis, we have studied three strategies to improve the dry period based on the administration of the proteins Mammary Serum Amyloid A3 (M-SAA3) and Matrix Metalloproteinase-9 (MMP-9), and the ergolin Cabergoline.

### Mammary Serum Amyloid A3

M-SAA3 is an acute phase protein synthetized locally in the mammary gland. It is involved in the regulation of local immunity and the

| Active principle | Involution and immune response markers | | | | | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lf | Ct | Alb | SCC | MMPs | Apopt./ Prolif. | Apopt. genes | IgG | $Na^+$/ $K^+$ | leakage | Udder congestion | PRL | Proinfl. cytokines | |
| rBoIL-1β | ↑ | - | ↓ | - | - | - | - | - | - | - | - | - | - | Rejman 1995 |
| | - | - | - | ↑ | - | - | - | - | - | - | - | - | - | Wedlock 2008 |
| | ↑ | | - | ↑ | - | - | - | - | - | - | - | - | - | Wedlock 2004 |
| rBoGM-CSF | ↑ | ↓ | - | ↑ | - | - | - | - | - | - | - | - | - | Wedlock 2004 |
| | - | ↓ | ↓ | - | - | - | - | - | - | - | - | - | - | Rejman 1995 |
| rBoIL-2 | ↑ | ↓ | - | - | - | - | - | - | - | - | - | - | - | Wedlock 2004 |
| rBoIL-8 | - | - | ↑ | ↑ | - | - | - | ↑ | - | - | - | - | - | Watanabe 2008 |
| | ↓ | - | - | ↑ | - | - | - | - | - | - | - | - | ↑ | Watanabe 2012 |
| Serum Amyloid A3 | - | - | - | ↑ | ↑ | - | - | - | - | - | - | - | ↑ | Domenech 2014 |
| Cabergolin | - | - | - | - | - | | | - | - | ↓ | ↓ | ↓ | | Bach 2015 |
| Quinagolide | - | - | - | ↑ | ↑ | - | - | - | - | - | - | ↓ | - | Ollier 2013 |
| | - | - | ↑ | ↑ | ↑ | | | - | ↑ | - | - | ↓ | - | Ollier 2014 |
| Casein hydrolisate | ↑ | ↓ | ↑ | ↑ | ↑ | | | - | ↑ | - | - | - | | Ponchon 2014 |
| *Panax ginseng* extract | - | - | - | - | - | ↑ | ↑ | - | - | - | - | - | - | Dallard 2011 |
| | - | - | - | - | - | - | - | - | - | - | - | - | ↑ | Baraballe 2011 |
| | - | - | - | - | - | - | - | - | - | - | - | - | ↑ | Baravalle 2015 |
| Serotonin inhibitor | - | - | - | - | - | | | - | ↑ | - | - | - | - | Hernandez 2011 |
| *E. faecium* SF68 | ↑ | - | - | ↑ | ↑ | - | - | - | - | - | - | - | - | Tiantong 2015 |
| (SF68) ultrasonicated | - | - | - | - | ↑ | - | - | - | - | - | - | - | - | Peng 2013 |
| *Lb. Perolens* CRL 1724 | - | - | - | ↑ | - | - | - | - | - | - | - | - | - | Frola 2013 |

**Table 1.** Review of molecules tested in order to improve the dry period, and its effects on involution and immune response markers.

regeneration of the mammary gland. The secretion pattern of this protein includes two main peaks: one at calving and another at drying (Molenaar et al., 2009).

Recombinant M-SAA3 has shown anti-bacterial and immunostimulating potential before both *in vitro* and *in vivo* (Molenaar et al., 2009; Domènech et al., 2014).

A previous study using recombinant M-SAA3 at drying (Domènech et al., 2014) has shown that intra-mammary infusion of recombinant M-SAA3 in the cow acts as an activator of the involution process, mainly through the activation of endogenous MMP-9 activity, which is involved in ECM remodeling. It has also been proven that M-SAA3 modulates the innate immunity by increasing mediators of the immune response such as IL-8 to ultimately enhance neutrophil recruitment, maturation of dendritic cells, and the phagocytic activity by opsonization (Domènech et al., 2012, 2014).

**Matrix Metalloproteinase-9**

MMP-9 is the main tissue remodeling protein present in the mammary gland at drying. Its task is to disaggregate the ECM in order to allow the involution of the mammary gland (Khokha and Werb, 2011). When ECM is disaggregated, cells are detached and enter apoptosis (Boudreau et al., 1995) being an important factor for the involution progression of the mammary gland.

MMP-9 is physiologically secreted as a zymogen, and then activated by proteolysis (Benaud et al., 1998). The main effectors of this proteolysis in the

mammary gland still remain unknown. Its inactivation goes by the hand of the tissue inhibitor of metalloproteinase (TIMP) family. Moreover, MMP-9 promotes the release of growth factors and cytokines, which in turn modulate the immune system and the cellular growth during mammary gland involution and regeneration (Rabot et al. 2007).

**Cabergoline**

Cabergoline is a strong agonist of the D2 dopamine receptors, whose dopaminergic effects cause inhibition of PRL secretion (Romagnoli et al., 2009). Its administration at dry-off has been proven to diminish milk production in dairy cows as well as reducing udder engorgement, udder pressure, milk leakage, and udder pain in the first week after dry-off (Bach et al., 2015).

Several studies investigated the effect of cabergoline on milk secretion (Jöchle et al., 1987) and lactation in healthy bitches and those with pseudopregnancies (Arbeiter et al., 1988; Harvey et al., 1997). Furthermore, a case study (Arlt et al., 2011) mentioned the application of cabergoline in goats with inappropriate lactation syndrome (Bertulat et al., 2017).

Other researchers tried the same strategy of blocking the release of PRL with quinagolide, a molecule that also binds to D2 receptors but has shorter half-life, and found that quinagolide decreases milk production in both early- (Lacasse et al., 2011) and late-lactation (Ollier et al., 2013).

Furthermore, cabergoline is used in women to treat hyperprolactinemia (Crosignani, 2006) and suppress lactation (de Groot et al., 1998; Gobello, 2006; Boutinaud et al., 2016).

# CHAPTER II – Objectives

The main objective of this thesis was to optimize the dry period of the dairy cow with different local and systemic strategies, and to learn more about the changes at the tissular level in order to find new targets in the improvement of cow dry period.

Four specific objective were defined:

1. To test the potential of recombinant LPS-free M-SAA3 in the inhibition of bacterial infection of *E. coli*, *S. dysgalactiae*, *S. aureus*, and *S. uberis in vitro*.

2. To test the potential of recombinant MMP-9 in soluble and nanoparticled format in the stimulation of mammary involution *in vivo*.

3. To evaluate the effects at the tissue level of the systemic administration of cabergoline at dry off.

4. To analyze changes around the dry period at the tissue level and to compare the differences between primiparous and multiparous cows, and high productive and low productive animals.

To achieve these objectives, four studies were conducted:

**Study 1** "Recombinant mammary serum amyloid A3 as a new strategy to prevent intrammamary infections in dairy cows"

Production of LPS-free recombinant M-SAA3, test of its effect on immune stimulation on primary mammary cell cultures, and study of its potential in the inhibition of bacterial translocation in primary mammary cell cultures.

**Study 2** "Facilitating mammary involution at dry-off by intra-mammary infusion of Matrix Metalloproteinase-9"

Analysis of the effects of intramammary infusion of recombinant LPS-free MMP9 nanoparticles and its soluble counterpart on involution and regeneration markers.

**Study 3** "Effects of prolactin inhibition on cell recruitment, apoptosis, turnover, and senesce of mammary epithelial cells at dry-off"

Analysis of the effects of cabergoline administration in tissular markers of involution, proliferation, senescence, immunity and milk production.

**Study 4** "Involution, regeneration, and senescence in of the the mammary gland of dairy cows around dry-off"

Analysis of the differences between primiparous and multiparous cows, and high productive and low productive animals during dry off on involution, proliferation, senescence, and immunity tissular markers.

# CHAPTER III – Study 1:

# Recombinant mammary serum amyloid A3 as a new strategy to prevent intrammamary infections in dairy cows

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## Introduction

Dairy cows need a dry period between lactations to allow an adequate involution and regeneration of the mammary gland in order to sustain high milk production levels in the following lactation (Capuco et al., 1997). However, during the dry period, the udder is highly susceptible to suffer intra-mammary infections (IMI) that can persist throughout the dry period onto the next lactation, causing important economical losses (Oliver and Murinda, 2012) and discomfort to the animal. In highly productive animals, a large volume of milk remains in the gland at drying producing milk leakage and favoring the entrance and growth of microorganisms. Also, at dry-off, the immune system is not fully active against pathogens because it is still partially inhibited by pregnancy hormones. Moreover, an important part of phagocytic cells localized in the mammary gland is engaged removing fat and protein debris from the milk that accumulates in the udder after cessation of milking (Burvenich et al., 2007). At present, the most common strategy to prevent IMIs at dry-off is the administration of broad-spectrum antibiotics and/or the use of teat sealant at drying. However, the use of antibiotics is associated with an increasing number of antibiotic resistant bacteria, which is becoming a global public health concern. In the context of bovine IMI, the enhancement of the immunity of the mammary gland, and the acceleration of the involution process using active molecules could become promising alternatives to the use of preventive antibiotics at dry-off.

M-SAA3 is an important protein produced locally and involved in the regulation of the local immunity and the regeneration of the mammary gland. Briefly, M-SAA3 is an acute phase protein whose concentrations reach peak levels in colostrum, in milk during IMI, and at dry-off (Molenaar et al., 2009). A previous study (Domènech et al., 2014) has shown that intra-mammary infusion of recombinant M-SAA3 in the cow acts as an activator of the involution process, mainly through the activation of endogenous MMP-9 activity, which is involved in ECM remodeling. It has also been proven that M-SAA3 modulates the innate immunity by increasing mediators of the immune response such as IL-8 to ultimately enhance neutrophil recruitment, maturation of dendritic cells, and the phagocytic activity by opsonization (Domènech et al., 2012, 2014). However, the role of recombinant M-SAA3 in the inhibition of the infections caused by mastitic pathogens has not yet been studied in depth. Thus, the main objective of the present study was to explore the potential of M-SAA3 in the inhibition of the infection of primary bovine mammary epithelial cultures by common mastitis-producing pathogens including *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae*. For that, we first optimized the production and purification of recombinant M-SAA3 in *E. coli* BL21. It is important to point out that although recombinant proteins are usually produced in *E. coli* (Ferrer-Miralles et al. 2009; Ferrer-Miralles and Villaverde, 2013), the presence of lipopolysaccharide (LPS) in its outer membrane limits the in vivo applicability of recombinant products

obtained from this expression system (Garcia-Fruitós, 2012; Mamat et al., 2015). LPS traces remain in the final purified recombinant product and these can lead to endotoxic responses (Burvenich et al., 2007). Hence, in these situations it is necessary to apply extra purification steps to completely remove LPS from the recombinant product. This is a crucial point in the case of recombinant proteins that modulate the immune system, because LPS traces could interfere in the evaluation of their activity. Alternatively, ClearColi® bacteria could be used, since it is a new *E. coli* strain genetically engineered to express a modified LPS named Lipid $IV_A$. This modified LPS does not trigger the endotoxic TLR4-dependant response in human cells (Planesse et al., 2015). Thus, in this study we compared the yield and purity of M-SAA3 obtained from both BL21 and ClearColi® strains to select the best strategy to minimize the effect of LPS traces on M-SAA3 function.

## Materials and Methods

### Recombinant M-SAA3 production and purification

The *E. coli* BL21 Star (DE3) and ClearColi® (Lucigen, Middleton, WI, USA) strains transformed with pET101/D-TOPO vector containing the goat M-SAA3 sequence isolated in a previous study (Domènech et al., 2012) were used for recombinant protein production. Microbial cells were grown in 400 ml of LB-Amp media at an initial $OD_{600}$ of 0.05 until log phase was achieved. Recombinant expression was induced by 0.1 mM IPTG at $OD_{600}$ of 0.9-1 for 1 h and 20 min in BL21 strain and at 0.6-0.7 for 2 h in ClearColi®. Cell pellet was obtained by centrifugation at 6,000 x *g* for 10 min, and frozen at -80°C until use. Bacterial pellets resulting from 1.3 l of production were lysated for 25 min at room temperature (RT) and 250 rpm in 36 ml tris-buffered saline lysis buffer containing 1x Bugbuster® (Merk-Millipore, Billerica, MA, USA), 0.1% v/v benzonuclease (Sigma-Aldrich, Saint Louis, MO, USA), and 1x cOmplete® EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Lysated product was centrifuged for 15 min at 20,000 x *g* at 4ºC. Supernatants containing protein were then used to obtain purified protein with the Amicon Pro System from Merck-Millipore (Billerica, MA, USA) and the Clontech (Mountain View, CA, USA) His60 agarose bead slurry (60 mg/ml binding capacity). First, we washed 200 µl resin by centrifugation at 1,000 x *g* for 1 min in the Amicon Pro. Then we added 500 µl binding buffer (Tris buffered saline + 80 mM imidazole) and centrifuged with the same

protocol discarding the flow through. After that, we incubated 9 ml of sample per Amicon for 30 min at RT and 200 rpm, and centrifuged to discard supernatant, followed by a wash step with 1.5 ml binding buffer. To elute the sample, we added 0.5 ml elution buffer (Tris buffered saline + 300 mM imidazole) and incubated for 3 min at RT and 200 rpm prior to centrifugation in a new collector tube at 1, 000 x *g* for 1 min in a basculating rotor. We eluted twice to make sure there was no protein left in the resin. The purified protein was dialyzed overnight at 4°C against phosphate buffered saline (PBS) and was further quantified using spectrophotometry ((A280×Molecular weight)/Extinction coefficient; (mg/ml)). The theoretical molecular weight of the recombinant version of M-SAA3 used in this study was 17.8 kDa.

**LPS quantification and removal from recombinant M-SAA3**

LPS and Lipid $IV_A$ were quantified by Microcoat Company using LAL Kinetic chromogenic assay. The removal of LPS from M-SAA3 produced in *E. coli* BL21 was carried out with the Mustang Q Acrodisc membrane (Pall Corporation, New York, USA) following manufacturer's instructions. Briefly, all samples and buffers were filtered through a 0.22 μm filter before punching into the membrane. Then, the membrane was washed with 5 ml 1 M NaOH and equilibrated with 1 ml 1 M NaCl and 5 ml PBS before running the sample.

## SDS-PAGE, Coomassie, and Western Blot

Protein production was assessed by SDS-PAGE in 12% polyacrylamide gels. Before loading, samples were diluted 1:1 in Laemmli 2x (Sigma-Aldrich, Saint Louis, MO, USA) and boiled for 10 min. Gels were then run for 60 min at 200 V and proteins were either stained for 1 h with Coomassie brilliant blue or transferred to Immuno-Blot PDVF membranes (60 min, 100 V) for Western Blotting. Then, membranes were blocked with 5% Skimmed milk in Tris-NaCl buffer and incubated with the Monoclonal Anti-polyHistidine, Clone HIS-1 (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:3,000 in tris buffered saline + 0.05% tween 20 (TBST) for 1 h at RT with gentle agitation. Secondary antibody, Anti-Mouse IgG (whole molecule) Alkaline Phosphatase Conjugate (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:20,000 in TBST was incubated for 30 min at RT with gentle agitation. The substrate used to develop the Western Blot was the BCIP/NBT solution, premixed (Sigma-Aldrich, Saint Louis, MO, USA).

## Mass spectrometry (MS) analysis

Mass spectrometry analyses were carried out in the Proteomics facility from Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain). Bands were excised from coomassie gel, washed, and destained in ammonium bicarbonate 50 mM / acetonitrile (ACN) 50%. Then, a reduction in DTT 10 mM and an alkylation with iodoacetamide 25 mM was performed. An in-

gel trypsin digestion was done using the Sequencing Grade Modified Trypsin Kit (Promega, Madrid, Spain). After elution in 50% ACN +0.2% TFA, protein was mixed with a matrix of 2.6 dihydroxyacetophenone (1:1), and 1 µl of the mixture was deposited on a ground steel plate. Samples were analyzed using a lineal method in an UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) with ion acceleration of 25 kV.

**Mammary epithelial primary cell cultures**

Mammary epithelial primary cultures were obtained as described in Domènech et al. (2014). Briefly, bovine mammary tissue from healthy cows was obtained at the slaughterhouse and transported in chilled PBS containing antibiotics and antifungals. In the laboratory, tissue was cut into small pieces and incubated in Hanks balanced solution with EDTA and DTT for 30 min with gentle agitation. Supernatant was discarded and tissue was incubated in RPMI 1640 + 0.05% collagenase for 30 min. Cells were pelleted by centrifugation at 800 x *g* for 5 min. The incubation and centrifugation steps were repeated three times.

Final cell pellets were resuspended in cell culture medium composed by DMEM/F-12 medium with 10% fetal bovine serum (FBS), 8 µg/ml bovine insulin, 10 µg/ml gentamycin, 50 µg/ml hydrocortisone, 100 µg/ml streptomycin, 100 U/ml penicillin, 2.5 µg/ml amphotericin, and 5 ng/ml EGF. After that, samples were incubated at a density of 80,000 cells/$cm^2$

in flasks until expansion. Epithelial cell phenotype was confirmed by immunofluorescence staining against anti-cytokeratin antibodies (Sigma-Aldrich, Saint Louis, MO, USA), as previously described in Hashim et al., 2004.

**Induction of IL-8 expression in primary mammary cell cultures**

Primary mammary epithelial cultures were challenged with LPS (Lipopolysaccharides from *E. coli* O111:B4 purified by phenol extraction; Sigma-Aldrich, Saint Louis, MO, USA), Lipid $IV_A$ (Lipid A 406, LA-14-PP, Precursor Ia; Peptanova, Sandhausen, Germany), or recombinant M-SAA3. The doses tested were 10,000, 5,000, 2,000, 1,000, 500 and 50 ng for LPS and Lipid $IV_A$, and 160, 90, 30, 10, and 3 μg for recombinant M-SAA3.

The protocol was carried on as it follows. Mammary epithelial cells from primary cultures were seeded in 24-well plates at 44,000 cells/well. After 24 h incubation at 37°C and 5% $CO_2$, wells were washed twice with warm PBS to remove antibiotics, and treatments were applied in a final volume of 100 μl of PBS + 400 μl of antibiotic-free medium (DMEM/F-12 medium with 8 μg/ml bovine insulin and 50 μg/ml hydrocortisone) in the case of LPS and Lipid $IV_A$ or in 600 μl PBS + 400 μl of antibiotic-free medium in the case of recombinant M-SAA3. After 3 h of incubation at 37°C, 5% $CO_2$, cells were gently washed with PBS, and 500 μl of Trizol reagent (Thermo Fisher Scientific, Madrid, Spain) were added to each well to

collect and lysate the cells. Cell lysates were kept frozen at -80°C until RNA extraction.

**RNA extraction and quantitative RT-PCR**

The extraction of RNA was performed using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed with Nanodrop 1000 instrument (Thermo Scientific, Barcelona, Spain) at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Quantitative PCR was performed for genes encoding for IL-8 (Primer sequences: Fw: 5'-TTGAGAGTGGGCCACACTGTG-3', Rv: 5'-TGCACCCACTTTTCCTTGG-3') and ACTB (Primer sequences: Fw: 5'-CTGGACTTCGAGCAGGAGAT-3', Rv: 5'-CCCGTCAGGAAGCTCGTAG-3') using a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan). Quantitative PCR conditions for each set of primers were individually optimized (ACTB: Annealing temperature = 57ºC, primer concentration = 0.125 μM; IL-8: Annealing temperature = 55ºC, primer concentration = 0.5 μM). The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8% DNA agarose gel and a single peak in the qPCR melting curves. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was

plotted to obtain the efficiency, which is calculated using the formula E=10^(-1/slope), with an acceptable range of 1.8-2.2 (Chow et al, 2010). A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBR Green Fluorescent (Bio-Rad), and the optimized primer concentration for each gene. The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. Relative gene expression was calculated using the 2ΔCt method with ACTB as reference gene.

**Obtention of pathogenic bacterial strains**

Pathogenic *E. coli*, *S. uberis*, *S. aureus*, and *S. dysgalactiae* were isolated from mastitic milk kindly provided by ALLIC (Associació lletera interprofessional de Catalunya, Laboratori interprofessional lleter de Catalunya, Cabrils, Spain). All strains were grown in brain-heart infusion (BHI) media and plated onto Tripticasein Soy agar plates (*S. dysgalactiae*, *S. uberis*) or BHI agar plates (*E. coli*, *S. aureus*). Single colonies were grown in 10 ml of BHI media overnight at 37°C in static conditions. A cell pellet was obtained by centrifugation at 6,000 x *g* for 10 min at 4°C and kept frozen in 10% glycerol upon use. Before infection experiments, bacterial pellets were thawed at RT and grown O/N in 10 ml of BHI at 37ºC in static conditions.

**Inhibition of mastitic infections by M-SAA3 and direct effect on bacteria**

Bacterial concentrations were determined by spectrophotometric quantifications according to previously established colony-forming unit (CFU) counts. Equivalence between $OD_{600}$ and CFU/ml was the following: *E. coli* $OD_{600}$: 5.00 = $5x10^9$ CFU/ml, *S. uberis* $OD_{600}$: 0.50 = $8.5x10^8$ CFU/ml, *S. aureus* $OD_{600}$: 5.00 = $5.1x10^9$ CFU/ml, and *S. dysgalactiae* $OD_{600}$: 0.50 = $6.2x10^7$ CFU/ml. Infective bacterial doses were obtained by resuspending and diluting the cell pellet in antibiotic-free medium. Mammary epithelial cells were seeded in 24-well plates at a density of 44,000 cells/well in cell culture medium (concentrations described above). After 24 h cells were washed twice with PBS and incubated with recombinant M-SAA3 for 1 h in a total volume of 600 μl PBS + 400 μl antibiotic-free medium. Negative control consisted of 600 μl PBS + 400 μl antibiotic-free medium. After 1 h incubation at 37°C and 5% $CO_2$ cells were infected with $10^6$ CFU/well. Cells were incubated in the same conditions for two more hours. After incubation, cells were gently washed with PBS twice and 0.5 ml of 0.9% NaCl was added. Recovered cells were serially diluted and seeded in agar plates. Colonies were counted after 24-48 h of growth at 37°C. The experiment was repeated three times with 6 replicates per treatment.

Also, direct bacterial killing activity of recombinant M-SAA3 was tested against *S. aureus*. A suspension containing $1x10^6$ CFU was incubated with

PBS with or without 160 μg/ml of recombinant M-SAA3 for 3.5 h and 5.5 h at 37°C. After incubation, viable bacteria were quantified by plating the suspension in agar plates.

**Statistical analyses**

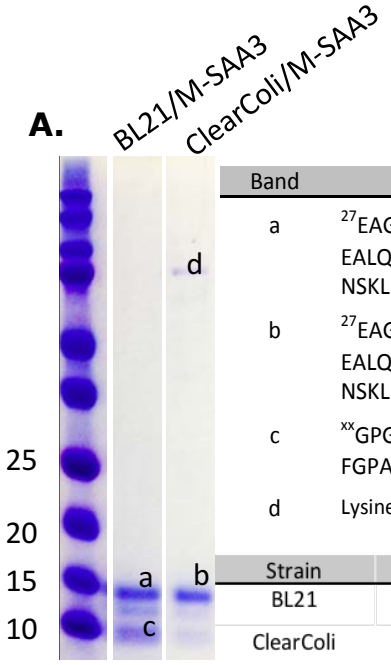
Data were transformed to achieve a normal distribution when necessary prior to statistical analyses. All data were analyzed using an analysis of variance that accounted for the effect of treatment using M-SAA3, LPS, or Lipid $IV_A$.

## Results

### M-SAA3 production in E. coli BL21 and ClearColi® strains

The M-SAA3 yields obtained after protein expression in *E. coli* BL21 and ClearColi® were determined by spectrophotometry obtaining a total of 1.8 and 1.33 mg/l culture, respectively (Figure 1C).

**A.**




**B.**

| Band | M-SAA3 sequence |
|---|---|
| a | $^{27}$EAGQGAKDMWRAYRDMKEANYKGADKYFHARGNYDAAQRGPGGVWAAEVR EALQGITDPLFKGMTRPQVREDTKADQFANEWGQSGKDPNHFGPAALPDKYKGEL NSKLEGKPIPNPLLGLDSTRTGHHHHHH |
| b | $^{27}$EAGQGAKDMWRAYRDMKEANYKGADKYFHARGNYDAAQRGPGGVWAAEVR EALQGITDPLFKGMTRPQVREDTKADQFANEWGQSGKDPNHFGPAALPDKYKGEL NSKLEGKPIPNPLLGLDSTRTGHHHHHH |
| c | $^{xx}$GPGGVWAAEVIREALQGITDPLFKGMTRPQVREDTKADQFANEWGQSGKDPNH FGPAALPDKY |
| d | Lysine decarboxylase CadA from *E.coli* |

**C.**

| Strain | M-SAA3 yield | Purity | LPS / Lipid $IV_A$ |
|---|---|---|---|
| BL21 | 1.80 mg/l | 98% | 0.08 ng LPS (0.8 EU)/µg M-SAA3 |
| ClearColi | 1.33 mg/l | 95% | 76 ng Lipid $IV_A$ (0.48 EU)/µg M-SAA3 |

**Figure 1. A.** Coomassie staining of SDS-PAGE with recombinant M-SAA3 produced in *E. coli* BL21 or ClearColi®. **B.** Peptide sequences obtained by Mass Spectrophotometry analyses of bands excised from Coomassie gel. **C.** Yield, purity and associated amount of LPS/Lipid $IV_A$ of recombinant M-SAA3 produced in *E. coli* BL21 or ClearColi®.

Despite the fact that we could produce recombinant caprine M-SAA3 in both systems, ClearColi® strain had very variable and inconsistent production rates (data not shown), which made it difficult to work with. The purity of recombinant M-SAA3 obtained from both expression systems was similar (Figure 1C). As assessed by Coomassie staining, the

purity was 98 and 95% for M-SAA3 produced in E. coli BL21 and ClearColi®, respectively. The two main bands of 15 kDa and 10 kDa observed by Coomassie staining were identified as M-SAA3 by western blot using anti-His antibodies (data not shown) and MALDI-TOF analyses (Figure 1B). Also, a band corresponding to lysine decarboxylase CadA from E. coli was identified in M-SAA3 samples purified from ClearColi® (Figure 1B). The inclusion of a second purification step based on a cation-exchange chromatography for M-SAA3 obtained from ClearColi® allowed to remove this contaminating protein. However, although the addition of this extra purification step increased the purity of M-SAA3, it also implied a loss of 70% of M-SAA3 yield (data not shown).

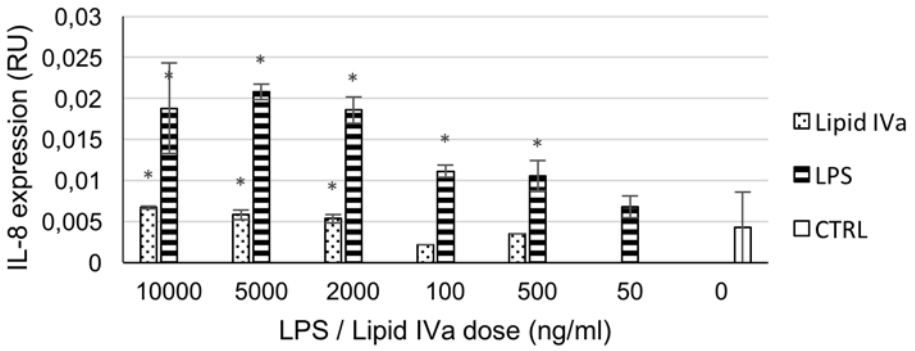
Surprisingly, the amount of Lipid $IV_A$ associated to 1 µg of M-SAA3 produced in ClearColi® was much greater than the amount of LPS traces in 1 µg of M-SAA3 produced in BL21 strain (76 ng or 54 pmols of Lipid $IV_A$ compared with 0.08 ng or 0.016 pmols of LPS, Figure 1C). In terms of endotoxic activity measured by LAL Kinetic chromogenic assay, as expected, Lipid $IV_A$ was less active than LPS. One Endotoxic Unit (EU) corresponded to 158 ng (112.39 pmols) and 0.1 ng (0.019 pmols) of Lipid $IV_A$ and LPS, respectively.

**Induction of IL-8 expression in mammary cultures**

The stimulation of IL-8 expression in Lipid $IV_A$ treatments was similar to the control cells. There was only a slight increase in those samples

incubated with the highest Lipid $IV_A$ concentration (Figure 2A). On the other side, LPS induced the expression of IL-8 in primary bovine epithelial mammary cultures in a dose-dependent pattern (Figure 2A).

**A.**




**B.**

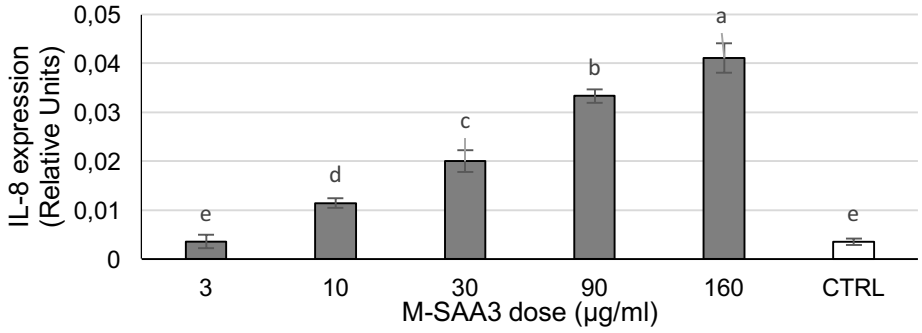



**Figure 2. A.** Expression of IL-8 in primary mammary cell cultures treated with LPS (lined bars), Lipid $IV_A$ (dotted bars), or control (white bar) expressed in relative units (RU). Asterisks indicate differences from control. **B**. Expression of IL-8 in primary mammary cell cultures treated with different concentrations of recombinant M-SAA3 expressed as relative units (RU). Different letters indicate differences between treatments.

Recombinant M-SAA3 (LPS-free) obtained after Mustang Q XT filtration, increased the expression of IL-8 in bovine primary mammary cells in a dose-dependent manner (Figure 2B). Expression of IL-8 increased ($P < 0.0001$) up to 13-fold in M-SAA3 treatments compared with control in a

dose-dependent manner, being 10 μg/ml the lowest dose with a significant effect.

**Inhibition of intramammary infections by M-SAA3**

A clear effect at reducing *E. coli*, *S. aureus*, and *S. uberis*, infection was seen with all concentrations tested (Figure 3B, 3C, 3D).

A reduction of 50% of *S. aureus* infection was achieved at 160 μg/ml. From a general point of view, the best concentration of caprine M-SAA3 at inhibiting infections was the lowest concentration tested (10 μg/ml). In the case *of S. dysgalactiae* and *E. coli* (Figure 3A, 3B) this concentration gave the best efficiency of inhibition, being 67% and 51 %, respectively. However, in the case of *S. aureus* and *S. uberis* (Figure 3C, 3D), although 10 μg/ml of SAA3 inhibited 23% and 29% of infection respectively, greater concentrations of M-SAA3 (up to 160 μg) increased the antimicrobial potential to 50%. Intriguingly, high M-SAA3 concentrations were not as efficient as low M-SAA3 concentrations in *E. coli* and *S. dysgalactiae*. In contrast, the greatest concentrations of M-SAA3 performed better with *S. aureus* and *S. uberis* than with *E. coli.*

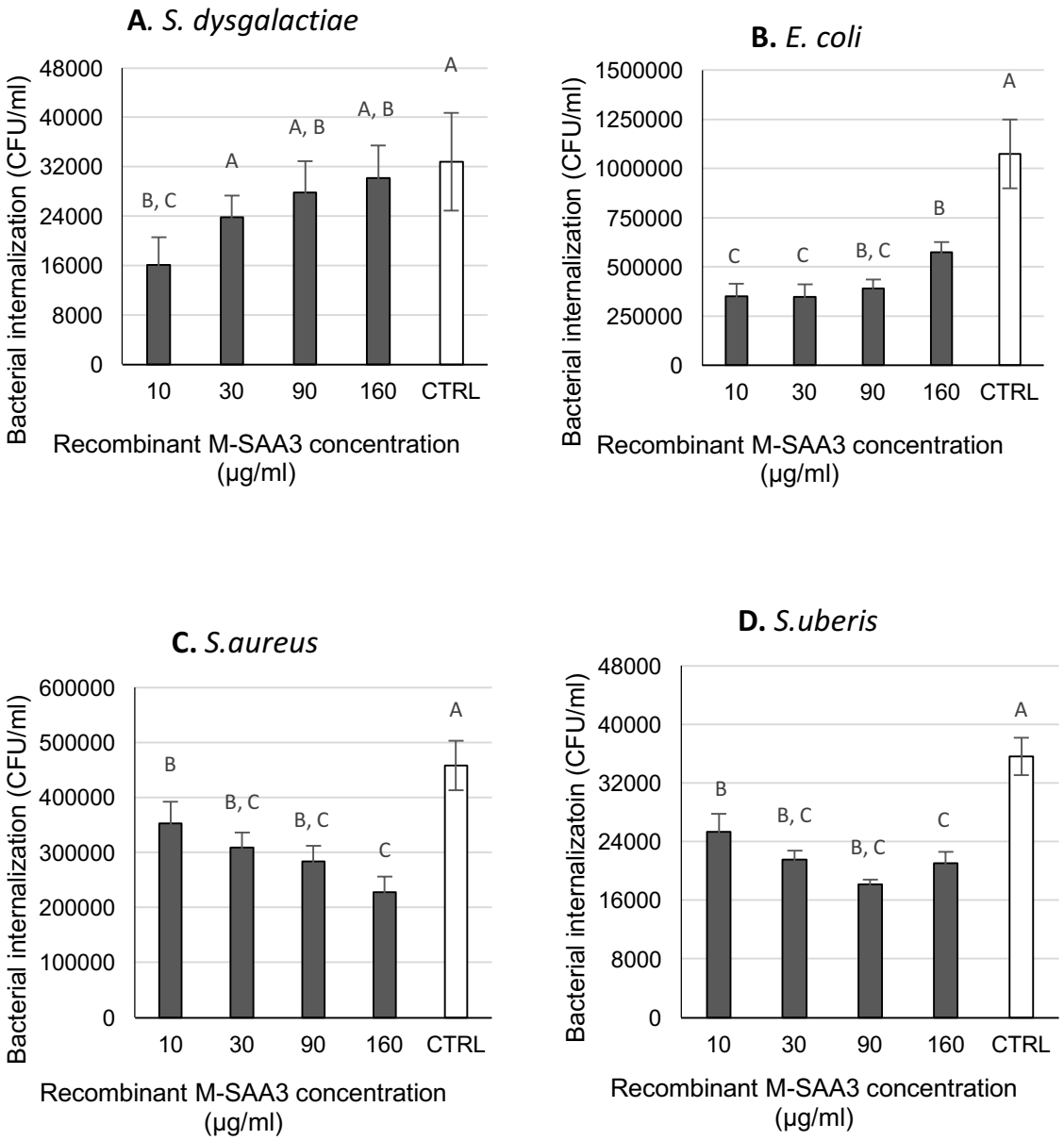



**Figure 3**. Bacterial counts expressed as CFU/ml of mammary primary cell cultures infected with $10^6$ CFU/ml of (**A**) *S. dysgalactiae*, (**B**) *E. coli,* (**C**) *S. aureus*, or (**D**) *S. uberis* and treated with different concentrations of recombinant M-SAA3. Different letters indicate differences between treatments.

Regarding the direct bacterial killing of *S. aureus* assay, there was no effect on the viability of *S. aureus* in presence of caprine M-SAA3 (Figure 4).

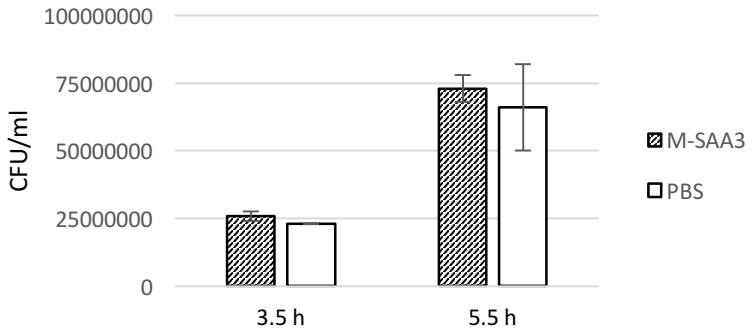



**Figure 4**. Bacterial counts expressed as CFU/ml of S. aureus treated with PBS containing or not 50 μg/ml of recombinant M-SAA3.

## Discussion

The recombinant production of M-SAA3 was successfully achieved in both BL21 and ClearColi®, but even if the latter system was more promising beforehand, we ended up choosing the first one. The inconsistent production rates and strong presence of Lipid $IV_A$ in the recombinant product made us opt for the *E. coli* BL21 followed by a LPS removal system.

We obtained a purified form of M-SAA3 of 15 kDa, instead of the expected 17.8 kDa form. That leads us to think that M-SAA3 protein was cleaved to be activated. It has been previously described that the members of the SAA family contain an 18 amino acid signal peptide that is cleaved post-translationally (Uhlar and Whitehead, 1999; McDonald et al., 2001). Besides, minor degradation forms of M-SAA3 were also observed by western blot and MALDI-TOF analyses in agreement with results shown in previous studies (Domènech et al., 2012).

The large amounts of Lipid $IV_A$ present in the recombinant product could be due to the smaller size of the molecule, which makes it more releasable from the cell wall; or to a greater affinity for M-SAA3 compared with LPS. Either way, although 6,000 molecules more of Lipid $IV_A$ than LPS would be required to observe the same level of endotoxic response, purified recombinant M-SAA3 contained a similar amount of endotoxic units (EU) according to LAL assay independently whether produced in *E. coli* BL21 or in ClearColi® strain (Figure 1C).

Although it has been described that Lipid $IV_A$ does not trigger the TLR4-based responses in human cells, it has also been proven that there is a specie-specific recognition and stimulatory activity of lipid $IV_A$. Specifically, the interaction of bovine cells with LPS was previously explored by Sauter et al. (2007) and they observed that human embryo kidney cells stably transduced with both bovine TLR4 and bovine MD-2 responded to LPS, and at less extent to Lipid $IV_A$ by increasing IL-8 production. In agreement with this, when we studied the endotoxic effect of LPS and Lipid $IV_A$ in bovine epithelial mammary cells we observed an IL-8 stimulation but only when using high doses of Lipid $IV_A$ (Figure 2A). Moreover, since the amount of Lipid $IV_A$ traces was high in the purified M-SAA3 protein (Figure 1), the use of high M-SAA3 concentrations (around 100 μg) in bovine cell culture experiments and further in vivo assays would drag a sufficient amount of Lipid $IV_A$ to stimulate IL-8 expression itself, which would interfere in the analysis of M-SAA3 activity. In this context, and taking into account that the yields of the purified form of M-SAA3 were greater in the BL21 strain than in ClearColi® strain, we decided to continue this study with M-SAA3 purified from BL21 and removing LPS from purified M-SAA3 using Mustang Q XT Acrodisc membrane (Pall Corporation, New York, USA). This system allowed to significantly reduce (800-fold) the amount of LPS traces (<0.001 EU (0.0001 ng LPS) /μg of M-SAA3). This amount of LPS should not interfere in the study of M-SAA3 functionality.

The stimulation of IL-8 expression in primary mammary cell cultures by recombinant LPS-free M-SAA3 showed potential for recombinant M-SAA3 to act as an activator of the immune system. Previous work with goat M-SAA3 in bovine mammary cells showed only an effect on mammary cultures at a single concentration of 30 μg/ml and without removing LPS traces (Domènech et al., 2014). Hence this is the first time that a clear modulation of immune system by increasing doses of recombinant M-SAA3 has been demonstrated. IL-8 is the main chemotactic cytokine released by mammary gland that is responsible for the recruitment of immune cells such as neutrophils and macrophages. Thus, it could be concluded that administration of M-SAA3 in the mammary gland could represent a promising approach to fight bacterial infections *in vivo* at dry-off. Moreover, we investigated the direct potential of M-SAA3 to fight infectious bacteria. Recombinant caprine M-SAA3 killing effect was not previously investigated. However, we were not able to demonstrate direct killing activity of *S. aureus*, which is one of the most important pathogens involved in IMI in dairy cows (Rainard, 2005). Molenaar et al. (2009) reported a moderate activity at 18 μg/ml and a complete killing activity of *E. coli* at concentrations ≥63 μg/mL of bovine M-SAA3. This could be explained because M-SAA3 can potentially have a more effective direct antimicrobial effect on Gram-negative microorganisms, due its binding affinity to OmpA (Hari-Dass et al., 2005; Molenaar et al., 2009). Alternatively, the differences observed between studies could be due to the sequence of the M-SAA3 protein used. Bovine or caprine M-SAA3

forms share the 91.3% of nucleotide and 86.3% of amino acid sequence, but they are not identical molecules. Moreover, the recombinant M-SAA3 produced herein lost a 9 amino acid peptide at the N-terminus mature form, which might be involved in this direct killing activity.

Domènech et al. (2014) had previously demonstrated a slight decrease of *S. aureus* infection in mammary cultures using caprine M-SAA3 at 30 μg/ml. Thus, we decided to explore the effect of four different concentrations of M-SAA3 on the reduction of bacterial infection by *S. aureus*, *S. dysgalactiae*, *S. uberis*, and *E. coli* in mammary cultures. We found a decrease in bacterial infections with all four strains and, interestingly, the response to the different doses varied among the pathogens, which could be explained by the different mechanisms used by bacteria to elicit an inflammatory response.

It has been previously described that M-SAA3 binds to MD-2 to activate p38 and NF-κB pathways in a MyD88-dependent manner (Deguchi et al., 2013). This regulates not only the stimulation of immune modulators such as several cytokines, but also the synthesis of antimicrobial peptides, tight junctions, and other inflammation components that allow epithelial cells control the infection (Tak and Firestein, 2001; Zarubin and Han, 2005). Moreover, the M-SAA3 increases the expression of mucins in several tissues (Tashiro et al. 2017). Mammary gland tissue expresses Mucin1 as a protective molecule (Patton et al.1995; Pallesen et al., 2001)

and also LAP antimicrobial peptide that could have a role at inhibiting the bacterial infections (Swanson et al. 2004).

*E. coli* (as a Gram-negative bacteria) stimulates the inflammatory response of epithelia via TLR4 activation like M-SAA3. However, *S. aureus* does not activate this receptor but activates TLR2 instead (Fu et al., 2013). A possible explanation to this issue could be related with a simultaneous immune stimulation by bacteria and M-SAA3 on the mammary cells that can lead to an over-stimulation of TLR causing negative effects on the tissue (Blach-Olszewska et al., 2007). Otherwise, a possible competitive effect for TLR4 between bacteria (*E. coli* / *S. dysgalactiae*) and M-SAA3 cannot be discarded. Although further research is needed to elucidate the exact mechanisms, it is important to emphasize that the recombinant M-SAA3 used herein represents a plausible strategy to prevent IMI in dairy cows.

Overall, these results show a clear potential of recombinant M-SAA3 preventing IMI in dairy cows. We have demonstrated the clear effects on mammary epithelia reducing the infections of four relevant mastitic pathogens, which coupled with the potential to recruit immune cells into the mammary gland, increase phagocytic activity and dendritic cells maturation (Domènech et al., 2014, 2012) making the use of M-SAA3 a plausible alternative to the administration of antimicrobial agents to prevent IMI in cattle.

## Acknowledgements

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# CHAPTER IV – Study 2:

# Facilitating mammary involution at dry-off by intra-mammary infusion of Matrix Metalloproteinase-9

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## Introduction

At the end of lactation and as calving approaches, dairy cows need a non-lactating (dry) period to optimize milk production in the subsequent lactation. During the dry period, mammary epithelial component regresses, proliferates, and differentiates to allow optimal milk production. Tissue regeneration of the mammary gland is necessary, and the omission of a dry period reduces milk production in the following lactation (Bachman and Schairer, 2003). However, the strong presence of galactopoietic hormones due to a concomitant pregnancy hampers the beginning of the involution and delays the activation of the immune system (Noble et al., 1999; Pezeshki et al., 2010). Furthermore, today's dairy cows are dried while producing ~25 kg/d of milk with some animals producing >35 kg/d (Bach et al., 2015). These copious amounts of milk remaining in the mammary gland exert high intra-mammary pressure and may cause discomfort (O'Driscoll et al., 2011) and milk leakage (Gott et al., 2016). In addition, high-producing dairy cows are more susceptible to intra-mammary infections during the early stages of the dry period (Rajala-Schultz et al., 2005).

Involution of the mammary gland starts with a complex signaling pathway of cell factors, hormonal changes, and immune stimulation. Activation of the immune system at the beginning of the dry period recruits blood leukocytes that progressively colonize the mammary gland after dry-off and phagocytize and destroy microorganisms (Burvenich et al 2007).

However, phagocytic activity against pathogens is diminished at dry-off as phagocytes engulf milk fat, cell debris, and other compounds derived from milk accumulation, and their activity is not fully focused in the fight against pathogenic bacteria (Burvenich et al., 2007). In fact, it is assumed that the immune system does not reach effective protective levels until 8 d after dry-off (Oliver and Sordillo, 1989), and thus, the health of the mammary gland is compromised during this period. To reduce risk of mastitis, antibiotics are infused routinely into the mammary gland at dry-off. However, this practice may be challenged due to the emergence of antibiotic resistances and social pressure. MMP-9 is a tissue-remodeling enzyme that is physiologically released in the mammary gland and is responsible of breaking the ECM (Khokha and Werb, 2011), which triggers a signal to the detached cells to enter into apoptosis (Boudreau et al., 1995). Moreover, MMP-9 promotes the release of growth factors and cytokines, which in turn modulate the immune system and the cellular growth during mammary gland involution and regeneration (Rabot et al. 2007). Thus, we hypothesized that the intramammary administration of recombinant MMP-9 could represent an effective strategy to accelerate tissue involution and boost tissue regeneration at dry-off. Moreover, it could boost the infiltration of immune cells into the mammary gland and stimulate other active immune factors, reducing the need to use antibiotics at dry-off.

Traditionally, recombinant proteins have been produced and purified as soluble forms. Soluble forms can sometimes be unstable, because other

enzymes from the tissue can degrade them. Also, the production and downstream processes to purify them are usually expensive. In order to overcome these obstacles, some research groups have produced protein nanoparticles in form of self-organizing bacterial functional amyloids, also known as inclusion bodies or nanopills (Cano-Garrido, 2016; Gifre et al., 2017), which represent a low-cost source of slowly-releasable, highly-stable functional proteins (Villaverde et al. 2012; Céspedes et al. 2016). Thus, the objective of this study was to determine the potential of an intra-mammary infusion of soluble and nanostructured recombinant forms of MMP-9 to accelerate the immune activity and the involution and regeneration of the mammary gland of dairy cows after dry-off.

## Materials and Methods

### Bacterial strains, plasmids, recombinant proteins and growth conditions

The production of recombinant MMP-9 was performed using *Lactococcus lactis* strain NZ9000 clpP- htrA- (clpP-htrA; EmR) (Poquet et al., 2000; Cortes-Perez et al., 2006) (kindly provided by INRA, Jouy-en-Josas, France; patent nº EP1141337B1). The sequence of the catalytic domain of the bovine (*Bos taurus*) MMP-9 (from Phe107 to Pro449 NM_174744.2) was codon optimized for the expression in *L. Lactis.* Moreover, a lysine and a histidine tag were added at the C-terminal end to assist protein detection and purification (GeneArt, Invitrogen by Thermo Fisher Scientific, USA). The recombinant active fragment of MMP-9 (39.6 KDa) was produced by expressing the encoding gene from the CmR pNZ8148 plasmid (NIZO) under the nisA promoter control (Mierau et al., 2005). This strain was cultured in flasks at 30 ºC without shaking in M17 Broth with 0.5 % glucose supplemented. Antibiotics were used at the following concentrations: chloramphenicol (5 µg/ml) and erythromycin (2.5 µg/ml). Recombinant gene expression was induced by 12.5 ng/ml nisin (Sigma-Aldrich, Barcelona, Spain) during 3 h.

**Purification and quantification of soluble and nanostructured MMP-9 proteins**

Pellets of *L. lactis* cells were resuspended in phosphate-PBS in the presence of protease inhibitors (Complete EDTA-Free, Roche) and frozen at -80 °C. After thawing, cells were disrupted at 1,500 psi (4 rounds for soluble protein and 3 for protein nanoparticles) in a French press (Thermo FA-078A). For the soluble MMP-9, 0.05 mg/ml lysozyme was added and the resulting mixture was incubated at 37 ºC with shaking for 2 h. The lysate was centrifuged at 15,000 x *g* for 45 min. After that, soluble MMP-9 was recovered from the pellet as described in Peternel et al. (2008) and filtered through a 0.22 µm filter. The protein was purified by His-tag affinity chromatography using HiTrap Chelating HP 1 ml columns (GE Healthcare, Barcelona, Spain) with an ÄKTA purifier FPLC System (GE Healthcare). The purified soluble MMP-9 was analyzed by both SDS electrophoresis/Coomassie Brilliant Blue staining and Western blotting. Concentration was determined by Bradford's assay (BioRad). The isolation of MMP-9 protein nanoparticles was conducted following Cano-Garrido et al. (2016). The yield of MMP-9 nanoparticles was determined by both SDS electrophoresis/Coomassie Brilliant Blue staining and Western blotting, using a standard curve of known amounts of a GFP protein. Densitometry analyses were performed with the Quantity One software (BioRad). For Western blotting, a commercial monoclonal antibody against the

polyhistidine-tag (#A00186-100 Genescript, Piscataway, USA) and an anti-mouse secondary antibody (#170-6516 Bio Rad) were used.

## Electron microscopy

Nanoparticles were detected by field emission scanning microscopy (FESEM) and transmission electron microscopy (TEM). In the first technique, sample microdrops were deposited 2 min on silicon wafers (Ted Pella Inc., Redding, CA, USA) and then air-dried. Nanoparticle micrographs were acquired at a nearly native state with a high-resolution in-lens secondary electron detector in a FESEM Zeiss Merlin (Zeiss, Oberkochen, Germany) operating at 2 kV. For TEM, nanoparticle samples were fixed with aldehydes and osmium, dehydrated and embedded in Epon resin. Ultrathin sections were deposited on copper grids and, after contrast, observed with an electron microscope TEM Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan).

## Analysis of MMP-9 activity

Metalloproteinase activity of recombinant MMP-9 (soluble MMP-9 and MMP-9 protein nanoparticles -39,6 KDa-) and endogenous MMP-9 -92 KDa- present in mammary secretion samples were analyzed and compared by zymography as described elsewhere (Domènech et al., 2014).

**Intra-mammary infusions**

This experiment was performed under the evaluation and permission of the Ethical Committee of IRTA, protocol number 8788. Twelve lactating Holstein cows (210-220 d pregnant, producing >20 kg/d of milk during the last 3 d preceding dry-off, and with milk SCC <200,000 cells/ml at dry-off) were enrolled in this study at dry-off. All cows were dried abruptly with no dietary intervention before dry-off and no change in milking routine or frequency. At drying time, cows received an intra-mammary infusion of either 0.75 mg of soluble recombinant MMP-9 or 100 mg of MMP-9 nanoparticles in a total volume of 10 ml of 0.9% NaCl sterile commercial saline solution. The actual enzymatic activity of the soluble and the nanostructured MMP-9 doses used were identical as assessed by zymography. Treatments were randomly assigned to front or rear quarters to avoid potential milk dilution effects. Also, a negative control consisting of 10 ml of 0.9% NaCl sterile commercial saline solution (Braun, Barcelona, Spain) was infused in the respective contralateral quarter. Then, broad-spectrum antibiotics (Mamyzin secado®, Boehringer Ingelheim, Barcelona, Spain) were locally administered following common production practices. No teat sealant was used. Milk or mammary secretion samples were obtained at days 0 (before last milking), 1, 2, 3, 6 and 7 post-drying at 08:00 h by manual milking. A fraction of mammary secretion samples was kept refrigerated until analyzed for SCC, and the remaining fraction was kept frozen at -20 ºC until subsequent analyses.

Mammary biopsies from the rear quarters (n=12) treated with MMP-9 nanoparticles were obtained at day 9 and conserved in RNAlater (Invitrogen, Madrid, Spain) for 24-72 h at 4 °C prior freezing at -80°C until subsequent RNA extraction.

**Biopsy procedure**

To perform the biopsies, cows were partially immobilized in a head bail. The rear part of the mammary gland was hair clipped and washed with a commercial povidone/iodine solution. All procedures beyond this point were carried in maximum sterility conditions possible. The selected biopsy area was a midpoint on a rear quarter. The incision area was locally anesthetized with a subcutaneous injection of 1.5 ml (30 mg) lidocaine solution (Procamidor, Richter Pharma, Austria). An incision of 1.5-2.5 cm was made with a scalpel through the skin and gland capsule, avoiding large blood vessels. Then, a 6-mm trocar was introduced about 10 cm deep into the mammary gland through the incision, the interior punch removed, and biopsy forceps introduced through the trocar canal into the mammary gland to collect a piece of tissue of about 3 $mm^2$. Pressure was applied to the biopsied area after the procedure for about 3-5 min. Lastly, skin was sutured with a non-absorbable multifilament suture (KRUUSE silk, Langeskov, Denmark). Samples were washed in PBS, and conserved in 1 ml of RNAlater (Invitrogen) for transportation.

## RNA extraction and qPCR

The extraction of RNA was performed using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed with Nanodrop 1000 instrument (Thermo Scientific, Barcelona, Spain) at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Reaction of qPCR was performed in a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan) and under the conditions and primers specified in Table 1.

| Gene | Primer Sequences | Amplicon Size (bp) | Annealing temperature (Celsius) | Primer concentration (μM) |
|---|---|---|---|---|
| *RPS9* (HK) | Fw: 5'-CCTCGACCAAGAGCTGAAG-3'<br>Rv: 5'-CCTCCAGACCTCACGTTTGTTC-3' | 63 | 57 | 0.125 |
| *RPS15* (HK) | Fw: 5'-GCAGCTTATGAGCAAGGTCGT-3'<br>Rv: 5'-GCTCATCAGCAGATAGCGCTT-3' | 150 | 57 | 0.125 |
| *CASP3* | Fw: 5'-AAGCCATGGTGAAGAAGGAA-3'<br>Rv: 5'-GGCAGGCCTGAATAATGAAA-3' | 134 | 55 | 0.5 |
| *MMP-9* | Fw: 5'-TCGACGTGAAGACACAGAGGT-3'<br>Rv: 5'-TGATCCTGGCAGAAGTAAGCTTTC-3' | 126 | 57 | 0.125 |
| *BAX* | Fw: 5'-AGTGGCGGCTGAAATGTT-3'<br>Rv: 5'-TTCTTCCAGATGGTGAGCG-3' | 287 | 60 | 0.5 |
| *MKi67* | Fw: 5'-AACACCCAGTCGTGTTCGTT-3'<br>Rv: 5'-GAGCCTTCGGTTCTTCACGA-3' | 130 | 62 | 0.5 |

**Table 1.** Primer sequences and amplification conditions for gene expression analysis

The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8 % DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which was calculated using the formula E=10^(-1/slope), with an acceptable range of 1.8-2.2. A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBER green fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95 °C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. The resulting Ct values were used to calculate the relative expression of selected genes by relative quantification using *RPS9* and *RPS15* as housekeeping genes.

**Mammary secretion analyses**

SCC were analyzed in fresh milk (last milking before dry-off) or mammary secretion samples within 4 h after extraction using the Scepter® cell counter (Merk-Millipore, Madrid, Spain) and 40 µm sensors. Briefly, 0.5 ml of mammary secretion was diluted 1:1 in PBS and cells were pelleted by centrifugation at 1,000 x g for 2 min. Cream and supernatant were discarded. Pellets were then washed 3 times in PBS (without

resuspending the pellet), and then resuspended in 0.5 ml of PBS. In some cases, samples had to be diluted in PBS prior to analysis.

Lactoferrin concentration in skimmed milk (last milking before dry-off) and mammary secretions was measured by ELISA, using the commercial bovine lactoferrin ELISA kit (Bethyl Laboratories Inc., Montgomery, TX). The absorbance for each sample was measured at 450 nm using a Model 680 microplate reader (Bio-Rad).

The concentration of bovine serum albumin (BSA) in milk and mammary secretions was analyzed by a colorimetric assay as previously described (Ponchon et al., 2014), with some modifications. Briefly, 200 µl of skimmed milk or mammary secretion were mixed with 450 µl of distilled water and 450 µl of a solution containing 1 volume of 1.2 mM of bromocresol green dissolved in 5 mM NaOH, 3 volumes of 0.2 M succinic acid (pH 4.0), and 0.8 % Brij 35 detergent. After mixing by inversion and centrifugation at 1,900 x *g* for 10 min at RT, 150 µl of the supernatant were added to a 96-well microplate and the optical density was read at 655 nm using a Model 680 microplate reader (Bio-Rad).

Gelatinase activity was analyzed by zymography as described above. Samples were skimmed by centrifugation at 2,700 x *g* for 10 min. The fat layer was discarded with a swab and supernatant was diluted between 1:20 to 1:250 in PBS and mixed 1:1 with the loading buffer.

Sodium and potassium concentrations in milk secretion were analyzed by inductively coupled plasma-Optical emission spectrometry (ICP-OES) using an ICP-OES Perkin-Elmer Optima 4300DV after dilution with Triton X-100 0.1 % (v/v) instead of digested in a microwave oven as described in Murcia et al. (1999). The method was validated before analyzing all samples by evaluating the repeatability of the results from 5 samples that were analyzed after digestion with microwaves or dilution in 0.1 % (v/v) Triton X-100.

**Statistical analysis**

Each mammary quarter was an experimental unit. All data, except that pertaining to gene expression in mammary tissue at day 9 after dry-off, were analyzed using a mixed-effects model that accounted for the random effects of quarter within cow, cow, and block (enrollment week) and the fixed effects of treatment, day of sampling, and their 2-way interaction. Sampling time was entered in the model as a repeated measure using an autoregressive covariance matrix. Data were previously transformed to achieve a normal distribution when necessary. Data pertaining to gene expression in mammary tissue at day 9 after dry-off was analyzed with a similar model as described above but without the fixed effect of sampling time and the random effect of quarter within cow.

## Results and Discussion

### Recombinant protein production and activity

A good strategy to accelerate the cow dry period would be to amplify the effects of some of the proteins that play important roles in the involution and remodeling processes through their exogenous administration. MMP-9 is the most important metalloproteinase involved in the involution of the cow mammary gland at dry-off. Although physiologically in the mammary gland, MMP-9 is secreted by the local immune cells (Khokha and Werb, 2011), MMP-9 can also be produced by recombinant DNA technologies. In this study, the recombinant production of two different MMP-9 formats (soluble and nanostructured) was done in *L. lactis* to ensure that MMP-9 would be free of LPS and would not elicit any endotoxic response in the animal (Garcia-Fruitós, 2012). The morphology of MMP-9 nanoparticles produced herein was round, compact, and with a smooth surface and a 431.58 nm diameter (Figure 1).

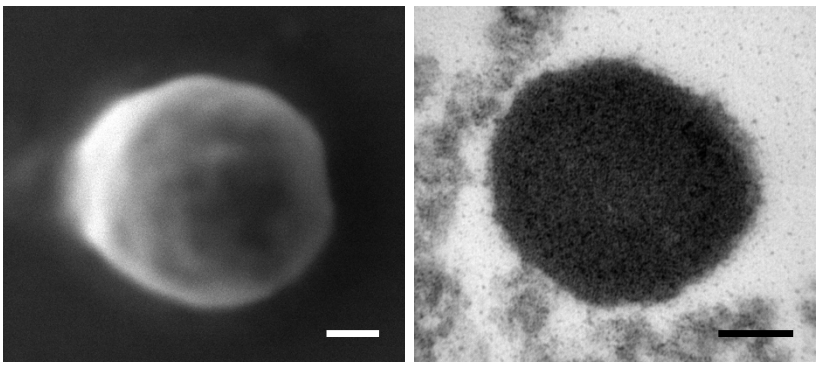

**Figure 1.** Field emission scanning electron microscopy (FESEM) micrographs of Matrix Metalloproteinase-9 (MMP-9) nanoparticles (left panel) and transmission electron microscopy (TEM) micrographs of MMP-9 nanoparticles (right panel). Scale bars: 100 nm.

The value of the Z potential was highly negative, indicating that the MMP-9 nanoparticles were very stable as it is the case of most recombinant proteins produced in this form (Cano-Garrido et al., 2014; Villaverde et al., 2015; Cano-Garrido et al., 2016). When comparing the MMP-9 activity by zymography the soluble form showed about 130-fold greater activity than their nanostructured counterpart (data not shown). Although MMP-9 is secreted as a zymogen physiologically, and then activated by proteolysis (Benaud, 1998), we have proven that it is possible to recombinantly produce an active MMP-9 domain in both soluble and nanostructured formats.

**Response of the immune system of the mammary gland after intra-mammary administration of recombinant MMP-9**

SCC in mammary secretions progressively increased ($P < 0.0001$) as time elapsed since dry-off increased (Figure 2A). However, the increase in SCC was greater at all sampling times ($P < 0.0001$) in the quarters treated with MMP-9 nanoparticles than in the other 2 treatments (Figure 2A), but SCC in milk secretion from quarters treated with soluble MMP-9 and control (saline) did not differ (Figure 2A, inset). From day 1 to 7, SCC increased ($P < 0.001$) to 157-fold in the mammary secretion from quarters infused with nanoparticles, whereas in the quarters treated with control or soluble MMP-9 the increase was 16- and 9-fold, respectively.

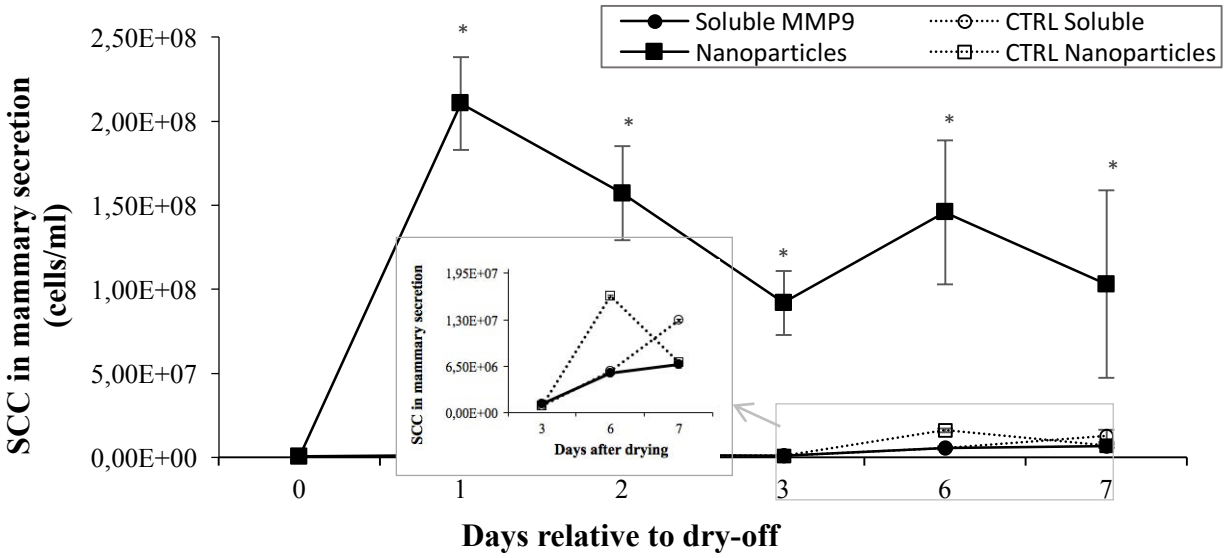


**Figure 2. A**. Evolution of somatic cell counts (SCC) in mammary secretion around dry-off.

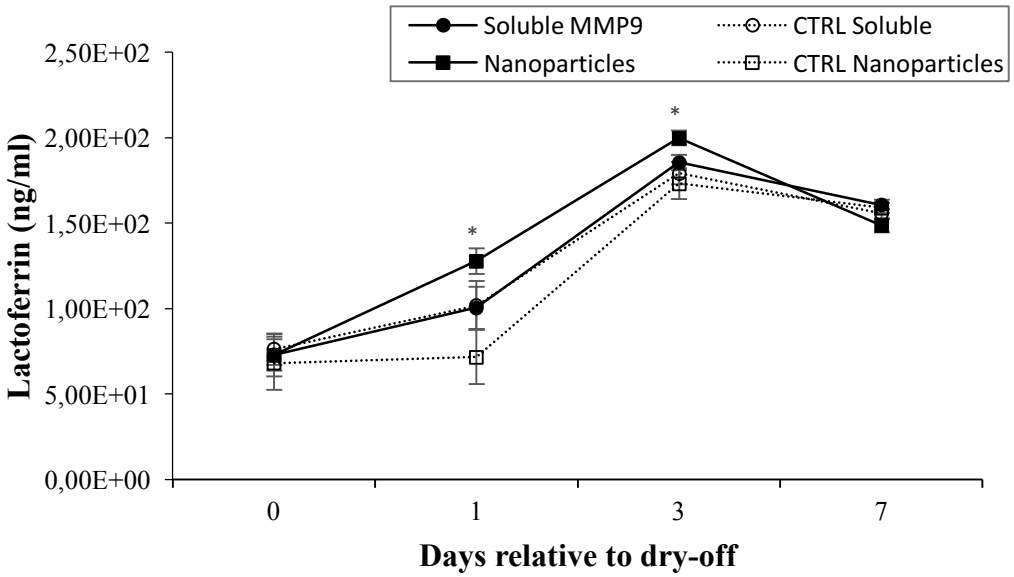


**Figure 2. B**. Lactoferrin concentration in mammary secretion. Continuous lines depict matrix metalloproteinase-9 (MMP-9) treatments, discontinuous lines indicate controls (saline). Circles correspond to soluble MMP-9 treatment and its control, squares represent MMP-9 nanoparticle treatment and its control. Asterisks indicate significant differences ($P < 0.05$) between treatment and control.

The more sustained recruitment of SCC in milk secretion observed with the nanostructured MMP-9 might be due, in part, to a more sustained and stable release of MMP-9 into the mammary gland. It has been previously shown that nanostructured recombinant proteins are much more stable than their soluble counterparts (Cano-Garrido et al., 2016) and, when exposed into a physiological environment, they show a slow-release pattern, which results in a long-term effect (Villaverde et al., 2012; Céspedes et al., 2016; Seras-Franzoso et al., 2016; Rinas et al., 2017;).

Lactoferrin concentration in mammary secretions increased ($P < 0.0001$) over time after dry-off (Figure 2B). Overall, mammary secretion from quarters treated with MMP-9 nanoparticles had greater ($P < 0.05$) lactoferrin concentration than that from quarters on control or treated with soluble MMP-9. There was also a positive interaction between treatment and time ($P < 0.0001$), with quarters treated with nanostructured MMP-9 showing greater values of lactoferrin in mammary secretion at days 1 and 3 after dry-off than that in other treatments. Lactoferrin concentration in mammary secretions from quarters treated with control or with the soluble form of MMP-9 did not differ at any sampling time during the week after dry-off. The appearance of immune cells and concomitant increase of lactoferrin concentration in the mammary secretion was very clear at day 1 after dry-off in the quarters treated with nanostructured MMP-9, but in the soluble or controls quarters the increase started at day 3 achieving levels of SCC much lower (100 X) than those seen with MMP-9 nanoparticles. These results indicate

that quarters treated with nanostructured MMP-9 had an earlier activation of the immune system, compared with the soluble or control treatments. The rise in SCC in mammary secretion indicates a hastened recruitment of immune cells and the increase in lactoferrin concentration reflects a stimulation of other effectors of the innate immune system. The increased lactoferrin concentration in mammary secretions may have resulted from either an increased synthesis by mammary epithelial cells, or from a release by infiltrated somatic cells (Pecorini et al., 2009).

Several parameters reflecting the tissular involution of the mammary gland were evaluated (Figure 3). The sodium/potassium ($Na^+/K^+$) ratio in mammary secretion increased ($P < 0.0001$) as days since dry-off increased (Figure 3A).

There was an interaction between treatment and time with mammary secretion from quarters treated with nanostructured MMP-9 having a greater $Na^+/K^+$ ratio at day 1 compared with those treated with saline solution, but at days 3 and 7 the $Na^+/K^+$ ratio in mammary secretion from quarters treated with nanostructured MMP-9 was lower than in controls. No differences in the $Na^+/K^+$ ratio were detected in mammary secretion from quarters treated with soluble MMP-9 or control. The presence of BSA in mammary secretion followed a similar pattern than $Na^+/K^+$ ratio (Figure 3B) and increased ($P < 0.0001$) within days after treatment. Concentration of BSA in mammary secretion from quarters treated with nanostructured MMP-9 was greater ($P < 0.0001$) than in the other two

treatments on days 1, 2, and 3 after dry-off. Tissue remodeling in the mammary gland is accompanied by a breakup of the tight junctions, increasing the permeability between milk and blood stream. As a consequence, BSA and $Na^+$ from blood can be present in mammary secretion and are commonly used as involution markers (Ponchon et al., 2014).

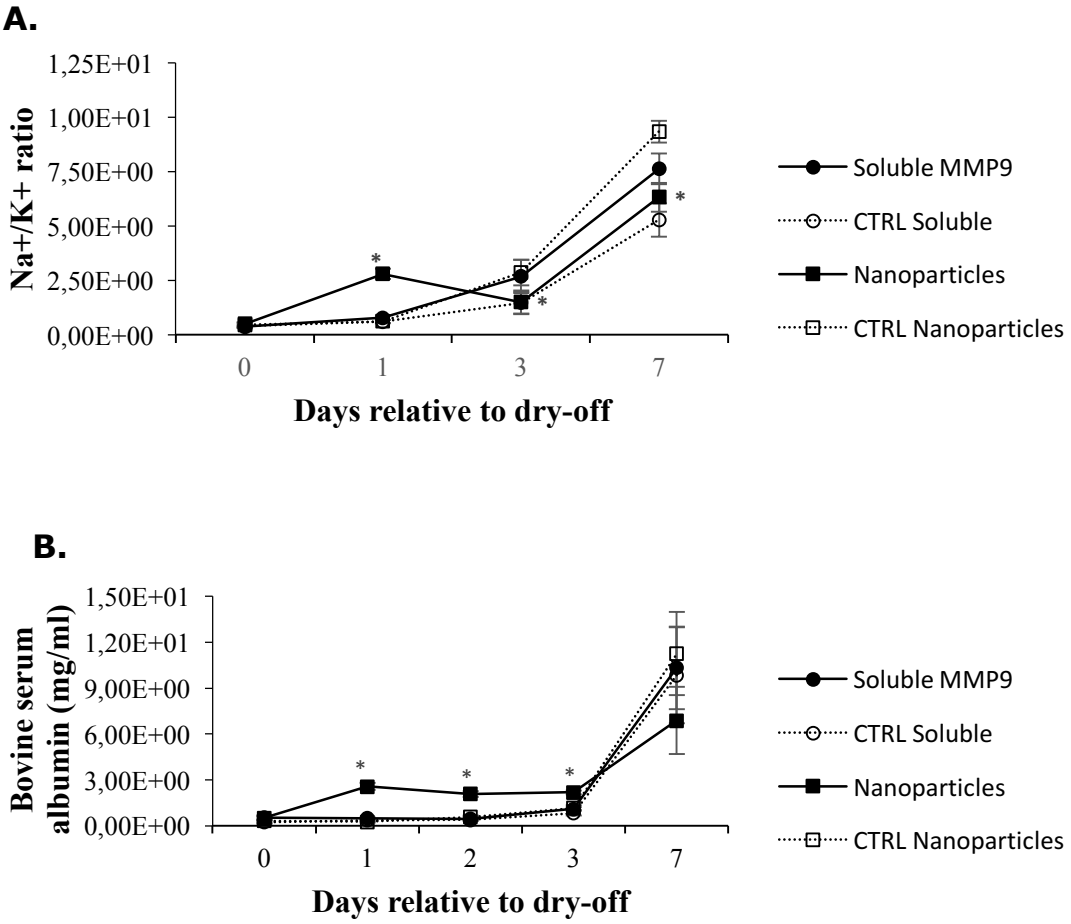


**Figure 3.** Sodium/potassium ($Na^+/K^+$) ratio in mammary secretion (**panel A**) and bovine serum albumin (BSA) concentration (**panel B**) in mammary secretion around dry-off. Continuous lines indicate matrix metalloproteinase-9 (MMP-9) treatments and discontinuous lines depict controls. Circles correspond to soluble MMP-9 treatment and its control, squares represent MMP-9 nanoparticle treatment and its control. Asterisks indicate differences ($P < 0.05$) between treatment and control.

Thus, the accelerated increase in both $Na^+/K^+$ ratio and BSA in mammary secretion from quarters treated with nanostructured MMP-9 indicated that the involution was initiated earlier than with the soluble MMP-9 form and control.

Lastly, endogenous metalloproteinase activity in mammary secretion was evaluated by zymography (Figure 4). The activity of infused recombinant MMP-9 (soluble or nanostructured) was easily differentiated from the activity corresponding to the endogenous MMP-9 due to differences in the molecular weight between the 2 forms (92 kDa and 39.6 kDa, respectively). In fact, we could only find activity from the endogenous MMP-9 in the mammary secretion samples. Overall, endogenous metalloproteinase activity increased as days since dry-off increased ($P < 0.001$). At days 1 and 3, the activity was greater in soluble and nanostructured MMP-9 treatments compared with their saline controls. The activity in quarters treated with soluble MMP-9 was greater than in the quarters treated with nanostructured MMP-9 at day 1, but at day 3 this activity was reversed ($P < 0.0001$), which could probably be due to a more slowly and stable release of MMP-9 in the nanostructured than in the soluble form.

We do not know yet why soluble recombinant MMP-9 induced endogenous metalloproteinase activity compared with controls quarters but failed in affecting any other involution parameters. One potential reason could be the activation of endogenous pro-MMP-9. MMP-9 is secreted as a

zymogen, and later activated by catalytic function or by other active metalloproteinases such as metalloproteinase-3 (Ramos-DeSimone et al., 1999).

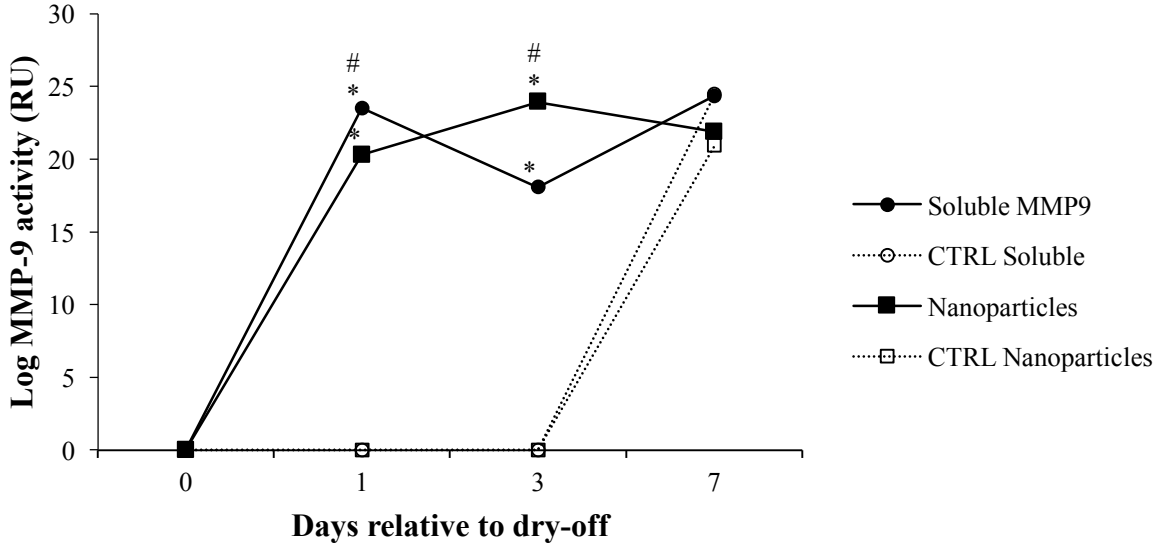


**Figure 4.** Endogenous matrix metalloproteinase-9 (MMP-9) activity in mammary secretions analyzed by zymography. Continuous lines indicate MMP-9 treatments, discontinuous lines depict controls. Circles correspond to soluble MMP-9 treatment and its control, squares represent MMP-9 nanoparticle treatment and its control. Asterisks indicate significant differences ($P < 0.05$) between treatment and control whereas pound sign indicates differences between treatments.

Recombinant MMP-9 could exert a catalytic effect on endogenous pro-MMP-9 increasing the final metalloproteinase activity in the tissue. However, we cannot discard that the composition and profile of recruited SCC could differ between quarters treated with saline or with soluble MMP-9, which could also result in different amounts of endogenous active MMP-9 released.

Overall these results indicate that, even though we selected a dose of the two protein formats that showed equivalent metalloproteinase activity by zymography, the main in vivo effects on involution markers in mammary secretion were observed with the nanostructured form of MMP-9 (Figures 2 and 3), which is in agreement with previous studies reporting that when protein nanoparticles are placed in a physiological environment, there is an in situ release of active molecules (Céspedes et al., 2016; Seras-Franzoso et al., 2016).

**Cell survival and apoptosis**

Expression of *MMP-9* in mammary tissue tended to increase in quarters treated with the nanoparticle form compared with control ($P = 0.081$) (Figure 5).

Gene expression analyses at day 9 (Figure 5) showed differences between nanoparticle and control treatments in the expression of the apoptosis marker *CASP3* and proliferation marker *MKi67*, which were increased by 1.4-fold ($P < 0.05$) and 1.9-fold ($P < 0.05$), respectively, when quarters were treated with MMP-9 nanoparticles. Also, a tendency ($P = 0.06$) towards an increased expression of the apoptosis marker *BAX* was observed in quarters treated with the nanostructured form of MMP-9 in comparison with the saline control.

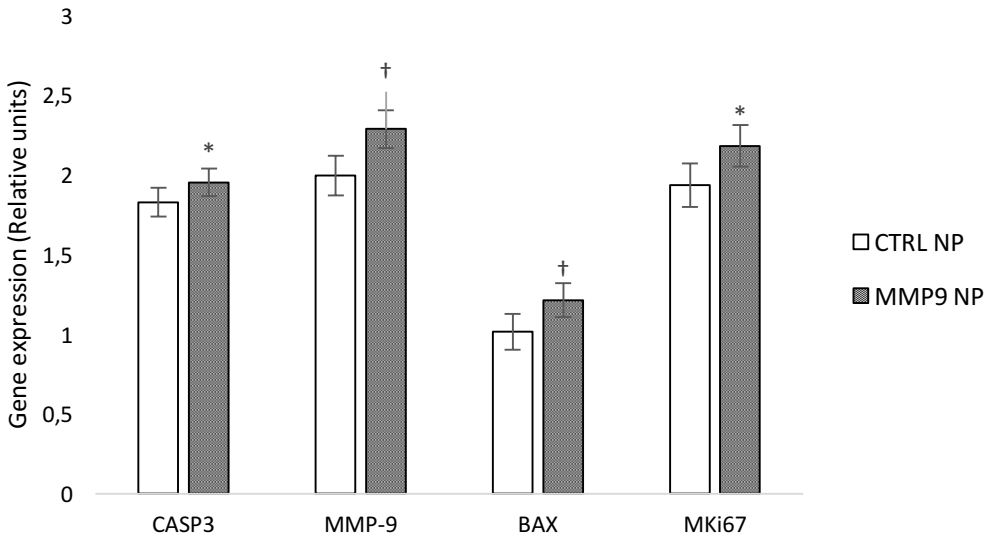


**Figure 5.** Gene expression in mammary tissue at day 9 after dry-off. White bars correspond to controls and textured bars correspond to mammary quarters treated with matrix metalloproteinase-9 (MMP-9) nanoparticles. Asterisks indicate significant differences ($P < 0.05$) between treatment and control. † indicates a tendency to differ ($P < 0.10$) between treatment and control.

In line with the observations in mammary secretions, and based on the expression these 2 markers, it could be concluded that cellular apoptosis and proliferation were greater in quarters treated with nanostructured MMP-9 than in controls. Furthermore, the increase in local expression of endogenous *MMP-9* and greater infiltration of blood and $Na^+$ also supports a more active involution in quarters treated with MMP-9 nanoparticles.

In conclusion, the infusion of nanoparticles of recombinant bovine MMP-9 into the mammary gland at dry-off has potential for accelerating the involution of the mammary gland by stimulating the local immune response. In contrast with the infusion of recombinant soluble MMP-9, intra-mammary infusions of nanostructured MMP-9 elicit a sustained

(slow-release) and stable response. Therefore, this new format of recombinant MMP-9, based on nanoparticles produced in *L. lactis*, could become a plausible alternative to optimize dry-off and potentially contribute to reduce the use of preventive antibiotics.

## Acknowledgements

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# CHAPTER V – Study 3:

# Effects of prolactin inhibition on cell recruitment, apoptosis, turnover, and senesce of mammary epithelial cells at dry-off

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## Introduction

As calving approaches, dairy cows need a non-lactating (dry) period to optimize milk production in the subsequent lactation (Capuco and Ellis, 2013). During the dry period, the mammary epithelial component regresses, proliferates, and differentiates to allow optimal milk production in the subsequent lactation. Tissue regeneration of the mammary gland between lactations is necessary, and the omission of a dry period typically reduces milk production in the following lactation (Bachman and Schairer, 2003).

The drying process is mediated by cell signaling and immune mechanisms. The first stages are considered reversible, and lactation could be restored by reinitiating milk extraction, and the second stage is considered irreversible. There are two main processes described to occur at dry-off: 1) involution, which takes place mainly by apoptosis, and 2) regeneration of the mammary epithelial component, although not much is known about how exactly the cell turnover occurs during the dry period (De Vries et al., 2010, Pezeshki et al. 2010).

With progressively increasing milk yields, the dry-off has become a challenging period for dairy cows. Furthermore, today's dairy cows are dried while producing ~25 kg/d of milk with some animals producing >35 kg/d (Bach et al., 2015). These copious amounts of milk remaining in the mammary gland exert high intra-mammary pressure and may cause discomfort and milk leakage after dry-off (Gott et al., 2016), and may

result in increased risk of contracting intramammary infections (Eberhart, 1986; Stefanon et al., 2002). In addition, the strong presence of galactopoietic hormones, consequence of a concomitant pregnancy, hampers the beginning of the involution of the mammary gland and delays the activation of the immune system (Noble and Hurley, 1999; Pezeshki et al., 2010).

A potential method to facilitate dry-off would consist of inducing a direct cessation of milk production by interfering with hormonal signals (Lacasse et al., 2011; Ollier et al., 2014; Bach et al., 2015). Recent evidence supports the galactopoietic role of prolactin (PRL), as this hormone is essential for maintaining lactation, and suppressing it strongly inhibits lactation in ruminants (Lollivier et al., 2015; Lacasse et al., 2016). Blocking the release of PRL by binding dopamine D2 receptors using quinagolide has been shown to decrease milk production in both early- (Lacasse et al., 2011) and late-lactation (Ollier et al., 2013), and to hasten the involution of the mammary gland at dry-off (Ollier et al., 2014). A similar strategy would consist of administering cabergoline at dry-off (Bach et al., 2015; Bertulat et al., 2017). Cabergoline is an ergot derivative with high affinity for the D2 dopamine receptors, whose dopaminergic effects cause inhibition of PRL secretion (Romagnoli et al., 2009).

Previous studies using cabergoline at dry-off (Bach et al., 2015; Bertulat et al., 2017) showed that cabergoline reduced udder engorgement, udder

pressure, milk leakage, and udder pain in the first week after dry-off. However, those studies did not evaluate the effects of cabergoline and PRL blocking on hormonal and cellular factors controlling the final cell turnover of the bovine mammary gland. Therefore, the objectives of the current study were to determine the cellular changes in the mammary gland and specifically cell recruitment, apoptosis, turnover, and senescence of epithelial cells from cows with normal levels of PRL or reduced concentration of PRL induced by cabergoline administration at dry-off.

## Materials and Methods

### Animals and experimental design

This experiment was performed under the evaluation and permission of the Ethical Committee of IRTA, protocol number 8788. Twenty-four lactating Holstein cows, 6 primiparous (PMC) and 18 multiparous (MPC), (210-220 d pregnant, producing an average >25 kg/d of milk during the last 3 d preceding dry-off, and with milk somatic cell counts <200,000 cells/ml at dry-off) were enrolled in this study at dry-off. All cows were dried abruptly with no dietary intervention before dry-off and no change in milking routine or frequency. At dry-off, cows were blocked by parity (PMC or MPC) and half of the cows (n=12) received one dose of 5.6 mg of cabergoline intra-muscular in 5 ml total volume (Velactis, Ceva Santé Animale, Libourne, France), and the remaining 12 cows were considered controls. Also, broad-spectrum antibiotics (Mamyzin secado®, Boehringer Ingelheim, Barcelona, Spain) were locally administered following common production practices.

All cows in this study underwent a mammary gland biopsy at -10, 9, and 21 d relative to dry-off, and -10 and 23 d relative to calving (a total of 5 biopsies per animal). Also, blood samples were collected at the same time points that biopsies were performed.

**Biopsy procedure**

To perform the biopsies, cows were partially immobilized in a head bail. The rear part of the mammary gland was hair clipped and washed with a commercial povidone/iodine solution. All procedures beyond this point were conducted in maximum sterile conditions possible. The biopsy area selected was at a midpoint on a rear quarter (alternating left and right quarters between consecutive biopsies). The incision area was locally anesthetized with a subcutaneous injection of 1.5 ml (30 mg) lidocaine solution (Procamidor, Richter Pharma, Austria). An incision of 1.5-2.5 cm was made with a scalpel through the skin and gland capsule, avoiding large blood vessels. Then a 6-mm trocar was introduced about 10 cm deep into the mammary gland through the incision, then removed, and biopsy forceps introduced through the trocar canal into the mammary gland to collect two tissue pieces of about 3 $mm^2$. Pressure was applied to the biopsied area after the procedure for about 3-5 min. Last, skin was sutured with a non-absorbable multifilament suture (KRUUSE silk, Langeskov, Denmark). Samples were washed in PBS, and then conserved in 1 ml of RNAlater (Ambion, USA) for subsequent gene expression analyses or in 10 ml of 10% neutral buffered formalin solution (NBF) for subsequent histological assessment.

## RNA Extraction and RT-qPCR

RNA was extracted using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed by Nanodrop 1000 instrument (Thermo Scientific, Barcelona, Spain) at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Reaction of RT-qPCR was performed in a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan) and the conditions and primers specified in Table 1.

The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8 % DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which was calculated using the formula 10^(-1/slope), with an acceptable range of 1.8-2.2. A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBR green fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 1).

| Gene | Primer Sequences | Amplicon Size (bp) | Annealing temperature (Celsius) | Primer concentration (μM) |
|---|---|---|---|---|
| *RPS9* | Fw: 5'-CCTCGACCAAGAGCTGAAG-3'<br>Rv: 5'-CCTCCAGACCTCACGTTTGTTC-3' | 63 | 57 | 0.125 |
| *RPS15* | Fw: 5'-GCAGCTTATGAGCAAGGTCGT-3'<br>Rv: 5'-GCTCATCAGCAGATAGCGCTT-3' | 150 | 57 | 0.125 |
| *MMP9* | Fw: 5'-TCGACGTGAAGACACAGAGGT-3'<br>Rv: 5'-TGATCCTGGCAGAAGTAAGCTTTC-3' | 126 | 57 | 0.125 |
| *BAX* | Fw: 5'-AGTGGCGGCTGAAATGTT-3'<br>Rv: 5'-TTCTTCCAGATGGTGAGCG-3' | 287 | 60 | 0.5 |
| *CASP3* | Fw: 5'-AAGCCATGGTGAAGAAGGAA-3'<br>Rv: 5'-GGCAGGCCTGAATAATGAAA-3' | 134 | 55 | 0.5 |
| *MKI67* | Fw: 5'-AACACCCAGTCGTGTTCGTT-3'<br>Rv: 5'-GAGCCTTCGGTTCTTCACGA-3' | 130 | 62 | 0.5 |
| *C-MYC* | Fw: 5'-GTGTCTACCCATCAGCACAA-3'<br>Rv: 5'-AACTGTTCTCGCCTCTTCTG-3' | 58 | 376 | 0.25 |
| *P16* | Fw: 5'-GGTGATGATGATGGGCAGCG-3'<br>Rv: 5'-ACCAGCGTGTCCTGGAAGC-3' | 62 | 134 | 0.5 |
| *SIRT1* | Fw: 5'-GGAGCAGATTAGTAAACGCCT-3'<br>Rv: 5'-CTTTCATCCTCCATGGGTTC-3' | 58 | 188 | 0.25 |
| *CK18* | Fw: 5'-TCGATGACACCAATGTCACCC-3'<br>Rv: 5'-ACCAGTACTTGTCCAGCTCC-3' | 60 | 249 | 0.25 |
| *IGFB5* | Fw: 5'-GTTTGCCTGAACGAAAAGAGCTA-3'<br>Rv: 5'-CGAGTAGGTCTCCTCTGCCATCT-3' | 57 | 104 | 0.5 |
| *IGF-1* | Fw: 5'-TCGCATCTCTTCTATCTGGCCCTGT-3'<br>Rv: 5'-GCAGTACATCTCCAGCCTCCTCAGA-3' | 57 | 239 | 0.125 |

**Table 1.** Primer sequences and amplification conditions for gene expression analysis.

The RT-qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C. The resulting Ct values were used to calculate the relative expression of selected genes by relative quantification using housekeeping genes *RPS*9 and *RPS*15 (Bionaz and Loor, 2007).

## Histological techniques: immunohistochemistry

All histological techniques were performed at the Morphology Laboratory at CBATEG (Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain). Tissue samples were fixed in 10% neutral buffered formalin solution for 48 h at 4ºC with gentle agitation. After incubation, samples were dehydrated in an ascending alcohol gradient and embedded in paraffin to acquire tissue sections. Immunohistochemistry stainings for Sirtuin 1 (SIRT1), p16, and Ki-67 were performed separately in 3 micron sections with the SIRT1 H-300 (Santa Cruz), p16 M-156 (Santa Cruz), and the purified Mouse Anti-Ki-67 (Bd biosciences) antibodies respectively. Biotinylated goat anti-rabbit IgG antibody (Vector Laboratories) and M.O.M. Kit (Vector laboratories) were used as secondary antibodies. Sections were counterstained with hematoxylin to perform nuclei count. All nuclei from one representative section at 40X were counted for each sample, and the results expressed as the ratio between antibody stained and total nuclei.

## Blood parameters

Blood samples were analyzed by ELISA for IGF-1 and progesterone concentrations and read in an EMS Reader MF V.2.9-0 plate reader. For the analysis of IGF-1, a sandwich ELISA was carried on using the IGF-I ELISA kit (Mediagnost, Germany). Progesterone concentrations were analyzed with the Progesterone ELISA Kit (DRG Instruments, Marbug,

Germany). Also, PRL and GH concentrations were analyzed by RIA at the facilities of the Endocrinology and Radioimmunoanalysis service at UAB. Synthetic bovine PRL and GH (National Hormone and Peptide Program, NHPP; Torrence, CA) were iodinated with [125I] Iodine (PerkinElmer, Waltham, MA, USA) following the chloramine T method (Greenwood et al., 1963) and used as the tracer. Rabbit sera against bovine PRL and GH (NHPP, Torrence, CA) were used at dilution 1:3750 and 1:15000 respectively. Samples and standards were incubated with tracer hormones and antibodies for 18h at 4ºC. Then, a rabbit serum anti IgG (AbD serotec) was added for 4 h at 4ºC to separate the bound fraction by centrifugation at 4700 x *g* for 30 min. Wallac Wizard 1470 Gamma Counter (GMI Inc, Ramsey, MN, USA) was used to quantify the gamma emissions of the pellet.

**Statistical Analysis**

Data were analyzed using a mixed-effects model. The model included the random effect of cow and the fixed effects of treatment, parity (PMC or MPC), and days relative to dry-off, plus their 2- and 3-way interactions. Sampling day was included as a repeated measure using the compound symmetry variance-covariance matrix.

## Results and Discussion

### Tissular factors involved in dry period

A set of tissue involution factors was analyzed for gene expression by qPCR (Table 2) and protein quantification by immunohistochemistry (Table 3) to potentially identify factors that were more relevant during the evolution of the dry period.

| | Treatment | | | P-value | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Cabergoline | SEM | Treatment (T) | Parity (P) | Time (t) | TxP | Txt |
| *IGF-1* | 1.32 | 1.37 | 0.23 | 0.24 | 0.27 | 0.25 | <0.05 | 0.39 |
| *IGFBP5* | 6.02 | 4.60 | 0.11 | <0.05 | 0.33 | 0.59 | 0.30 | 0.67 |
| *MMP-9* | 1.29 | 3.23 | 0.65 | 0.51 | 0.10 | 0.09 | 0.62 | 0.17 |
| *MKI67* | 505.6 | 684.2 | 221.2 | 0.61 | <0.01 | <0.01 | 0.05 | 0.32 |
| *P16* | 3.85 | 10.53 | 1.83 | 0.13 | 0.06 | <0.05 | 0.15 | 0.93 |
| *SIRT1* | 39.68 | 72.09 | 14.50 | 0.08 | 0.21 | <0.05 | <0.05 | 0.57 |
| *C-MYC* | 6.44 | 7.82 | 1.88 | 0.94 | 0.09 | <0.05 | 0.69 | 0.55 |
| *CK18* | 67.2 | 13.2 | 1.29 | 0.35 | 0.06 | 0.77 | 0.25 | 0.32 |
| *BAX* | 0.36 | 1.03 | 0.20 | 0.26 | 0.35 | <0.0001 | 0.35 | 0.48 |
| *CASP3* | 0.59 | 0.52 | 0.05 | 0.82 | 0.47 | 0.09 | 0.80 | 0.44 |

**Table 2:** Gene expression in the mammary gland as affected by treatment, parity, sampling day and their interactions

We observed changes through time in the expression of the apoptotic genes *BAX* and *CASPASE3* (CASP3) ($P < 0.0001$ and 0.09 respectively), the marker of proliferation *MKI67* ($P < 0.05$), the cell senescence marker *P16* ($P < 0.05$), the cell survival marker *SIRT1* ($P < 0.05$), the *C-MYC* gene ($P < 0.05$), which plays a role in cell cycle progression and cellular transformation, and the tissue remodeler *MMP-9* ($P = 0.09$) (Figure 1).

| | Treatment | | | P-value | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Cabergoline | SEM | Treatment (T) | Parity (P) | Time (t) | TxP | Txt |
| SIRT1 | 74.4 | 78.5 | 3.69 | 0.18 | 0.11 | <0.05 | <0.01 | 0.75 |
| Ki67 | 5.01 | 4.72 | 1.04 | 0.26 | 0.73 | <0.01 | 0.90 | 0.47 |
| p16 | 24.1 | 31.6 | 5.77 | 0.84 | 0.68 | <0.05 | 0.96 | 0.83 |

**Table 3:** Percentage of cells positive for immunostaining against Sirt1, Ki67 and P16 proteins

However, no differences were found in the expression of *IGF-1*, *IGFBP5*, or the epithelial marker *CK18* along the evolution of the dry period (Table 2). No significant interaction was found between parity and sampling day in any of the analyzed factors.

On the other hand, in the immunostainings, we found differences throughout the dry period in abundance of SIRT1, Ki67 and p16 ($P < 0.05$, $P < 0.01$ and $P < 0.05$ respectively) (Figure 2). The proportion of cells positive for SIRT1 in the mammary epithelium, a factor involved in cellular metabolism and cell survival (Rahmann and Islam, 2011), was higher at mid-dry period compared to the end of lactation. The results from immunostaining for Ki67 and SIRT1 did not concur with those from gene expression.

For both factors, the immunostaining results showed lesser levels during lactation (before drying and after calving) and greater levels during the dry period, whereas results from gene expression followed an opposite pattern.

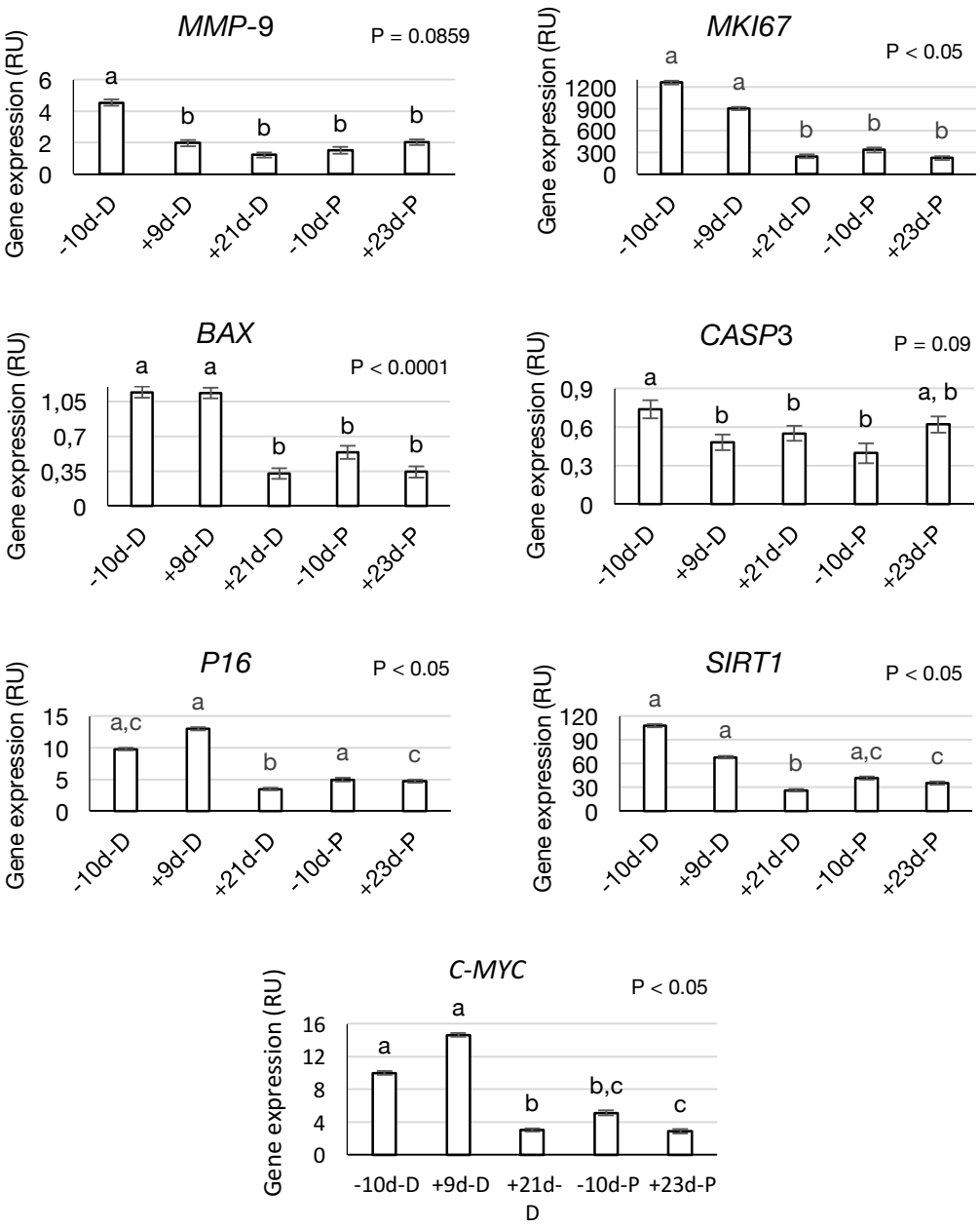


**Figure 1**. Gene expression expressed as relative units (RU) of *MMP-9*, *MKI67*, *BAX*, *CASP3*, *P16*, *SIRT1* and *C-MYC* in mammary gland tissue at -10, 9 and 21 d relative to dry-off, and -10 and 23 d relative to parturition. Different letters indicate differences between samples.

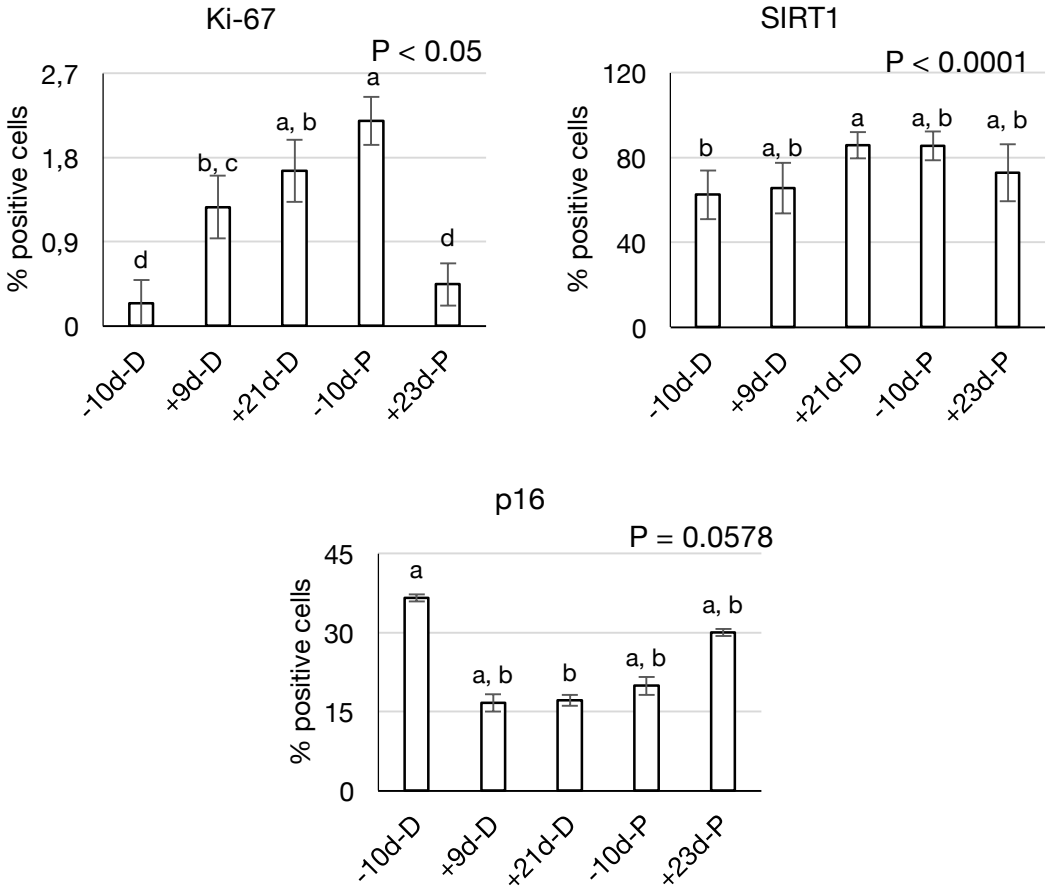


**Figure 2**. Immunostaining results of Ki67, SIRT1 and p16 expressed as percentage of positive cells from total cells at -10, 9 and 21 d relative to dry-off, and -10 and 23 d relative to parturition. Different letters indicate differences between samples.

Results from qPCR reflect mRNA expression whereas immunostaining quantifies the protein in the cells, thus, even at the same time point, the results for specific genes/proteins can differ substantially. The complicated pathways from gene to protein and post-transcriptional and post-translational regulation steps, such as alternative splicing, activity of polyribosomes, and micro-RNAs, could account for the lack of correlation herein. In fact, Sinn et al. (2017) compared the results from gene expression and immunohistochemistry analyses of Ki-67 and found little

correlation between the two techniques. As expected, the results indicated a greater number of proliferating cells (% Ki-67 positive cells) at the end of the dry period (-10 d before parturition, Figure 2), which coincides with the greatest amount of regenerated cells (Sorensen et al. 2006; Norgard et al. 2008). The lowest percentage of senescent cells (p16-positive cells) and also the lowest levels of expression of p16 were found at the middle of the dry period (21 d after dry off). This observation would be linked with the high apoptotic activity in the epithelium of the mammary gland after dry-off, which probably removed a large proportion of senescent cells. Consistent with this finding, the greatest expression levels of apoptotic factors *BAX* and *CASP3* were found at the end of lactation and at the beginning of the dry period (-10 and 9 d relative to dry-off). Puvogel et al. (2005) also described greatest levels of apoptosis in the epithelium of the mammary gland during early stages of the dry period. Several studies have also described high apoptosis rates during the first or second week of lactation (Sorensen et al., 2006; Capuco et al., 1997). Herein, we did not sample tissue until day 23 of lactation, where we did not detect differences in expression of apoptotic genes in the mammary epithelium.

The greatest levels of *MMP-9* expression, involved in ECM remodeling, cell detachment, and apoptosis (Boudreau et al., 1995) were found at the end of lactation. From our knowledge, apoptosis has not been previously described at the end of lactation so this is an interesting fact to be confirmed in future experiments. Nevertheless, MMP-9 is secreted as a

zymogen (Benaud et al., 1998), and then locally activated by other enzymes so we can not discard that although the expression is high 10 d before drying the metalloproteinase activity in the mammary gland is concentrated right after dry off where it has been described high metalloproteinase activity in mammary secretion (Ponchon et al., 2014).

Expression of *C-MYC* in the mammary epithelium was increased during the dry period, which coincides with previous observations (Norgaard et al., 2008). Growth factors initiate post-translational modification of transcription factors for early response genes such as C-FOS, C-JUN, and C-MYC. These genes induce transcription of other factors initiating cell cycle progression (Sherr, 1995). The increase of *C-MYC* expression at the beginning of the dry period (Figure 1) preceded the increase in proliferating cells detected towards the middle and the end of the dry period (Figure 2). However, C-MYC is a pleotropic factor and it has also been described that can be a central mediator of apoptotic signaling in the mammary gland, being a direct target of Stat3 and inducing expression of pro-apoptotic genes (Hynes and Stoelzle, 2009). Hence, we can not discard its involvement in apoptosis also at the beginning of dry period.

**Blood hormones and dry period**

Blood concentrations of GH, PRL, progesterone, and IGF-1 were analyzed through the dry period. Progesterone, PRL, and IGF-1 induce growth of

mammary epithelial cells and consequently, an increase in milk production. Also, PRL, GH and progesterone stimulate IGF-1 activity (Ha et al., 2016). As expected, blood concentrations of all these hormones changed as the dry period progressed (Table 4 and Figure 3), but no significant interactions with parity and sampling day were found. The blood levels of PRL were reduced when milking was stopped at dry-off ($P < 0.005$).

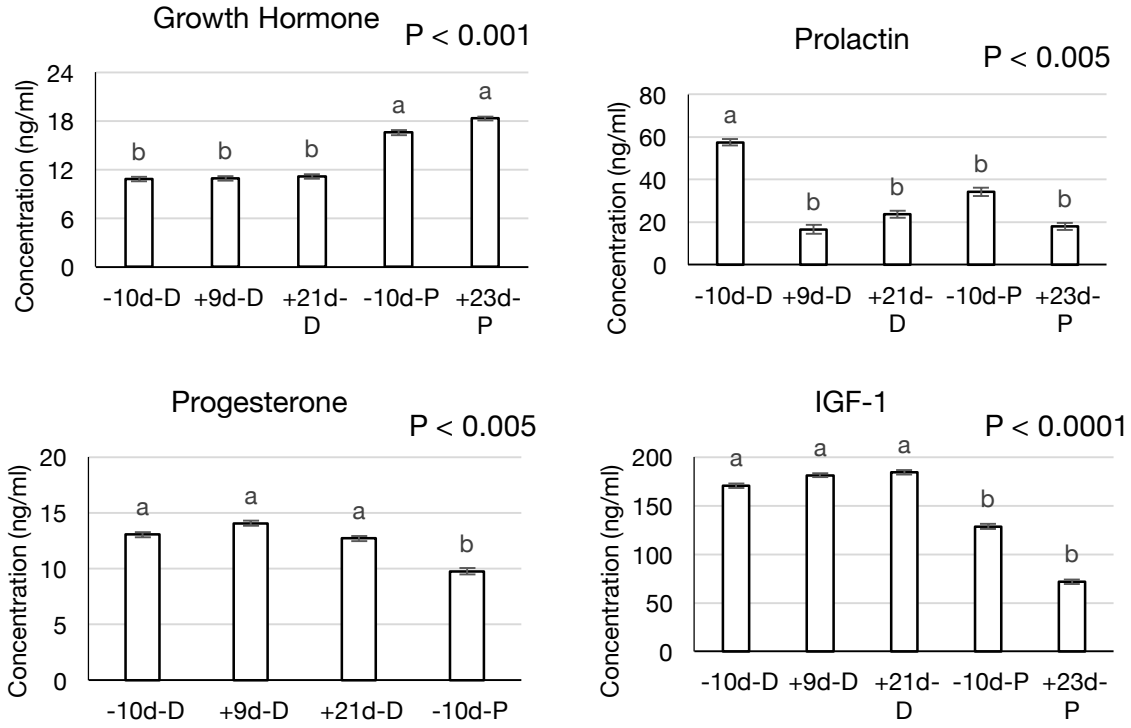


**Figure 3**. Blood concentration (ng/ml) of GH, prolactin, progesterone, and IGF-1 at -10, +9 and +21 d relative to dry-off and at -10 and +23 d relative to calving. Different letters indicate differences between samples.

The concentration of IGF-1 in blood was maintained high until mid-dry period ($P < 0.001$) coinciding with the expression pattern of early response genes such us *C-MYC*, probably promoting cell proliferation.

When the concentration of IGF-1 in blood was high, blood GH concentration was low, and when blood IGF-1 concentration decreased, blood GH increased, probably because of the feedback pathway between both hormones (Romero et al., 2012). Coinciding with previous studies (Sorensen et al., 2006), blood concentrations of IGF-1 were lower in early lactation and greater during the dry period ($P < 0.001$), whereas blood GH concentration was greater ($P < 0.001$) during the late-dry period and early-lactation than during late-lactation and in the early-dry period.

| | Treatment | | | P-value | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Cabergoline | SEM | Treatment (T) | Parity (P) | Time (t) | TxP | Txt |
| GH | 13.46 | 13.65 | 1.23 | 0.57 | 0.99 | <0.0001 | 0.26 | 0.75 |
| Prolactin | 36.36 | 23.55 | 0.82 | 0.07 | 0.07 | <0.001 | 0.25 | 0.32 |
| IGF-1 | 151.8 | 142.71 | 12.53 | 0.61 | 0.71 | <0.0001 | 0.05 | 0.22 |
| Progesterone | 11.60 | 13.184 | 1.29 | 0.35 | 0.05 | <0.05 | 0.37 | 0.72 |

**Table 4:** Blood concentration of selected hormones (ng/ml) as affected by treatment, parity, sampling time and their interactions.

Blood progesterone concentrations are commonly maintained high during pregnancy and decrease before calving (Bernier-Dodier et al., 2011). Hence, we only detected a small reduction in the blood levels of progesterone in the samples collected 10 d before calving ($P < 0.005$).

## Cabergoline effects on mammary epithelial involution and proliferating factors

Applying cabergoline at dry-off tended ($P = 0.07$) to reduce blood PRL concentrations (Table 4). Previous studies (Bach et al., 2015; Boutinaud

et al., 2016) have shown a decrease in blood PRL after administration of cabergoline. This decrease has also been obtained with the use of quinagolide (Ollier et al., 2013), though it required several daily treatments instead of one single injection as in the case of cabergoline (Boutinaud et al., 2016).

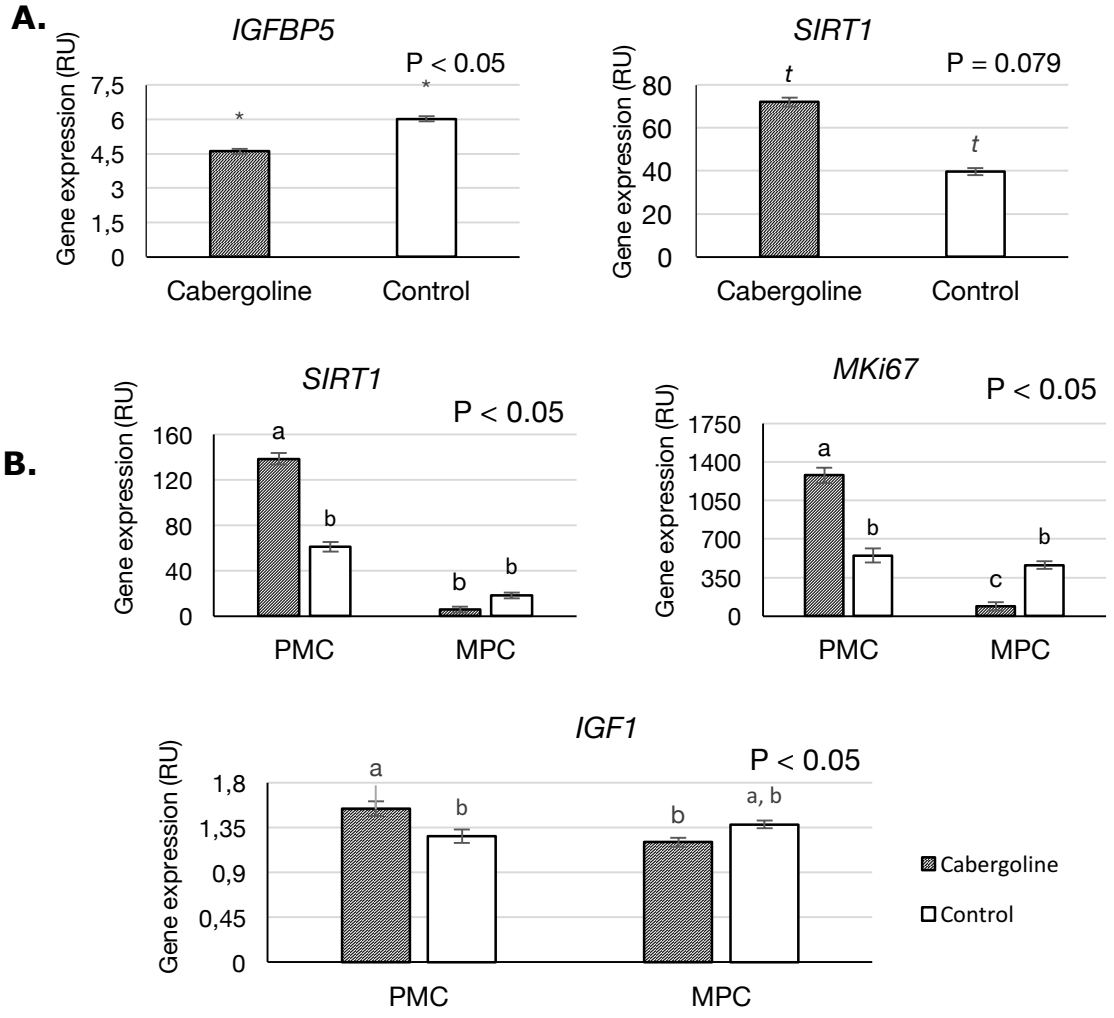


**Figure 4. A**. Gene expression expressed as relative units (RU) of *IGFBP5* and SIRT1 in the mammary gland of cows treated with cabergoline (textured bar) or control (open bar). Asterisks indicate a difference between treatments. Letter t indicates a tendency. **B**. Gene expression expressed as relative units (RU) of SIRT1, MKI67, and IGF-1 in primiparous and multiparous cows treated with cabergoline (textured bar) or control (open bar). Different letters indicate differences between treatments.

However, to our knowledge, the potential effects of inhibiting PRL at dry-off on cellular factors and hormones controlling involution and regeneration of the mammary gland have not been investigated.

In this study we observed an interaction between PRL inhibition and parity (P = 0.05) resulting in an increase in plasma IGF-1 concentrations in PMC treated with cabergoline compared with MPC both treated or untreated with cabergoline (Figure 5B).

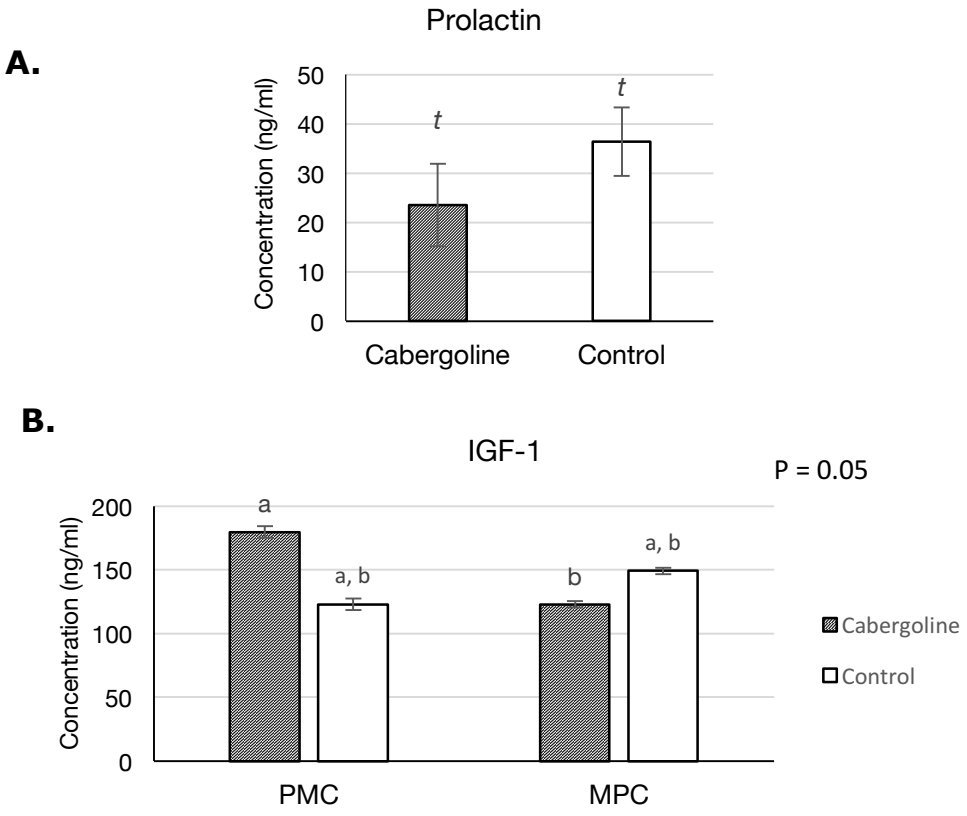


**Figure 5. A.** Blood concentrations (ng/ml) of prolactin from animals treated with cabergoline (textured bar) or control (white bar). Letter t indicates a tendency. **B.** Blood concentrations (ng/ml) of IGF-1 in primiparous and multiparous cows treated with cabergoline (textured bar) or control (open bar). Different letters indicate differences between treatments.

This interaction was confirmed with gene expression analyses of *IGF-1* at the mammary level (Figure 4B). There is strong evidence supporting the role of IGF-1 in the stimulation of cell proliferation in the mammary gland (Weber et al., 1999; Knight, 2000); thus, it seems that cabergoline and the subsequent PRL inhibition increase the proliferating capacity of epithelial cells of the mammary gland in PMC, but not in MPC. In agreement, the expression of *MKi67* in the mammary epithelial cells of PMC was also increased, but no changes in the counts of proliferating cells (Ki67 positive cells) were observed. Cabergoline treatment also reduced the expression of *IGFBP5*, a gene codifying for the IGF binding protein 5 (IGFBP5) that binds to IGF to inhibit its pro-survival activity in the mammary epithelium. The reduction of *IGFBP5* correlated with the increased systemic and tissular IGF-1 levels, but no interaction between treatment and parity or treatment by sampling day was found. In fact, IGFBP-5 is considered a proapoptotic factor in the mammary gland, where it has both IGF-1–dependent and IGF-1–independent effects (Allan et al., 2004). Overall it is difficult to interpret the consequences of inhibiting IGFBP5 because it has a dual role. On one hand, it reduces the availability of the survival factor IGF-1, and on the other it increases the degradation of ECM, thereby coordinating apoptosis and tissue remodeling (Tonner et al., 2000; Flint et al., 2005). Furthermore, PRL inhibits the expression of *IGFBP5* (Wilde et al., 1999). Hence, it was unexpected to observe a reduction of *IGFBP5* in animals treated with cabergoline compared to control animals. These results would indicate that PRL might not be the

only pathway modulating the expression of *IGFBP5* and the complexity of mammary involution processes.

Lastly, there was an interaction ($P < 0.05$) between treatment and parity for *SIRT1* expression. Recently, it has been demonstrated that SIRT1 modulates IGF-1 signaling in mice, critical for both growth regulation and mammary gland development (Li et al., 2007). A deficiency of SIRT1 deregulates the expression of IGF-1 binding protein-1 and attenuates the effect of IGF-1 signals, including estrogen-stimulated local IGF-1 signaling for the onset of ductal morphogenesis (Li et al., 2007). Therefore, it is possible that this mechanism is also present in bovine species and, in fact, it would in fact be in agreement with the greater blood IGF-1 concentration found in PMC cows treated with cabergoline at dry-off. No differences in the 3-way interaction of treatment x parity x sampling day were found in any of analyzed factors.

In conclusion, the inhibition of PRL secretion by cabergoline does not alter most cellular factors and hormones involved in the involution and regeneration of the mammary gland along the dry period. However, cabergoline exerted specific effect on PMC increasing IGF-1 and consequent increase in the expression of *MKi67*. This effect was probably mediated by SIRT1, which was also increased in PMC cows treated with cabergoline and has been previously related in other species with the stimulation of IGF-1 signaling in the mammary gland.

# CHAPTER VI – Study 4:

# Involution, regeneration, and senescence in the mammary gland of dairy cows at drying

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## Introduction

Dairy cows need a dry period between lactations to maintain adequate production levels in the following lactation, especially in PMC (Collier et al., 2012). However, the dry period is associated with an increased risk of mastitis, especially in cows with high milk yields (Rajala-Schultz et al., 2005), due to the copious amounts of milk remaining in the mammary gland at drying, which exert high intra-mammary pressure and may cause discomfort and milk leakage (Gott et al., 2016). An interesting strategy to diminish mastitis risk at dry-off consists of accelerating mammary involution. Several authors have proposed different strategies to achieve that, such as the administration of PRL inhibitors such as quinagolide or cabergoline (Ollier et al., 2014; Bach et al., 2015; Parés et al., unpublished data) or M-SAA3 (Domènech et al., 2014) among others. However, although the use of such approaches shows promising results, currently, the vast majority of cellular events and processes during the dry period are still not well described, limiting the achievement of a precise and appropriate modulation at a molecular level of the involution and regeneration of the mammary gland.

The dry period can be divided in two main phases, an initial phase of involution, followed by a second phase of tissue regeneration (De Vries et al., 2010; Domènech et al., 2014). It has also been described that involution of the mammary gland is mainly due to apoptosis (Wilde et al., 1997). Although it was initially postulated that all alveolar cells undergo

apoptosis after dry-off, it has been proven that in mammalian species a significant number of differentiated secretory cells remain in the gland after involution (Vonderhaar et al., 1978; Wagner et al., 2002). These permanently-differentiated cells are carried over into the next lactation, where they function as precursors, giving rise to clonal populations of alveolar cells during subsequent pregnancies (Wagner and Smith, 2005). Studies in laboratory species indicate that this differentiated alveolar cells are more responsive to hormonal stimuli than the undifferentiated cells found in the mammary gland (Wagner and Smith, 2005).

Milk yield is generally greater in MPC than in PMC, but the lactation curve for PMC and MPC differs also in lactation persistency, with PMC cows being capable of maintaining lactation for longer periods with minimal yield losses (Miller et al., 2006). It has been postulated that greater circulating levels of mitogenic or survival factors such as IGF-1 could result in improved lactation persistency. However, to our knowledge, no study has evaluated the potential differences at a molecular level during the dry period of PMC and MPC.

On the other hand, little is known on how apoptosis, regeneration, and senescence of the mammary gland progress throughout the dry period and which are the factors orchestrating this process. The first stages of mammary gland involution are considered reversible, and are triggered by local stimuli that initiate apoptosis, but the process can be reversed by

reinitiating milk removal. The second stage is irreversible and it is characterized by activation of proteases that destroy the lobular-alveolar structure of the gland by the ECM and basement membrane, as well as massive loss of alveolar cells (Capuco and Akers, 1999). Not much is known about cell recruiting and programmed cell death during this process, but a plausible hypothesis is that the mammary gland needs to suffer a minimum degree of pressure to induce sufficient 'damage' to the ECM to later foster renovation of epithelial cells in preparation for the next lactation. It has been shown that milk stasis and mechanical stress trigger cellular events associated with involution (Quaglino et al., 2009). Thus, the objective of this study was to assess which are the main changes in apoptosis, cell development, and senescence processes from the end of lactation and along the dry period, and to study the potential differences between PMC and MPC dried when producing either low or high levels of milk.

## Materials and Methods

### Animals and Experimental design

Twenty-four lactating Holstein cows (210-220 d pregnant, producing, and with milk somatic cell counts < 200,000 cells/ml at dry-off), 12 PMC and 12 MPC, were split in groups (6 cows in each) based on their average milk production during the last 5 d preceding dry-off, with cows producing ≥ 28 kg/d (average = 33.0 kg/d; SD = 4.8 kg/d) classified as high-producing (HP) and those producing ≤ 25 kg/d (average = 17.9 kg/d; SD = 5.3 kg/d) classified as low-producing (LP). Specifically, the average milk production 5 d before dry-off for PMC and MPC classified as HP or LP was 31.7±3.1, 23.1±3.7, 34.4±6.0, and 12.7±9.8 kg/d, respectively. All cows were dried abruptly with no dietary intervention before dry-off and no changes in milking routine or frequency. Broad-spectrum antibiotics (Mamyzin secado®, Boehringer Ingelheim, Barcelona, Spain) were administered at dry-off following common production practices.

A mammary biopsy of the rear quarter of all cows was performed at -10, 9, and 21 d relative to dry-off, and -10 and 23 d relative to calving (a total of 5 biopsies per animal).

This experiment was conducted under the evaluation and permission of the Animal Care and Ethical Committee of IRTA (protocol number 8788).

## Mammary Biopsies

To perform the mammary biopsies, cows were partially immobilized in a head bail. The rear part of the mammary gland was hair clipped and washed with a commercial povidone/iodine solution. All procedures beyond this point were carried in maximum sterility conditions possible. The biopsy area selected was at a midpoint on a rear quarter. The incision area was locally anesthetized with a subcutaneous injection of 1.5 ml (30 mg) lidocaine solution (Procamidor, Richter Pharma, Austria). An incision of 1.5-2.5 cm was made with a scalpel through the skin and gland capsule, avoiding the incision of large blood vessels. Then a 6-mm trocar was introduced about 10 cm deep into the mammary gland through the incision, then removed, and biopsy forceps introduced through the trocar canal into the mammary gland to collect two tissue biopsies of about 3 $mm^2$. Pressure was applied to the biopsied area after the procedure for about 3-5 min. Last, skin was sutured with a non-absorbable multifilament suture (KRUUSE silk, Langeskov, Denmark). Samples were washed in PBS, and then conserved in 1 ml of RNA later reagent (Ambion, USA) for subsequent determination of gene expression, or placed in 10 ml of 10% neutral buffered formalin solution (NBF) for subsequent immunohistochemistry analyses. Consecutive biopsies were performed in alternated rear quarters.

## Quantification of Gene Expression

RNA was extracted using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain) and retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed with a Nanodrop 1000 instrument (Thermo Scientific, Barcelona, Spain) at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Reaction of qPCR was performed in a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad, Barcelona, Spain) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan) and the conditions and primers specified in Table 1. The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8 % DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which was calculated using the formula 10^(1/slope), with an acceptable range of 1.8-2.2. A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBR green fluorescent (Bio-Rad, Barcelona, Spain), and the optimized primer concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95 °C, followed by 40 cycles of 10 s at 95°C, 15 s at optimized annealing temperature for each gene, 30 s at 72°C and a final extension of 10 min at 72°C.

| Gene | Primer Sequences | Amplicon Size (bp) | Annealing temperature (Celsius) | Primer concentration (μM) |
|---|---|---|---|---|
| *RPS9* (HK) | Fw: 5'-CCTCGACCAAGAGCTGAAG-3'<br>Rv: 5'-CCTCCAGACCTCACGTTTGTTC-3' | 63 | 57 | 0.125 |
| *RPS15* (HK) | Fw: 5'-GCAGCTTATGAGCAAGGTCGT-3'<br>Rv: 5'-GCTCATCAGCAGATAGCGCTT-3' | 150 | 57 | 0.125 |
| *MMP-9* | Fw: 5'-TCGACGTGAAGACACAGAGGT-3'<br>Rv: 5'-TGATCCTGGCAGAAGTAAGCTTTC-3' | 126 | 57 | 0.125 |
| *BAX* | Fw: 5'-AGTGGCGGCTGAAATGTT-3'<br>Rv: 5'-TTCTTCCAGATGGTGAGCG-3' | 287 | 60 | 0.5 |
| *CASP3* | Fw: 5'-AAGCCATGGTGAAGAAGGAA-3'<br>Rv: 5'-GGCAGGCCTGAATAATGAAA-3' | 134 | 55 | 0.5 |
| *MKI67* | Fw: 5'-AACACCCAGTCGTGTTCGTT-3'<br>Rv: 5'-GAGCCTTCGGTTCTTCACGA-3' | 130 | 62 | 0.5 |
| *C-MYC* | Fw: 5'-GTGTCTACCCATCAGCACAA-3'<br>Rv: 5'-AACTGTTCTCGCCTCTTCTG-3' | 376 | 58 | 0.25 |
| *P16* | Fw: 5'-GGTGATGATGATGGGCAGCG-3'<br>Rv: 5'-ACCAGCGTGTCCTGGAAGC-3' | 134 | 62 | 0.5 |
| *SIRT1* | Fw: 5'-GGAGCAGATTAGTAAACGCCT-3'<br>Rv: 5'-CTTTCATCCTCCATGGGTTC-3' | 188 | 58 | 0.25 |
| *CK18* | Fw: 5'-TCGATGACACCAATGTCACCC-3'<br>Rv: 5'-ACCAGTACTTGTCCAGCTCC-3' | 249 | 60 | 0.25 |
| *uPA* | Fw: 5'-GTCTGGTGAATCGAACTGTGGC-3'<br>Rv: 5'-GGCTGCAAACCAAGGCTG-3' | 510 | 57 | 0.125 |
| *IGFBP5* | Fw: 5'-GTTTGCCTGAACGAAAAGAGCTA-3'<br>Rv: 5'-CGAGTAGGTCTCCTCTGCCATCT-3' | 104 | 57 | 0.5 |
| *IFG-1* | Fw: 5'-TCGCATCTCTTCTATCTGGCCCTGT-3'<br>Rv: 5'-GCAGTACATCTCCAGCCTCCTCAGA-3' | 239 | 57 | 0.125 |
| *LALBA* | Fw: 5'- CTCTGCTCCTGGTAGGCATC-3'<br>Rv: 5'- ACAGACCCATTCAGGCAAAC-3' | 124 | 56 | 0.125 |
| *CSN2* | Fw: 5'- GCTATGGCTCCTAAGCACAAAGA-3'<br>Rv:5'- GGAAACATGACAGTTGGAGGAAG-3' | 172 | 56 | 0.125 |
| *TGFβ* | Fw: 5'- TGAGCCAGAGGCGGACTACT-3'<br>Rv:5'- TGCCGTATTCCACCATTAGCA-3' | 61 | 60 | 0.5 |
| *LF* | Fw: 5'- TGAAAGGGGAAGCAGATG-3'<br>Rv: 5'- AAGTCCTCACGATTCAAGTT-3' | 552 | 50 | 0.5 |
| *LIF* | Fw:5'- CAGGAGTTGTGCCCTTGCTGCTGCTTCT-3'<br>Rv:5'- CAGGGAGGCGCCCAGGTACGCGATGATG-3' | 331 | 66 | 0.125 |
| *BCL2* | Fw: 5'- GCCTTCTTTGAGTTCGGA-3'<br>Rv:5'- TTCAGAGACAGCCAGGAGA-3' | 209 | 55 | 0.25 |
| *OCLN* | Fw: 5'- ATCAACCCCGGTGCCGGAAG-3'<br>Rv:5'- GTGGTCTTGCTCTGCCCGCC-3' | 162 | 57 | 0.5 |
| *PRLR* | Fw: 5'- ATAGCATGGTGACCTGCATCC-3'<br>Rv:5'- TCTTCGGACTTGCCCTTCTC-3' | 90 | 57 | 0.125 |

**Table 1.** Primer sequences and amplification conditions for gene expression analysis

The resulting Ct values were used to calculate the relative expression of selected genes by relative quantification using the genes *RPS9* and *RPS15* for housekeeping.

## Immunohistochemistry

Tissue samples were fixed in 10% NBF for 48 h at 4ºC with gentle agitation. After incubation, samples were dehydrated in an ascending alcohol gradient and embedded in paraffin to acquire tissue sections. Immunohistochemistry stainings for SIRT1, p16, and Ki67 were performed separately in 3-µm sections with SIRT1 H-300 (Santa Cruz, Dallas, TX, USA), p16 M-156 (Santa Cruz, Dallas, TX, USA) and the Purified Mouse Anti-Ki-67 (Bd biosciences, Fanklin Lakes, NJ, USA) antibodies, respectively. As secondary antibodies, biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) and M.O.M. Kit (Vector laboratories, Burlingame, CA, USA) were used. Sections were counterstained with hematoxylin to count stained and untainted nuclei from one representative section at 40x.

## Statistical Analysis

Data were analyzed using a mixed-effects model that included the random effect of cow and the fixed effects of parity (PMC or MPC), level of production at dry-off (HP or LP) and days relative to dry-off, plus their 2-

and 3-way interactions. Sampling day entered the model as a repeated measure using a compound symmetry variance-covariance matrix

## Results

### Tissular Involution and Regeneration Factors

There was a 3-way interaction among parity, level of milk production at dry-off, and sampling time affecting the expression of *BAX* ($P < 0.05$), *CK18* ($P < 0.01$), *MKi67* ($P < 0.01$), cytokine leukemia inhibitory factor (*LIF*) ($P < 0.001$) and *IGF-1* ($P < 0.05$), and a tendency to be significant for B-cell lymphoma-2 (*BCL2*) ($P = 0.07$) and *C-MYC* ($P = 0.07$). In all cases the differences were detected at the end of lactation, 10 days before dry-off, where a different performance between PMC and MPC was observed depending on the level of milk yield at dry-off (Figure 1).

Particularly, MPC classified as LP had greater levels of expression of *BAX*, *CK18*, *LIF, Mki67, C-MYC, and BCL2* in the mammary gland at the end of lactation, compared to PMC classified as LP. Moreover, expression of *BAX*, *CK18*, *MKi67*, *LIF*, and *BCL2* also differed between PMC classified as LP and PMC classified as HP, being the latter the ones showing greater expression levels. In the case of *IGF-1* those differences were inverted, being PMC classified as LP the ones showing greater expression levels compared to PMC classified as HP. In addition, *LIF*, *IGF-1*, and *BCL2* presented differences in the expression levels between MPC classified as HP and PMC classified as LP, being greater in PMC classified as LP for *IGF-1* and *BCL2*, and lower for *LIF.* In the case of *LIF*, differences were also observed between MPC classified as HP and MPC classified as LP, with the

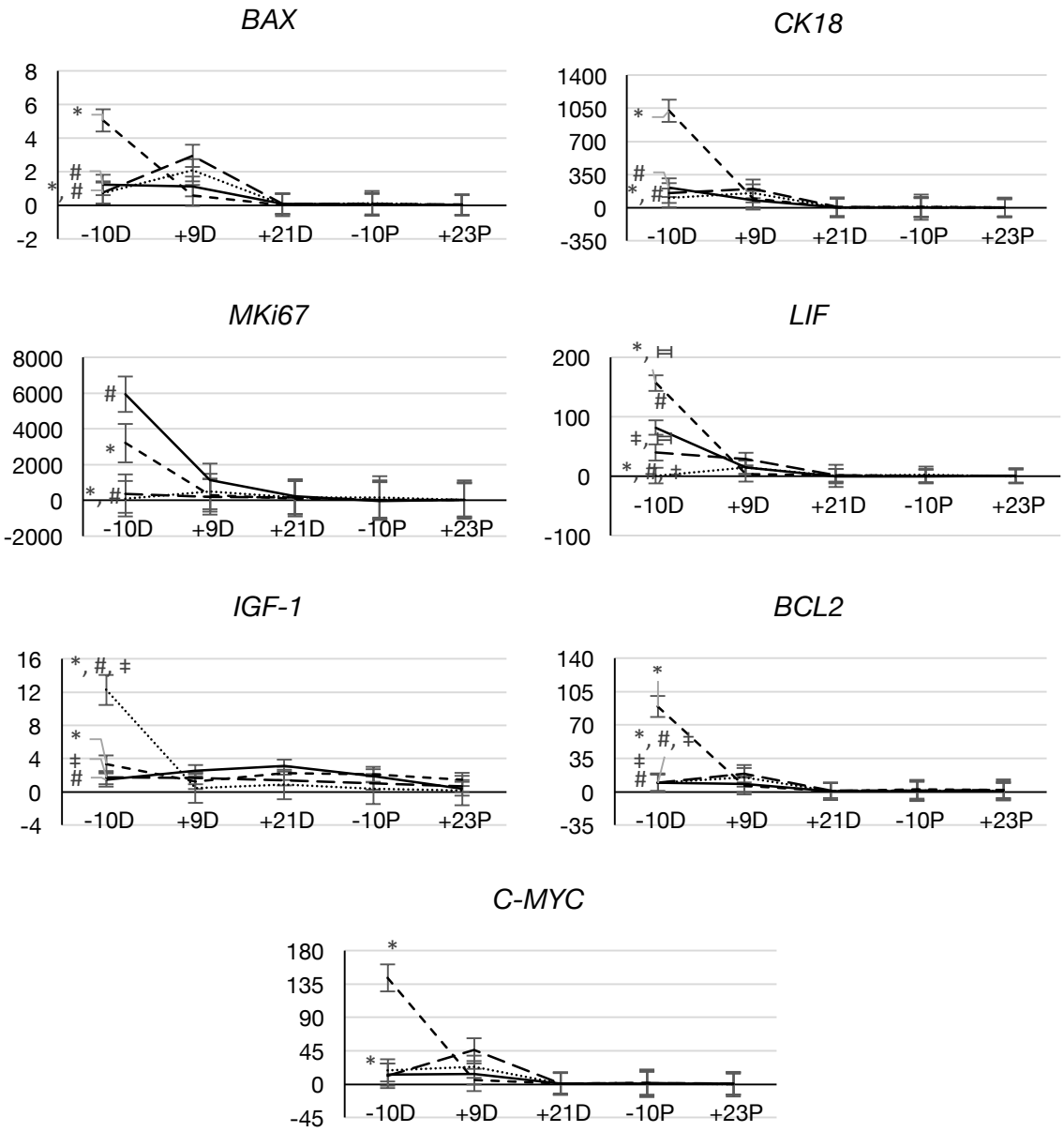



**Figure 1.** Three-way interaction between sampling, parity and production level for the expression of the tissular involution and regeneration markers *BAX*, *CK18, MKi67, LIF, IGF-1, BCL2*, and *C-MYC* in the mammary gland throughout late lactation, dry period, and early subsequent lactation represented as relative units (RU). The symbols in the graph denote differences between: *: primiparous (PMC) low-productive (LP) animals and multiparous (MPC) LP animals; #: PMC LP cows and PMC high-productive (HP) cows. ⋿: MPC LP and MPC HP, ǂ: PMC LP and MPC HP. Long-dashed lines correspond to MPC HP, short-dashed lines correspond to MPC LP, dotted lines correspond to PMC LP, and solid lines correspond to PMC HP.

first ones showing greater expression levels. No differences were found in the expression of the apoptotic factor *CASPASE-3*. At dry-off, the expression levels of all these genes was no longer different, and the expression for all these genes remained low during mid and late dry period and the onset of the following lactation.

Besides, the expression of genes encoding for *SIRT1*, *IGFBP5*, occludin (***OCLN***), *TGF-β1,* lactoferrin (***LF***), *P16*, and *MMP-9* was affected ($P < 0.05$) by sampling time independently of parity and milk production level at dry-off (Figure 2A). Expression of *SIRT1, OCLN, TGF-ß1, LF, P16* and *MMP-9* was greater at the end of lactation and right after dry-off compared with the samplings performed during the dry period. In the case of *IGFBP5* the expression pattern was the opposite. Moreover, there was a tendency towards a greater expression of *OCLN* in PMC compared with MPC ($P = 0.07$), and in LP compared with HP ($P = 0.07$). The expression of Uroplasminogen activator (***uPA***) in the mammary gland was greater ($P < 0.05$) in MPC than in PMC and tended ($P = 0.06$) to be greater in MPC at the beginning of the subsequent lactation (Figure 2B).

**2. A.**

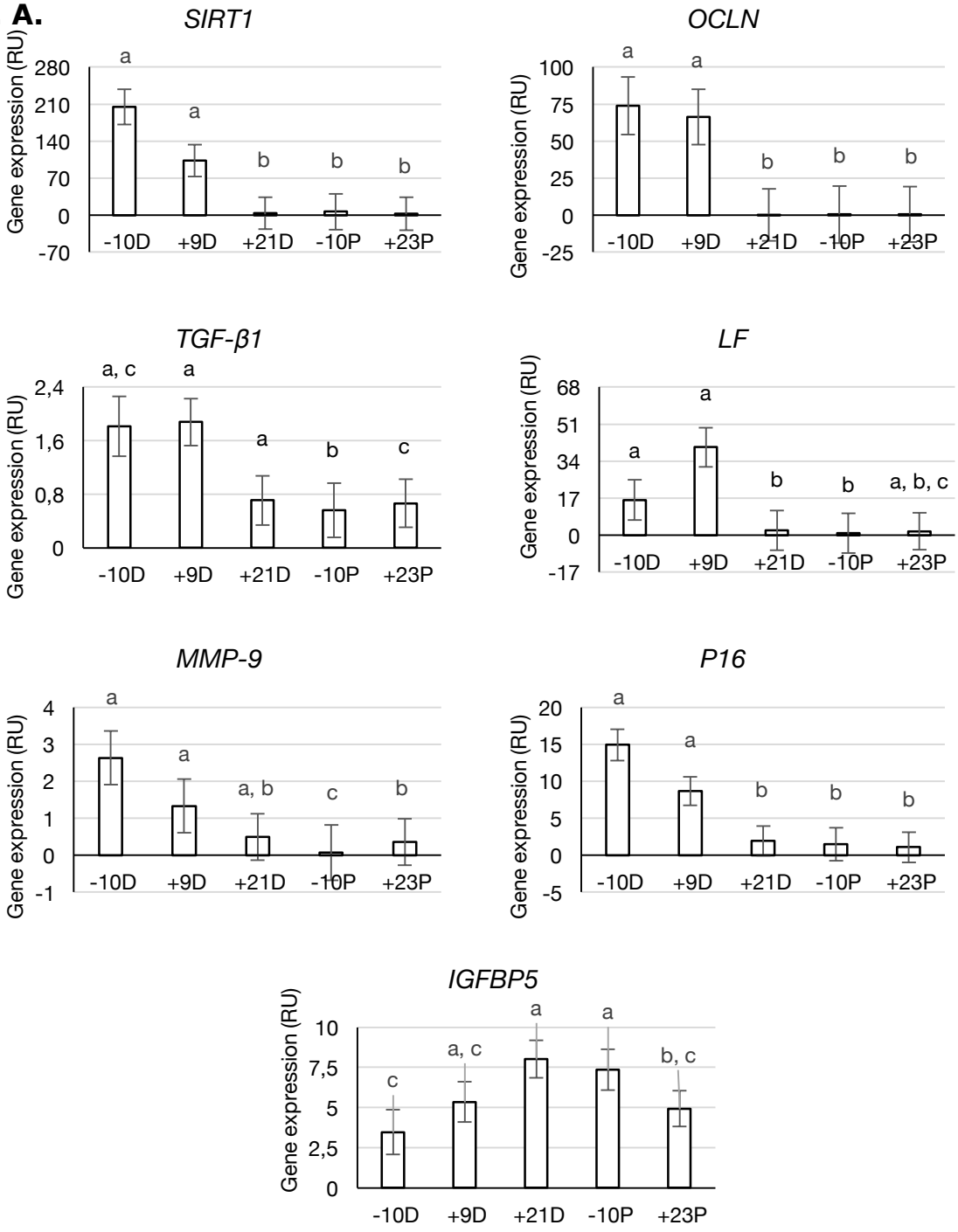



**Figure 2. A.** Expression throughout late lactation, dry period, and early subsequent lactation of the markers *SIRT1, OCLK, TGF-β1, LF, MMP-9, P16*, *and IGFBP5* in the mammary gland represented as relative units (RU).

**2. B.**

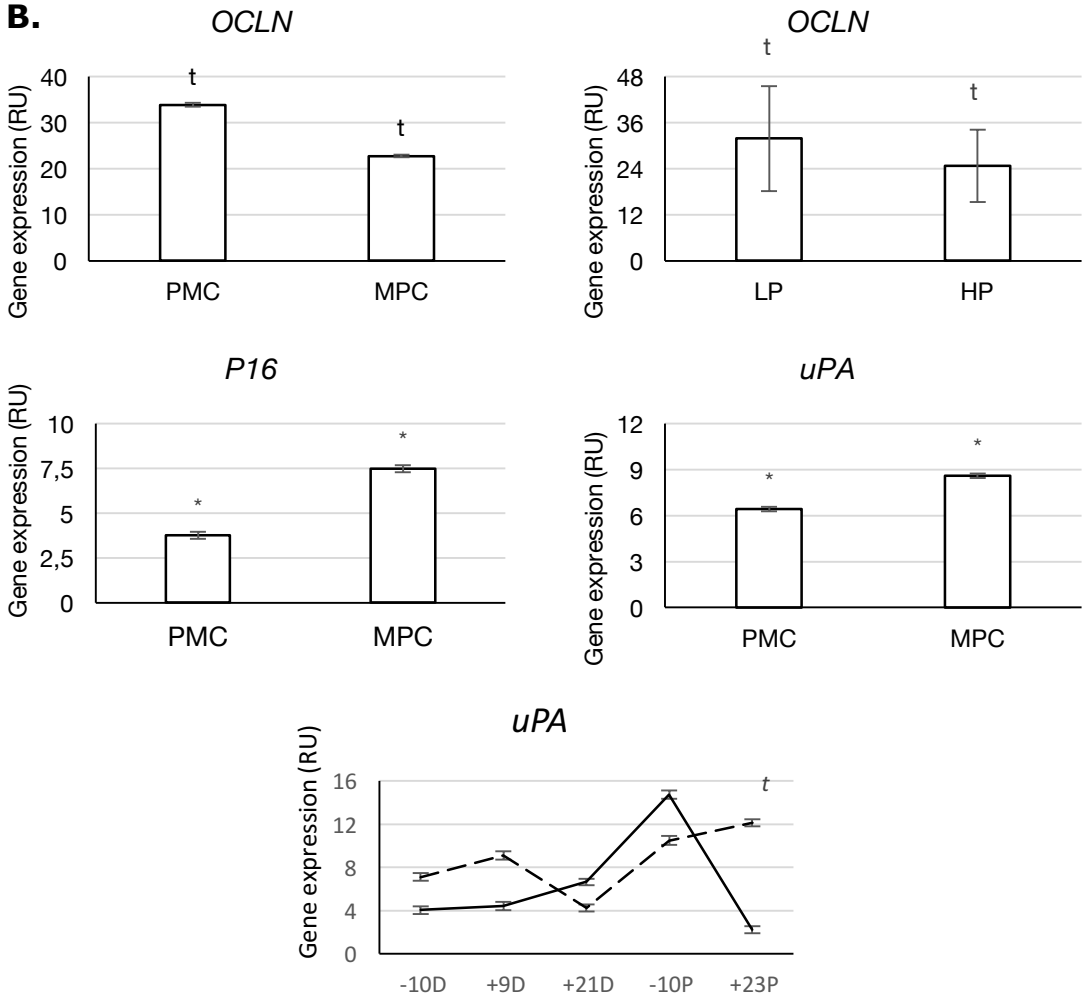



**Figure 2. B.** Expression of *OCLN* and *uPA* in the mammary gland of primiparous (PMC) and multiparous (MPC) cows and/or low productive (LP) and high productive (HP) animals expressed as relative units (RU); and interaction between parity and sampling for the expression of *uPA* expressed as RU. Solid line corresponds to PMC, dashed line corresponds to MPC.

The levels of p16 in the mammary gland did not change as assessed by immunohistochemical analyses, but the expression of *P16* was greater ($P < 0.005$) at -10 and 9 d relative to dry-off (Figure 2A). Moreover, *P16* expression was increased ($P < 0.01$) in MPC compared to PMC.

An increased ($P < 0.01$) proportion of Ki67-positive cells was detected by immunohistochemistry at mid- and late-dry period and the number of

cells positive for SIRT1 did not change along the dry period, although the levels tended (P = 0.07) to be greater during the dry period than at the onset of the subsequent lactation (Figure 2C).

**2. C.**

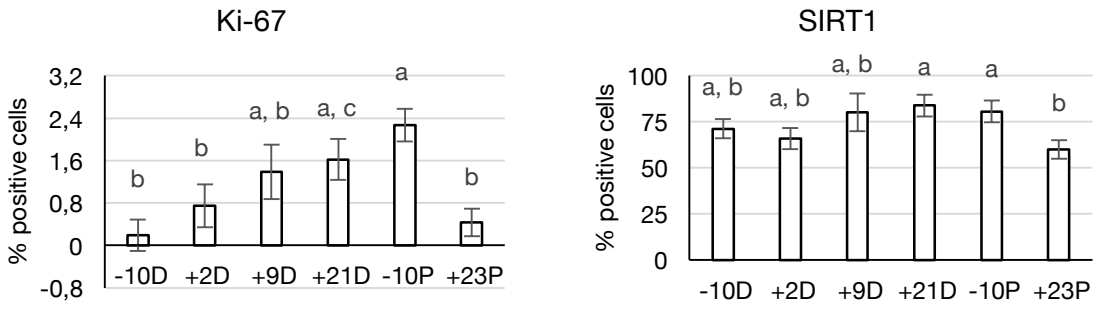
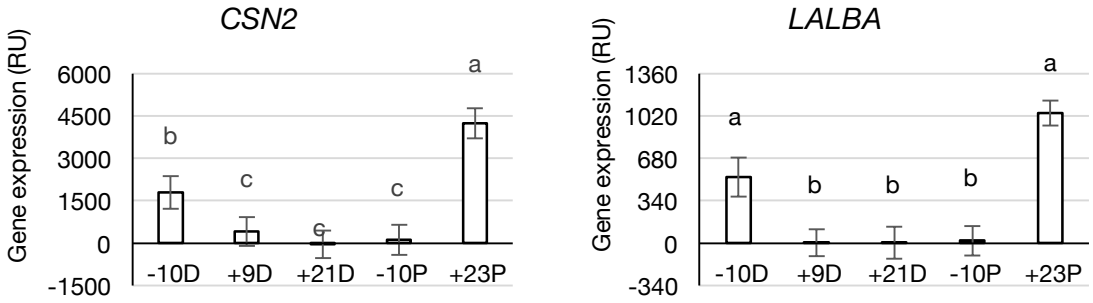



**Figure 2. C.** Ratio of immunopositive cells throughout late lactation, dry period, and early subsequent lactation of Ki67 and SIRT1.

**<u>Mammary Gland Secretion related factors</u>**

The expression of β-casein (*CSN2*) and α-lactalbumin (*LALBA*) in the mammary gland was greater at the beginning of lactation, followed by the expression levels prior to dry-off, with the lowest (P < 0.001) values observed during the dry period (Figure 3A). An interaction between sampling time and parity was found (P < 0.005) for the expression of *LALBA*, which was also elevated at the end of lactation and after calving in all cows, but it was greater in MPC than in PMC independently of milk production level at dry-off (Figure 3B).

**A.**




**B.**

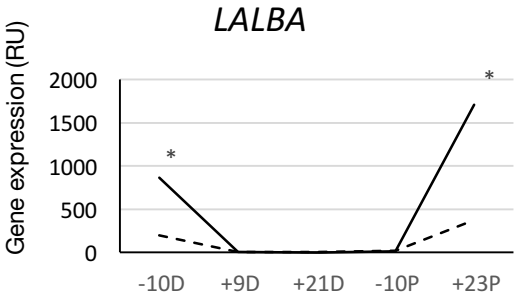
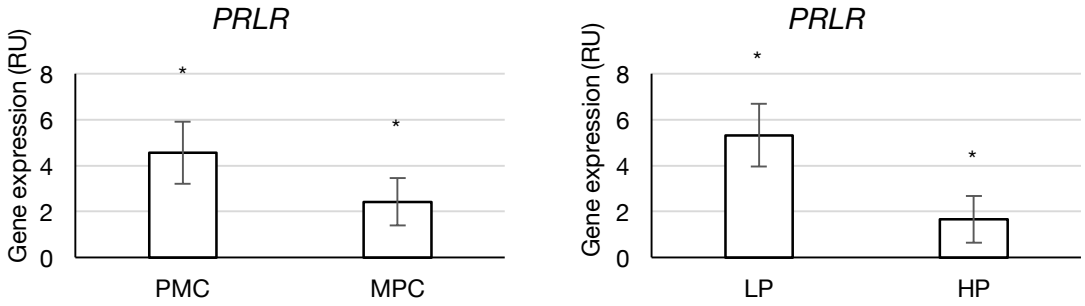



**Figure 3. A.** Expression throughout late lactation, dry period, and early subsequent lactation of the markers *CSN2* and *LALBA* expressed as relative units (RU) **B.** Interaction between parity and sampling for the expression of *LALBA* expressed as RU. Solid line corresponds to PMC, dashed line corresponds to MPC.

Lastly, the expression of prolactin receptor (*PRLR*) in the mammary gland was 2-fold greater in PMC compared with MPC ($P < 0.01$), and 3-fold greater ($P < 0.01$) in cows classified as LP compared with those classified as HP (Figure 4A); and tended ($P = 0.06$) to be superior at the end of lactation and at dry-off compared with the other sampling times (Figure 4B).

**A.**




**B.**

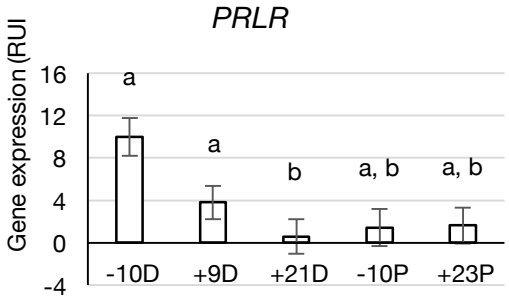



**Figure 4. A.** Expression of *PRLR* in the mammary gland of primiparous (PMC) and multiparous (MPC) cows and low productive (LP) and high productive (HP) animals expressed as relative units (RU). **B.** Expression throughout late lactation, dry period, and early subsequent lactation of *PRLR* expressed as RU.

## Discussion

Apoptosis is responsible for the loss of cells during mammary involution in ruminants (Quarrie et al. 1996; Wilde et al. 1997). The accumulation of BAX in organelle membranes precedes the activation of CASPASE-3, an apoptosis executor (Zhang et al., 1998). In the current study, there were differences in expression of *BAX* and, although we expected to find differences in expression of *CASP3* as reported elsewhere (Dallard et al., 2011), we could not observe any change in *CASP3* expression. A possible explanation could be, as it has been described previously, that apoptosis is initiated before the cells detach from the tissue but CASPASE-3 is not involved in the process until the cell has been detached from the epithelial layer (Watson, 2006). Differences were also found in the expression of *LIF*, *CK18*, *C-MYC*, and *OCLN*. Pleiotropic factor LIF is the physiological activator of STAT3 and is responsible for the signaling of apoptosis in the mammary gland (Kritikou et al., 2003). Previous studies demonstrated that implantation of LIF-containing pellets in lactating mammary glands resulted in a marked increase in epithelial apoptosis (Schere-Levi et al., 2003). Also, the increased expression of *CK18* could be related to reorganization of the cytoskeleton leading to an apoptotic process in epithelial cells (Linder, 2007). The role of C-MYC is to mediate apoptotic signaling in the mammary gland, being a direct target of STAT3 and inducing expression of pro-apoptotic genes (Hynes and Stoelzle, 2009). Occludins are transmembrane proteins present in the tight junctions and they play a role as signaling molecules inducing apoptosis when normal

tight junction protein interactions are disrupted (Beeman et al., 2012). Increased expression of all these apoptotic-related factors was observed, herein, at the end of lactation and at the beginning of dry period. Although some studies have also reported apoptosis at dry-off (Wilde et al., 1999; Boudreau et al., 1995), not many studies have found clear evidences of apoptosis at the end of lactation in dairy cows. Wareski et al. (2001) reported high apoptosis rates at the end of lactation and at the beginning of the dry period in goats. After peak of lactation, the mammary gland undergoes gradual regression through the process of apoptotic cell death. Although cell death exceeds cell proliferation, considerable turnover of mammary cells occurs during lactation (Capuco et al., 2001). In our study, highest expression levels of most apoptosis factors were found in MPC classified as LP, followed by PMC classified as HP and by PMC classified as LP. These data indicated that production levels were correlated to the apoptotic activity in the mammary gland of PMC at the end of lactation, being greater in those classified as HP than in those classified as LP. However, since production levels were not greater in MPC classified as LP than in PMC (regardless of their production level), it is possible that other factors such us senescence of the mammary gland of MPC exert greater effects on cell turnover at the end of lactation than production level itself. In this context, MPC presented more senescence rates than PMC, which would be in agreement with the fact that some secretory cells bypass apoptosis and are transferred to the subsequent lactation, increasing the senescence status of MPC's mammary gland at

the end of lactation, and perhaps being responsible for differences in the lactation curve between PMC and MPC. Not many differences were detected between MPC classified as HP and the rest of the cows, possibly indicating greater variability in MPC HP. The only differences were found in the expression of *LIF, IGF-1,* and a tendency in the expression of *BCL2*. Whereas the expression levels of *LIF* and *BCL2* in MPC classified as LP were 10 times greater than in PMC classified as LP, in the case of MPC classified as HP the levels were only slightly greater than in PMC classified as LP. It is possible that combination of senescence and high production levels result in cell signals that lead to less apoptosis and cell turnover at the end of lactation. It is also important to note that all differences in apoptotic and proliferation factors affected by parity and milk production level at the end of lactation disappeared after dry-off. This is an interesting result because it abolishes the hypothesis that greater mechanical stress produced by milk stasis in high producing animals triggers greater involution rates in the mammary gland (Quaglino et al., 2009).

Transforming growth factor-β1 is a multifunctional growth factor, affecting cell proliferation, apoptosis, and ECM homeostasis (Wu et al., 2008). Previous studies have revealed an inhibitory effect of PRL on the transcription of *TGF-β1*, which may explain the low synthesis of mRNA coding for *TGF-β1* during lactogenesis and galactopoiesis, and the increased expression in late lactation and during the dry period, when PRL

levels decrease (Motyl et al., 2000). An increase of *TGF-β1* expression in the mammary gland during late lactation may contribute to the decline in milk production in this period, as *TGF-β1* is known to slow down epithelial proliferation and it affects milk protein synthesis (De Vries et al., 2010; Wu et al., 2008). Although the expression levels of *TGF-β1* were similar in late lactation and at dry-off, it is possible that TGF-β1 in late lactation is in latent form, and later activated by proteases at dry-off (Plaut et al., 2003). In agreement, low *CSN2* and *LALBA* expression levels were detected after dry-off. Expression of *MMP-9*, involved in ECM degradation and cell apoptosis (Boudreau et al., 1995; Khokha and Werb, 2011), was also greater at the end of lactation and after dry-off. Matrix Metalloproteinase 9 is secreted as a zymogen (Benaud et al., 1998) and then locally activated by plasmin, other MMP, or other proteases abundant at dry-off. Hence, it is possible that, although the expression of *MMP-9* is high 10 d before drying, metalloproteinase activity is concentrated at dry-off. In fact, it has been demonstrated that MMP-9 activity in mammary secretion of cows increases drastically following milk-stasis, which is partially explained by the increase of both neutrophil infiltration and neutrophil degranulation (Yu et al., 2012). In the context of tissue remodeling and ECM degradation, MPC expressed more ($P < 0.05$) *uPA* than PMC (Figure 2B). Regardless of milk production level, expression of *uPA* tended to be greater in MPC at the beginning of the subsequent lactation, probably due to the greater amount of secretory tissue and complex stroma structure (Figure 2B).

Prolactin is the main hormone involved in orchestrating the initiation of milk production and sustaining it thereafter (Lollivier et al., 2015; Lacasse et al., 2016). Cows adapt to different circulating PRL concentrations by altering the relative levels of *PRLR* expression in target cells, especially when the changes in PRL concentrations are mediated by melatonin and different photoperiods (Auchtung et al., 2005). In fact, an inverse relationship between blood PRL concentrations and *PRLR* expression in the mammary gland has been previously described (Auchtung et al., 2005). This is in agreement with the fact that cows classified as LP expressed more *PRLR* than those classified as HP (Figure 4A). On the other hand, greater expression levels of *PRLR* in PMC than in MPC could be related to more persistent lactation curves in PMC. Greater *PRLR* expression agrees with the tendency to present greater expression of *OCLN* in PMC compared with MPC and in cows classified as LP compared with those classified as HP, because it has been demonstrated that PRL regulates *OCLN* expression (Barrington et al., 1997).

Surprisingly, *IGF-1* expression followed an inverted pattern compared with the other genes affected by 3-way interaction, and it was greater at the end of lactation in PMC classified as LP compared with other animals. This result is in agreement with those reported by Miller et al. (2006), concluding that high circulating levels of IGF-1 in PMC could explain better lactation persistency. It is possible that levels of PRL and GH, not studied herein, modulated the increased *IGF-1* expression but, unexpectedly, this

was not translated in greater expression of pro-survival factors such as *BCL2* or *MKI67.* However, in agreement with the high expression levels of *IGF-1* in PMC classified as LP, *C-MYC* expression tended to be lower in PMC classified as LP than in MPC classified as LP at the end of lactation. Further studies would be needed to assess the role of IGF-1 in PMC classified as LP. The expression of *IGFBP5* in the mammary gland increased at mid-dry period and remained elevated until calving independently of parity and production levels at dry-off. The function of IGFBP5 is to block IGF-1, as it acts as a local modulator of mammary growth during the dry period (Akers, 2006). There is evidence supporting the role of IGF-I in stimulation of cell proliferation in the mammary gland (Weber et al., 1999; Knight, 2000). The high expression levels of *IGF-1* until mid-dry period coexist and precede the increase in proliferating cells, as indicated by the increased proportion of Ki67-positive cells immunodetected at mid- and late-dry period. Sorensen et al. (2006) also found that the abundance of mRNA coding for *IGF-I* increased during the dry period, but in their study the expression levels were maintained until early lactation.

Cells positive for SIRT1, a factor involved in cellular metabolism and cell survival (Rahmann and Islam, 2011), were also quantified, but no important changes were found along the dry period, although the levels were greater during the dry period compared with the beginning of the new lactation. Immunostainings of Ki-67 and SIRT1 did not correlate with

gene expression analyses reflecting, perhaps, the complicated pathways involved in the translation of genes into proteins including a number of post-transcriptional and post-translational regulation steps.

Lastly, important changes in different components of the immune system were detected in this study. Some of them have been already mentioned as pleiotropic factors also involved in mammary development and regression such as LIF and TGF-β1. In addition, as expected, the expression of *LF* in the mammary gland was increased during early involution. Lactoferrin is an iron-binding protein with antibacterial properties, and it also plays a role as an antiviral, antioxidant and immunomodulator. Lactoferrin present in milk is, at least partly, originated in the neutrophils, but the mammary epithelium can synthesize it too (Davis and South, 2015).

In conclusion, higher expression of *P16* senescence marker was found in MPC cows, indicating that some cells bypass apoptosis in previous cycles increasing the pool of senescent cells in MPC mammary glands. Senescence in MPC combined with greater *IGF-1* and *PRLR* expression at the end of lactation of PMC could be one of the reasons for more persistent lactations in PMC compared with MPC.

## Acknowledgements

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# CHAPTER VII – General discussion

The goal of this thesis was to evaluate different strategies to improve the dry period in dairy cows, as well as to improve our knowledge of the cellular and tissular factors involved in this process in order to better define the approaches to optimize cow dry period and subsequent lactation.

To meet the growing global demand and to keep dairy farming profitable at the same time, average milk yield per cow, as well as average herd size, has increased in recent decades (De Vliegher et al, 2012). The high milk yields in dairy cows nowadays hinder involution and regeneration of the mammary gland at drying, and challenge animal health, productivity and welfare because there is a high risk to contract intramammary infections. The common practices to avoid infections at dry-off that could affect next lactation involve the use of antibiotics as a routine preventive treatment, contributing to the emergence of antibiotic resistances. In order to reduce or even avoid the use of antibiotics, it is important to further investigate alternatives that are efficient enough against pathogens. Moreover, the acceleration of involution and regeneration of mammary gland by new strategies could allow shortening the dry period and extending the productivity period of dairy cattle. In this thesis, we have worked on different strategies to improve the dry period and we have studied which are the factors orchestrating such complex processes of involution and remodeling of the mammary gland.

In **chapter III**, a strategy involving the stimulation of the immune system using the acute phase protein M-SAA3 was studied to reduce the risk of intramammary infections at drying. At dry-off, the hormones involved in pregnancy downregulate the immune system (Burvenich et al., 2007). However, the participation of the immune system is of crucial interest in order to control bacterial infections. From one hand, some cytokines and immune modulators, also involved in cell survival and development, are crucial to initiate cell-signaling cascades of apoptosis and involution. In fact, we have demonstrated in **chapter VI** the decisive role of cytokines TGF-β and LIF in the initiation of these cellular pathways. Also, the infiltration of macrophages and neutrophils in the mammary gland is of decisive importance to phagocyte milk debris, apoptotic cells, and fight against pathogens entering the mammary gland. However, it has been demonstrated that the immune system does not fully respond to this needs until one week after the beginning of the dry period (Oliver and Sordillo, 1989). Previous work in our laboratory demonstrated a key role of acute phase M-SAA3 in the innate immunity of the mammary gland and its involvement on fostering remodeling factors in the dry period such as Matrix Metalloproteinase-9 (Domènech et al., 2014). Hence, the administration of exogenous recombinant M-SAA3 could be a possible strategy to foster the immune system during the first week of the dry period and prevent intramammary infections. However, the real potential of M-SAA3 to fight against mastitic infections has not been deeply explored so far. To investigate that, we produced recombinant LPS-free

M-SAA3, so that endotoxin from *Escherichia coli* would not interfere in our immune system stimulation results. For this recombinant production, we tested an *E. coli* strain named ClearColi, which has its LPS modified to Lipid $IV_A$ in order to avoid the triggering of immune system in some mammalian species. However, our results showed that recombinant product produced in ClearColi dragged large amounts of Lipid $IV_A$. Even though Lipid $IV_A$ does not trigger an immune response as much as LPS, the large amounts present in the purified recombinant M-SAA3 made us choose a production system based in a regular BL21 *E. coli* strain, and further eliminate LPS with the Mustang XQ Acrodisc membrane. Several doses of M-SAA3 (3, 10, 30, 90, and 160 μg/ml) were tested in primary mammary cell cultures to evaluate how it affected the expression of IL-8, which is the main cytokine increased by M-SAA3 (He et al., 2003). We demonstrated a clear potential of M-SAA3 in the stimulation of the expression of IL-8 in primary mammary cell cultures. There was a clear dose-dependent effect, and the minimum dose of M-SAA3 that showed activity stimulating *IL-8* expression *in vitro* was 10 μg/ml whereas until now the lowest active dose demonstrated was 30 μg/ml. Cytokine IL-8 is an important immune modulator involved in neutrophil recruitment in the mammary gland. Thus, an *in vivo* administration of M-SAA3 would be of crucial importance against infections. Despite these effects, could M-SAA3 produce other direct effects by itself? Molenaar et al., (2009) demonstrated the capacity of bovine M-SAA3 to directly kill pathogens such as *Pseudomonas aeruginosa* and *E. coli*. Following this idea, we

tested our recombinant M-SAA3 against *S. aureus*, which is an important representative of mastitic pathogens, but we did not detect any effects on reducing the viability of *S. aureus in vitro*. However, in the context of mammary epithelial infection by *S. aureus*, we detected a clear potential of M-SAA3 in the inhibition of bacterial translocation in primary mammary cell cultures. This capacity was extended to other pathogens such as *E. coli*, *S. dysgalactiae* and *S. uberis*. From a general point of view, the best concentration of M-SAA3 at inhibiting infections *in vitro* was the lowest concentration tested (10 µg/ml). In the case of *E. coli* and *S. dysgalactiae* this concentration showed the best efficiency of inhibition, being 67% and 51 %, respectively. However, in the case of *S. aureus* and *S. uberis*, although 10 µg/ml of M-SAA3 inhibited 23% and 29% of infection respectively, greater concentrations of M-SAA3 (up to 160 µg/ml) increased the antimicrobial potential up to 50%.

The variability in the response to the several doses from different bacteria is probably due to their different mechanisms of infection. For example, *E. coli*, as a gram-negative bacterium, stimulates the inflammatory response of the epithelium via TLR4. However, *S. aureus* activates TLR2 instead (Fu et al., 2013). M-SAA3 also binds to TLR4 and maybe, in the case of *E. coli* infections, the combination of pathogen infections and high concentrations of M-SAA3 leads to an over-stimulation of TLR4, showing negative effects on the tissue (Blach-Olszewska et al. 2007). Consequently, it translates to lower effects of M-SAA3 regarding the

protection of the tissue against bacterial translocation. On the other hand, a possible competitive effect for TLR4 between pathogen and M-SAA3 cannot be discarded. Further research would be needed to assess those questions. One of the most important conclusions of this first study is that M-SAA3 is able to prevent and reduce infections by itself in the epithelia. Probably there is a stimulation of mechanisms such as secretion of mucins, antimicrobial peptides, or regulation of proteins involved in tight junctions, which form part of inflammatory response as a first defense barrier against pathogens (Roselli et al., 2007).

Therefore, M-SAA3 allows to fighting against bacterial infections at the epithelial level, and can recruit immune cell effectors via IL-8 and other immune modulators. Moreover, it has been previously described that this mammary acute phase protein acts as an opsonin increasing phagocytosis and it also induces maturation of dendritic cells, a key linker between innate and adaptive immunity (Banchereau and Steinman, 1998). On the other hand, studies from our group (Domènech et al., 2014) showed an effect of M-SAA3 in MMP-9 activity *in vivo*. The function of MMP-9 in the drying mammary gland is to disaggregate the ECM and thus, trigger an apoptotic response on epithelial cells. M-SAA3 increases MMP-9 activity in the mammary gland and produces a moderate effect on involution of mammary gland. This induced us to test a second strategy based on the acceleration of the involution process by the administration of recombinant MMP-9 (**Chapter IV**). Physiologically, MMP-9 is one of the

main proteins involved in ECM degeneration at drying (Khokha and Werb, 2011). The detachment from ECM leads epithelial cells to apoptosis (Boudreau et al., 1995). Moreover, MMP-9 promotes the release of growth factors and cytokines, which in turn modulate the immune system and the cellular growth during mammary gland involution and regeneration (Rabot et al. 2007). Hence, we hypothesized that the intramammary administration of recombinant MMP-9 could represent an effective strategy to accelerate tissue involution and boost tissue regeneration at dry-off.

In this study, we produced recombinant MMP-9 to be administrated exogenously into the mammary gland. Since in chapter III the use of ClearColi was not successful, we decided to test another LPS-free system that allowed us to administrate the protein *in vivo* without any endotoxin traces. *Lactococcus lactis* is a gram-positive lactic acid bacteria considered completely safe in the production of recombinant proteins (Garcia-Fruitós, 2012). Recombinant MMP-9 was produced in this system as a soluble form and also as a protein nanoparticle (inclusion body composed by insoluble forms of MMP-9). We were interested in comparing the activity of both formats since it has been previously demonstrated that protein nanoparticles, despite being less active than its soluble counterpart, suppose an interesting source of slow-release active protein (Villaverde et al. 2012; Céspedes et al. 2016). Recombinant LPS-free MMP-9 was infused in soluble or nanoparticled forms through the teat

canal at drying to twelve lactating Holstein cows, using each quarter as an experimental unit, and infusing saline solution as a negative control. The benefits of nanoparticles compared to soluble protein would be: a longer effect because of the slow-release pattern, and a higher stability of the protein in a hostile environment such as the mammary gland at dry-off.

Our results showed very promising effects of MMP-9 nanoparticles on tissue regeneration and stimulation of the immune response. We observed that the infusion of MMP-9 nanoparticles was able to stimulate endogenous MMP-9 activity. We could differentiate the endogenous protein from the recombinant one because of its molecular weight. Also, we saw an increase in other regeneration markers such as *MKi67* and *CASP3* and remodeling indicators such as BSA and $Na^+/K^+$ ratio, as well as immunity markers such as lactoferrin or SCC. This indicates an increase in apoptosis and tissue remodeling rates, as well as a stimulation of the immune system. We could not observe any effects of the soluble protein besides the increase of endogenous MMP-9 activity. This could be due to a quick degradation of the solubilized MMP-9 by proteases present in the mammary gland. Also, the fact that we observed differences in some parameters up to day 7 with MMP-9 nanoparticles would be in agreement with previous studies reporting that when protein nanoparticles are placed in a physiological environment, there is an in situ release of active molecules (Céspedes et al., 2016; Seras-Franzoso et al., 2016). Despite that, further research with inactive nanoparticles would be

needed in order to confirm that the effects we saw were due to the protein contained in the nanoparticles or to the nanoparticles itself.

Two phases have been described in the involution process of the mammary gland: a first reversible stage in which milk production can be restored if suckling is reinitiated, and that is mainly due to the local effects of intramammary pressure due to milk accumulation; and a second irreversible stage in which hormonal stimulus for milk production disappear (Capuco and Akers, 1999).

The third strategy assessed on this thesis (**Chapter V**) involved systemic regulation of lactation by hormonal control of lactation. As it is known, prolactin is the main hormone involved in lactation onset. Recent evidence supports its essentiality in lactation maintenance, and its suppression strongly inhibits lactation in ruminants (Lollivier et al., 2015; Lacasse et al., 2016). Cabergoline is an ergot derivative with high affinity for the D2 dopamine receptors whose dopaminergic effects cause inhibition of PRL secretion (Romagnoli et al., 2009). In previous studies done by our research group (Bach et al., 2015) we already demonstrated a positive effect of Cabergoline on the suppression of milk production in dairy cows reducing udder pressure and milk leakage which is an important risk factor for intramammary infections. Unfortunately, in that study we did not perform a deeper evaluation at the tissue level, in order to discard possible negative effects on the long term.

In order to assess that matter, we designed an experiment to evaluate the effects of Cabergoline injection at dry-off at the tissue level. For that, we took biopsies of twelve cabergoline treated animals and twelve controls at five time points from late lactation to the onset of the following lactation, and evaluated different parameters. We also took a blood sample at each time point to evaluate circulating hormone levels. Our results showed little differences between animals treated with Cabergoline and controls. However, we found an interaction between parity and treatment for the expression of *IGF-1*, a growth factor with a relevant role on cellular regeneration during the dry period; *SIRT1*, which is involved in metabolism and cell survival and whose expression is related to IGF-1 expression in mice (Li et al., 2007); and the proliferation marker *MKi67*. The expression of these genes was increased in primiparous cows treated with cabergoline, but remained unaltered in multiparous cows either treated with cabergoline or in the control group. These results suggest a possible role of cabergoline fostering mammary development in primiparous cows at dry-off, and given the fact that primiparous cows usually can maintain lactation for longer than multiparous cows (Miller et al., 2006), it is very promising that cabergoline is able to exert such effect.

As we observed some differences between primiparous and multiparous cows on the response to cabergoline treatment, we thought it would be interesting to further explore the differences between the two groups.

Thus, in the fourth study (**Chapter VI**), our objective was to delve into the processes of involution and regeneration at a molecular level and see if there were differences between primiparous and multiparous animals, or between high productive and low productive animals, in order to better define the strategies focused to the optimization of dry period.

As it is known, the lactation curve from primiparous and multiparous cows differs mainly in persistency. Primiparous cows can usually maintain lactation for longer, even though their peak milk yield is generally lower compared to multiparous cows (Miller et al., 2006). Also, high productive animals have an increased risk of suffering mastitis at dry-off because of the high amounts of milk remaining in the gland and increased milk leakage. That being so, it was interesting to explore if we could find any differences at the tissue level among these groups that can help finding new and more personalized strategies for each group of animals.

For this experiment, twenty-four animals divided in four groups of six animals each were used: 1) Low productive primiparous (LP PMC), 2) Low productive multiparous (LP MPC), 3) High productive primiparous (HP PMC), and 4) High productive multiparous (HP MPC). We took biopsies at 5 sampling points around dry-off and analyzed the expression of several genes by qPCR and also performed immunostainings for p16, SIRT1 and Ki67 proteins. Our results showed a 3-way interaction (parity x production level x time) in the expression of genes involved in both apoptosis and

proliferation of mammary gland (*BAX, CK18, MKI67, LIF,* and *IGF1*), and a tendency to be significant for *BCL2* and *C-MYC*. In all cases, the differences were detected at the end of lactation, 10 days before dry-off, where a different performance between PMC and MPC was observed depending on the milk yield. In *BAX, CK18, MKI67, LIF, and BCL2*, gene expression was higher in LP MPC compared to LP PMC. Also, HP PMC group showed intermediate expression rates compared with both LP groups. After dry-off, the expression levels for all genes decreased, and differences between groups were no longer seen. In the case of *IGF-1*, the expression pattern was opposite. In this case, LP PMC were the ones with the highest expression levels at -10 d relative to parturition. In Chapter V we did not take into account the milk yield at dry off in the statistical analyses so we could not find the 3-way interaction. However, we did not observe any interactions between parity and sampling time either, probably because the number of PMC animals was too low. From those interactions between parity and sampling time found in Chapter VI, it is important to highlight the expression of *PRLR* being higher in PMC compared to MPC, and in LP compared to HP. Cows adapt to different circulating PRL concentrations by altering the relative levels of PRLR expression in target cells, especially when the changes in PRL concentrations are mediated by melatonin and different photoperiods (Auchtung et al., 2005). Hence, the results indicate the need to increase the PRL sensitivity in those cases. Also, greater senescence rates (*P16* expression) were found at the end of lactation in MPC compared to PMC.

This finding combined with greater expression of *IGF-1* (which is involved in mammary development) in PMC could account for more persistent lactations in PMC compared to MPC.

On the other hand, the results of Chapter V and VI agree about the fact that the highest levels of apoptosis were found at the end of lactation and at the beginning of the dry period in both studies. Also the highest number of proliferating cells as assessed by immunohistochemistry was detected at the end of the dry period after the greatest expression of apoptotic factors.

A discordance between chapter V and VI was the differences observed for the expression of *CASP3*, that showed no changes in chapter VI whereas *CK18* showed no differences on chapter V. Regarding immunostaining results, the results of both experiments are also similar but for those of p16, for which we could not find any significant differences on chapter VI.

In both studies, the main differences between groups were found at the end of lactation for apoptosis and proliferation markers. These data indicate a correlation between production levels and apoptotic and proliferative activity in mammary gland of PMC at the end of lactation, showing greater expression levels in HP PMC than in LP PMC. This also reflects that there is a constant balance of apoptosis and cell regeneration in the lactation state as suggested previously by Capuco et al. (2001). It is important to point out that all these differences in apoptotic and

proliferation factors, affected by interaction of parity and milk production at the end of lactation, disappeared after dry-off. This is an interesting result, because it abolishes the hypothesis that greater mechanical stress produced by milk stasis in high producing animals triggers greater involution rates in the mammary gland (Quaglino et al., 2009).

In summary, we have studied three different promising strategies to improve the dry period. M-SAA3 could be a nice alternative to reduce or avoid the use of antibiotics and prevent infections since it controls the innate immunity of the mammary gland, and it could accelerate the immune response of epithelia and the recruitment of phagocytes during the first week after dry-off. Cabergoline is also partly involved in reducing intramammary infections because it decreases milk leakage, and after our study, we know that it does not exert any negative effects on the involution and regeneration of the mammary gland. On the contrary, further studies could confirm that the use of cabergoline in primiparous cows could exert a positive effect on mammary gland development before the subsequent lactation. Finally, concerning involution, the use of MMP9 has demonstrated very exciting effects. Further research on the application of these nanoparticles using high number of animals, with the objective of reducing dry period length, would be an interesting line of study. Also, testing the use of M-SAA3 or its combination with cabergoline as a potential preventive strategy against IMIs to avoid the use of antibiotics at dry-off would be of crucial interest. A deeper study of which

factors, others than milk yield at dry off, regulate the remodeling of mammary gland in MPC would also be an interesting line of study.

This thesis establishes the bases to improve the dry period of the dairy cow in a near future. Further research comparing these treatments alone or in combination would be required to define the best strategy and ascertain if the subsequent lactation is really improved, and if the elimination of antibiotic use could be done without affecting the risk to contracting new intrammamary infections.

# CHAPTER VIII – Conclusions

1. Recombinant LPS-free M-SAA3 stimulates IL-8 expression in bovine primary mammary cell cultures in a dose dependent manner.

2. Lipid $IV_A$ stimulates the expression of IL-8 in bovine primary mammary cell cultures at a lower extent than LPS.

3. The amount of Lipid $IV_A$ traces in recombinant M-SAA3 produced in *Escherichia coli* BL21 is 3000-fold greater than LPS traces present in recombinant M-SAA3 produced in Clearcoli, determining a possible endotoxic risk of M-SAA3 produced in Clearcoli strain.

4. Recombinant LPS-free M-SAA3 reduces the infection of bovine primary mammary cell cultures by *E.coli*, *S.dysgalactiae*, *S.uberis* and *S.aureus in vitro*.

5. Recombinant LPS-free M-SAA3 has no direct bacterial killing effect against *S. aureus*.

6. Recombinant LPS-free MMP-9, in a soluble or nanoparticulated format, stimulates endogenous MMP-9 activity when infused in the cow's mammary gland at dry-off, being the effect more sustained in the case of MMP-9 nanoparticles than with its soluble counterpart.

7. *In vivo* infusion of recombinant LPS-free MMP-9 nanoparticles in cow mammary gland at dry-off increases tight junction disruption, stimulates the immune system and accelerates mammary gland involution.

8. The administration of cabergoline as prolactin-inhibitor at dry-off in dairy cows does not impair the expression of involution and regenerating factors in mammary gland.

9. Cabergoline administration increases the expression of proliferating factors *IGF-1, SIRT1* and *MKi67* in primiparous cows.

10. High levels of expression of coexisting apoptosis and proliferation-related factors are found at the end of lactation and at the beginning of dry-off compared with the rest of the dry period and the beginning of subsequent lactation.

11. High milk production at the end of lactation correlates with greater apoptosis at the end of lactation and at dry-off in primiparous but not in multiparous cows.

12. The number of cells positive for cell survival SIRT1 factor and proliferation KI67 marker, was higher at mid and end-dry period, respectively, compared to lactation and dry-off.

13. The expression of *P16* senescence marker is greater at the end of lactation and right after dry-off, and it is increased in multiparous cows compared to primiparous cows.

14. Lactoferrin expression increases at dry-off and the expression of milk proteins is low during the dry period, and increased at the end and onset of lactation.

15. There is a greater expression of IGF-1 and PRLR in primiparous cows compared to multiparous.

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