LOCAL REGULATION OF MILK SYNTHESIS CAPACITY IN THE MAMMARY GLAND OF LACTATING DAIRY COWS

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ABSTRACT

Lactating dairy cows heavily rely on mammary gland functionality to maximize milk production. The number and activity of secretory mammary epithelial cells (**MEC**) plays a pivotal role in defining the synthesis potential of the gland. This dissertation aimed to investigate the effects of increased milking frequency (**IMF**), heat stress (**HS**), and cell heterogeneity as key contributors to the regulation of mammary gland milk synthesis capacity in lactating Holstein cows. The first study evaluated the implementation of IMF with 2× and 4× udder halves at early and mid-lactation for 21 and 20 d on milk yield (**MY**) and its association with changes in cistern and alveolar capacity. Results showed that udder halves milked 4× produced 2.27 kg more MY. Additionally, cows milked during early and mid-lactation had increased cistern capacity, while alveolar capacity remained unaffected. This suggests that increased cistern capacity may support MY enhancement through possible systemic responses caused by IMF. The second study examined the effects of 4 days of HS on mammary gland tissue structure, MEC number, and activity using a pair feeding model. Heat stress reduced MY of 4.3 kg/d. At the tissue level, HS decreased alveolar area and increased alveoli number and nucleated MEC per area. Gene expression analysis revealed unaffected activity-related targets but showed reduced phosphorylation of protein synthesis (pSTAT5) and cell survival (pS6K1) markers, as well as upregulation of an autophagosome-related protein (LC3 II). These findings indicate impaired pathways that could explain the reduction in MY after acute HS. The final study utilized single-cell RNA sequencing (**scRNA-seq**) to

characterize the heterogeneity of epithelial and immune cell subpopulations in milk. Analysis revealed multiple subpopulations with distinct gene expression profiles, including different subtypes of mammary epithelial cells expressing representative marker genes (*CSN3*, *CSN2*, *CSN1S1*, *CSN1S2*, and *LALBA*) and immune cell types such as T cells, granulocytes (including neutrophils), macrophages, and B cells. Understanding the populations of hematopoietic cells in milk provides valuable insights into mammary gland function during lactation. The investigation of factors influencing cell number and activity in MEC is crucial for optimizing milk production and maintaining udder health. By identifying and addressing these factors, dairy farmers and researchers can implement strategies to enhance mammary gland function, improve milk production efficiency, and ensure the overall well-being of dairy cows.

LOCAL REGULATION OF MILK SYNTHESIS CAPACITY IN THE MAMMARY GLAND OF LACTATING DAIRY COWS

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GENERAL AUDIENCE ABSTRACT

Milk production capacity in dairy cows relies on specialized cells in the mammary gland called secretory mammary epithelial cells (**MEC**). This study investigated how management practices, environmental factors, and individual cow factors affect the regulation of milk synthesis in Holstein cows. In the first study, we compared milking frequency in udder halves milked two times or four times per day during early and mid-lactation. The cows that were milked four times produced 2.27 kg/d of additional milk. This perhaps happened because the mammary gland's storage capacity increased with more frequent milking. Next, we studied the effects of short-term heat stress on the structure of the mammary gland tissue and the number and activity of MEC. Heat stress lowered milk production by 4.3 kg/d. We observed changes in the size and number of certain cells in the mammary gland, which likely affected the observed milk production findings. We also noticed differences in the activity of proteins related to protein production, cell survival, and the recycling of cell materials. In the final part of the study, we used single-cell characterization techniques to examine the different types of MEC and immune cells in milk. We found that there are various subgroups of MEC, as well as different types of immune cells such as T cells, neutrophils, macrophages, and B cells. Understanding the variety and abundance of these cell populations helps us learn more about how the mammary gland works during milk production. Studying the factors that influence the number and activity of MEC is essential for optimizing milk production in dairy cows. By identifying and addressing these factors, dairy farmers and

researchers can develop strategies to enhance mammary gland function, improve milk production efficiency, and ensure the overall well-being of dairy cows.

DEDICATION

To my loving family, who have done everything possible to support and inspire me to pursue my dreams, and who have always been my pillar of strength and unwavering support, I will always be grateful for all your sacrifices and love.

A mi amada familia, quienes han hecho todo lo posible para apoyarme e inspirarme a perseguir mis sueños, y quienes siempre han sido mi pilar de fortaleza y apoyo inquebrantable. Siempre estaré agradecida por todos sus sacrificios y amor.

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LIST OF ABBREVIATIONS

1×	Once-daily milking
2×	Twice-daily milking
3×	Three times daily milking
4×	Four times daily milking
4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
AF488	AlexaFluor 488
AKT	protein kinase B
ATG7	Autophagy-related protein 7
ATO	Atosiban
B2M	β-2-microglobulin
BSA	Bovine serum albumin
BTN	Butyrophilin 1A1
C	Control
CSN2	β-casein
CSN3	κ-casein
DIM	Days in milk
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
EIF3K	Eukaryotic translation initiation factor 3
ERK1/2	Signal-regulated kinase 1 and 2
FACS	Fluorescence-activated cell sorting
FASN	Fatty acid synthase
FSC	Forward scatter
GEM	Gel beads-in-emulsions
GLUT1	Glucose transporter 1
GLUT8	Glucose transporter 8
H&E	Hematoxylin and eosin

HS	Heat stress
HSP70	Heat shock protein family A
HSPA5	Heat shock protein family A5
HSPA8	Heat shock protein family A8
IMF	Increased milking frequency
JAK2	Janus tyrosine kinase 2
LALBA	**α**-lactalbumin
LC3	Lipidated microtubule-associated protein light chain 3
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MRLP39	Ribosomal protein L39
mTOR	Mammalian Target of Rapamycin
MY	Milk yield
NFDM	Non-fat dry milk
OT	Oxytocin
PCA	Principal component analysis
PE	IgG2a-phycoerythrin
PEG300	Polyethylene glycol 300
PI	Propidium iodide
PPIA	Peptidylprolyl isomerase A
PRLR	Prolactin
PRLR	Prolactin receptor
PVDF	Polyvinylidene difluoride
R	Repeated
RLP0	Ribosomal protein large P0
S6K1	Ribosomal protein S6 kinase β-1
scRNA-seq	Single-cell RNA sequencing
SCC	Somatic cell count
SCS	Somatic cell score
SLC7A5	Solute carrier family 7 member 5
SSC	Side scatter

STAR	Spliced Transcripts Alignment to a Reference
STAT5	Signal transducer and activator of transcription 5
TBST	Tris-buffered saline solution
THI	Temperature and humidity index
TMR	Total mixed ration
TN	Thermoneutral
TUNEL	Transferase-mediated dutp nick end labeling
Tween – 80	Polyoxyethylene sorbitan monooleate
UMI	Unique molecular identifiers

INTRODUCTION

The mammary gland, a specialized organ devoted to synthesizing milk, is distinctive of mammals. Its evolutionary significance resides in its fundamental role in the reproductive biology of all mammalian species, as milk provides essential nutrition for the survival, growth, and development of offspring after birth (Biswas et al., 2022). Mammary glands are usually located in pairs; cows have a total of four mammary glands, collectively termed "the udder". Each mammary gland is composed of non-secretory connective tissue and glandular secretory tissue containing non-epithelial components (i.e., fibroblasts, adipocytes, immune cells, endothelial cells) and epithelial components (i.e., mammary epithelial cells and myoepithelial cells).

The mammary epithelium is composed of a complex network of ducts, lobes, and alveoli. These structures house secretory mammary epithelial cells (**MEC**) which are responsible for the synthesis and secretion of milk in lactating females and myoepithelial cells, which aid in milk ejection due to their contractile properties. During pregnancy, MEC undergo extensive proliferation, differentiation, and metabolic adaptations in preparation for the demands of milk synthesis and secretion during lactation. In lactating mammals, the coordinated activity of MEC is crucial for the efficient incorporation and production of diverse milk components, including proteins, lipids, carbohydrates, and bioactive elements (i.e., vitamins, minerals, growth factors).

In the case of lactating dairy cows, the importance of milk production is further amplified due to its central economic and agricultural significance. Milk production in dairy cows is a highly regulated and complex process, requiring the coordination of numerous factors within each mammary gland. In the last 60 years remarkable improvements in milk production have been achieved in dairy cows through genetic selection (Oltenacu and Broom, 2010) and advances in

management practices (Capper et al., 2009) that maximize and optimize milk synthesis, secretion, and composition. However, additional factors can significantly impact milk synthesis at the mammary gland level by the regulation of MEC number and activity (Capuco and Choudhary, 2020). These factors can include hormonal signals, local growth factors (Zhou et al., 2008), nutrient availability (Burgos et al., 2010), immune factors (Wellnitz and Bruckmaier, 2021), management practices such as milking frequency (Stelwagen et al., 2013) and environmental conditions including temperature and humidity (Tao et al., 2018).

Understanding the factors that influence mammary epithelial cell number and activity is of great importance for maximizing milk synthesis, milk production efficiency, and ensuring the health and productivity of lactating dairy cows, and ultimately, maximizing the productivity and profitability of dairy farms. This dissertation aims to explore a diverse range of factors, such as increased milking frequency, heat stress, and cell heterogeneity that have been identified as key contributors to the regulation of mammary epithelial cell number and activity in lactating dairy cows. By examining the interactions between these components and the mammary gland, we can gain valuable insights into the intricate mechanisms underlying mammary gland function and explore potential strategies to enhance milk production in the dairy industry. The objectives of these studies were to:

1. Characterize the effects of increased milking frequency at early and mid-lactation on milk, protein and fat yields and its association with changes in cistern and alveolar capacity in multiparous Holstein cows.

2. Evaluate the impact of heat stress on secretory mammary epithelial tissue structure and cell losses in milk, and mammary epithelial cell number of lactating Holstein cows under heat stress and thermoneutral conditions using a pair-feeding model.
3. Identify and recognize functional diversity within the epithelial and hematopoietic cells subpopulations present on milk samples from healthy Holstein cows using single-cell RNA sequencing.

REVIEW OF LITERATURE

THE FOUNDATIONAL BASIS OF LACTATION

Mammary glands are complex and unique anatomic organs that differentiate mammals from all other animals. They are exocrine glands arranged in complex tubulo-alveolar structures and have the capacity to synthesize and secrete nutrient-rich milk. Mammary glands are located on ventral body surfaces of in most mammals, usually in pairs. Mammary gland origin has not been totally elucidated due to a lack of fossil structures. Nonetheless, the prevailing hypothesis suggests that eutherian mammary glands, found in placental mammals comprising approximately 98% of existing mammalian species, originated over 300 million years ago from ancestral apocrine glands that were connected to hair follicles (Oftedal, 2002). The most recognized function of the mammary gland is to execute the biological process of lactation - the synthesis, secretion, storage, and ejection of milk – a unique feature in mammals (Capuco and Akers, 2009). Lactation is a coordinated process involving several types of cells present in the mammary gland. The central features controlling milk synthesis in the bovine mammary gland and the positive and negative regulators of milk production and lactation persistency are concepts that will be examined in this review.

Bovine mammary gland development and endocrine regulation

The earliest sign of mammary gland formation matches the time of specialization and maturation of the embryonic mammalian primary germ layers of the mesoderm and ectoderm which give rise to the secretory and supporting portions of the gland. The ectoderm outlines mammary gland structural organization whereas mesenchymal contributions guide ectoderm modifications including mammary line formation and expansion, placode assembly, gland bud

formation and elongation, teat and sprout formation, development of the teat and gland cisterns, and development of the median suspensory ligament resulting in the development of a rudimental mammary gland before birth (Rowson et al., 2012; Slepicka et al., 2021). Later, at birth, mammary gland expansion continues with isometric and allometric growth, and a more complex ductal system is formed during puberty. The ductal system is the basis for the lobulo-alveolar mammary development that occurs during gestation, where lobulo-alveolar development is significantly accelerated in preparation for the coming lactation (Macias and Hinck, 2012; Akers, 2016). Finally, mammary gland development is finalized with the complete functional differentiation and production of milk by the epithelium. After lactation cessation, the mammary gland goes through a regression process known as involution, a complex process of controlled apoptosis and tissue remodeling. The involution process has been described mainly in rodents (Stein et al., 2004), and only some studies have been carried out in ruminants (Knight and Peaker, 1984; Capuco and Akers, 1999; Singh et al., 2016), displaying remarkable differences between these two species. Capuco and Akers (1999) reported that the involution of the mammary gland throughout the dry period is significantly more gradual in the bovine than in rodents. During the involution process, dairy cattle retain several alveolar structural characteristics (Hurley, 1989; Singh et al., 2005), and do not have sloughing of epithelial cells into alveolar lumina or detachment of cells from the basement membrane (Noble and Hurley, 1999; Akers, 2016).

Mammary gland development and ultimate cellular differentiation are driven by a combination of hormones with indirect and direct effects on the mammary gland. Follicle-stimulating hormone, luteinizing hormone, thyroid stimulating hormone, adrenocorticotropic hormone, growth hormone, and vasopressin play an indirect role, while prolactin and oxytocin are the two fundamental hormones directly regulating milk synthesis and milk ejection during

established lactation. Prolactin is a peptide hormone involved in hundreds of homeostatic and physiological functions including metabolism, reproduction, immune response, and lactation (Ben-Jonathan et al., 2008). Specifically, prolactin plays a fundamental role in lobuloalveolar development during pregnancy and mammary differentiation and lactogenesis (Akers et al., 1981; Wagner et al., 2004). Oxytocin is a peptide hormone synthesized in the hypothalamus and transported into the posterior pituitary, where it is released into the bloodstream. One of the main functions of oxytocin is to produce milk ejection from the mammary gland by triggering the contraction of myoepithelial cells surrounding the mammary alveoli (Lollivier et al., 2006a).

Anatomy of the bovine mammary gland: function and structure

The general structure of the mature bovine mammary gland is four individual glands known as quarters separated longitudinally in pairs by the intermammary sulcus. Internally within each individual gland, the main anatomical structures present are teat, teat meatus, teat cistern, annular ring, gland cistern, ductal network, and lobes formed by multiple glandular structures called lobules containing the alveoli, the basic structure of the lactating mammary gland (Figure 2-1). Although the main structures, development of the mammary gland, and the nutritional constituents of milk are conserved across species, some variation in the anatomical position and number of glands as well as the number of openings per teat and specific milk composition exists. These variations are the result of evolutionary adaptations and ecological factors that have shaped the reproductive strategies and survival needs of different species.

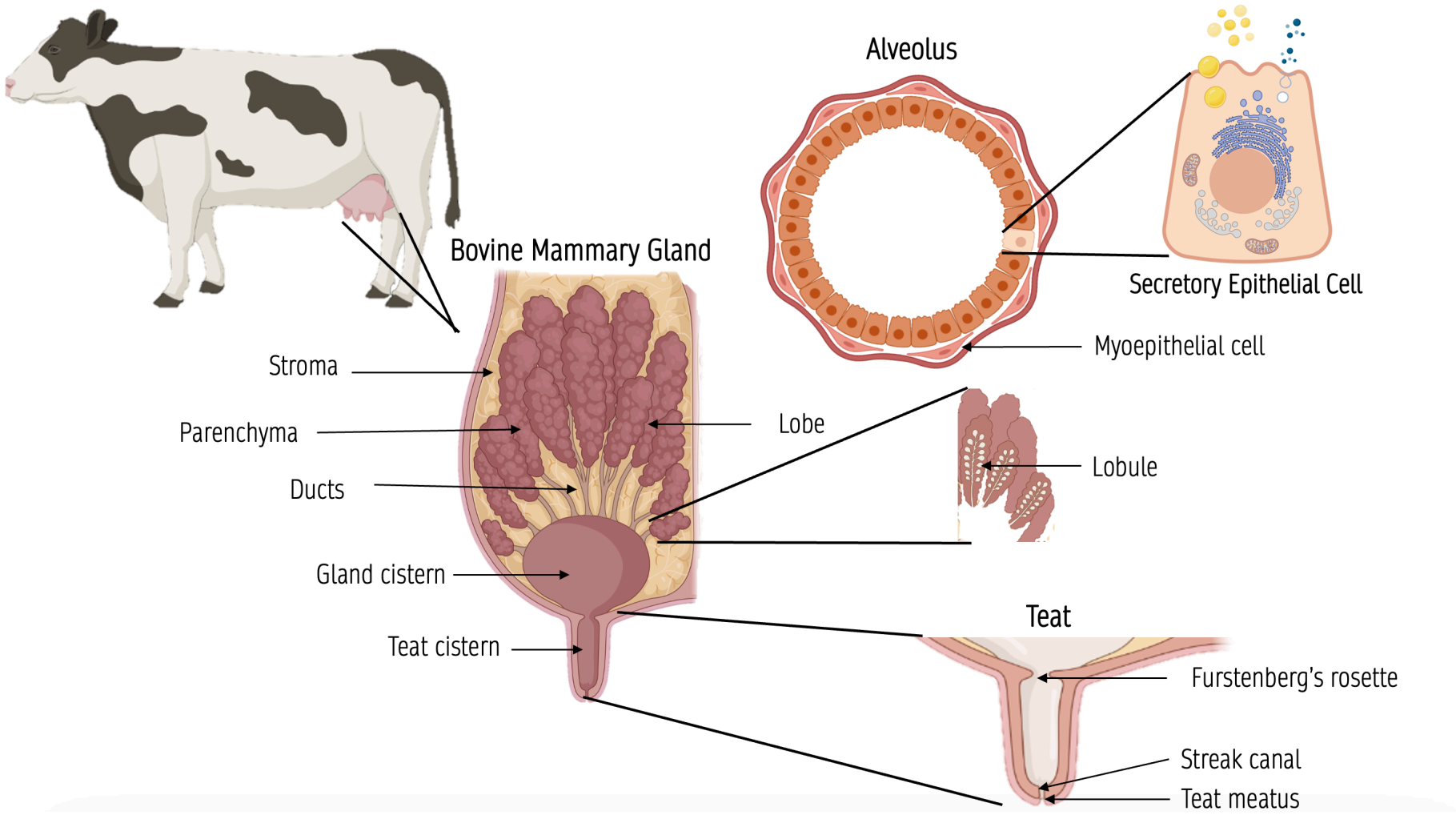

Figure 2.1. General anatomy of the bovine mammary gland

Constant milk synthesis and luminal secretion occur in the alveoli, small structures lined with milk-secreting epithelial cells connected to ducts that carry synthesized milk to the gland cistern. Shortly after milking, a small volume of the cistern is filled with residual milk not harvested at milking. As time passes, milk synthesis continues within the secretory tissue. As milk accumulates in the alveolar luminal space, the pressure on the epithelial lining causes the secretory cells to compress (Nickerson and Akers, 2011). Between 1 and 4 hours after milking, a significant pressure upsurge occurs increasing the flow of milk from the alveoli into the teat and gland cisterns where milk is stored between milkings (Schmidt, 1971; Knight et al., 1994). The alveolar and cistern proportions reported in the literature before milking are 60% and 40%, respectively (Davis et al., 1998; Nickerson and Akers, 2011).

At the cellular level, three major systems compose the mammary gland, a support and immune system including the skin, a non-secretory connective system known as stroma, and a glandular secretory system known as parenchymal tissue (Figure 2.1). The stroma or connective tissue is primarily composed of adipocytes, fibroblasts, immune cells, and nerves. The coordinated function of all these cell types provides nutrients, blood supply, physical structure, and immune defenses to the mammary gland (Watson and Khaled, 2008). Furthermore, cross-talk between stromal cells and the mammary epithelium coordinates the development and function of the glandular tissue (Sternlicht, 2005). The parenchymal or secretory tissue is the glandular epithelial tissue that is formed by the alveoli, duct system, and lobules and lobes. Within the epithelium, there are two main subtypes: the luminal epithelium which forms the inner layer that surrounds the hollow lumen, and the basal epithelium composed of myoepithelial cells (Biswas et al., 2022).

CELL HETEROGENEITY OF THE LACTATING MAMMARY GLAND

The cell types of the mammary gland coordinate their function to synthesize milk and they change across lactation and the reproductive state of the animal. This review will focus exclusively on changes and regulation playing a role in the mature lactating mammary gland. Although the traditional way to study cell function in the mammary gland is through mammary gland biopsies, multiple cell types from the mammary gland can be found in milk. These cells present in milk are known as somatic cells, and are mainly composed of immune cells (Sharma et al., 2011) and secretory mammary epithelial cells (Boutinaud and Jammes, 2002a). The diversity of cell subtypes within these two main subpopulations in milk make them a suitable sample that can be regularly obtained in a safe and approachable manner and these cells display the molecular and cellular features of the mammary cells allowing insight into the function of the gland (Martin Carli et al., 2020).

Identifying specific molecular features of cell subpopulations to understand the basis of milk synthesis and continuous milk production in cows has been challenging with the technology available until recently. Additionally, the study of the cellular composition of the mammary gland, a heterogeneous organ, faces challenges in terms of dissociating individual cell types and identifying minor subpopulations to investigate their contributions to the functionality of the bovine mammary gland due to the complex interaction between various cell populations, the dynamic nature of cellular interactions, and the limited availability of specific markers for certain subpopulations. Furthermore, cell dissociation should minimize cellular perturbation to mimic cell functionality in vivo (Cristea and Polyak, 2018). Use of techniques like fluorescence-activated cell sorting (**FACS**) and single-cell RNA sequencing (**scRNA-Seq**) has helped to overcome or minimize these challenges and reveal the heterogeneity of the mammary gland in greater detail.

Adipocytes, Fibroblasts, and Vascular cells

Adipocytes, fibroblasts, and vascular cells contribute to mammary cell functionality in diverse ways. Adipocytes or fat cells comprise primarily the stromal fat pad in the prepubertal non-lactating mammary gland. Additionally, mammary adipocyte activity increases during the transition from pregnancy to lactation and from lactation to involution (Hovey and Aimo, 2010). After lactation cessation, during the dry period, contrary to what happens in murine species, the bovine mammary gland does not resume lipid deposition. During lactation, the number of adipocytes and lipid content within the mammary gland is reduced likely due to the use of reservoir fat for milk production (Gregor et al., 2013). Furthermore, adipocytes function as endocrine cells that might regulate and control the communication of MEC (Hovey and Aimo, 2010). Stromal fibroblasts are embedded within the fat pad and synthesize multiple extracellular matrix components including collagens, proteoglycans, and fibronectin. In vitro models have proposed that fibroblast might play a chief role in supporting MEC survival (Makarem et al., 2013; Inman et al., 2015). Another crucial role of the stromal tissue is to provide a supportive framework for the constantly adapting vascular and lymphatic network. The intricate network of blood vessels and lymphatic vessels plays a vital role in maintaining the proper functionality of the mammary gland. The primary function of the vascular system is to transport oxygen and essential nutrients including glucose, amino acids, and lipids to the mammary gland. Without an efficient vascular network, the mammary gland would not receive an adequate supply of oxygen and nutrients, compromising its ability to produce milk. In particular, research has shown that the development of the lymphatic and blood vasculature occurs parallel to the mammary epithelial and myoepithelial cells (Betterman et al., 2012). This coordinated development suggests a close

interplay between the stromal tissue and the vascular network, highlighting their reciprocal influence on each other's function and regulation.

Immune cells

Immune cells can be found within the stroma surrounding the alveolus and participate in mammary gland development and immune response regulation. Immune cell profile and function have been extensively reported during mammary gland development and post-lactation regression, where macrophages, eosinophils, and mast cells contribute to branching morphogenesis and dead cell removal (Gouon-Evans et al., 2000; Lilla and Werb, 2010; O'Brien et al., 2012). However, immune cell function in healthy mammary glands during lactation has not been extensively investigated but has been proposed to participate in epithelial cell proliferation and differentiation in preparation for lactation. Knock-out studies in mice have shown impaired lactation, decreased MEC functional differentiation, decreased overall litter size, and increased pup mortality when macrophage (CSF1) and eosinophil (IL-5) related genes were silenced (Pollard and Hennighausen, 1994; Colbert et al., 2005). Other immune cells such as B and T cells are recruited to the mammary gland during lactation to participate in transfer of passive immunity to the newborn (Reed and Schwertfeger, 2010). Specifically, B cells infiltrate the mammary gland during lactation and contribute to the secretion of immunoglobins including immunoglobin A in milk and colostrum (Bourges et al., 2008). In the bovine mammary gland, leucocytes (lymphocytes, granulocytes, monocytes, and macrophages) participate in the defense against pathogen infection by playing an important role in the inflammatory response (Leitner et al., 2003). In particular, activated macrophages in milk or tissue recognize the invading pathogens and release pro-inflammatory cytokines that induce neutrophil recruitment to eliminate the pathogens from the mammary gland (Oviedo-Boyso et al., 2007).

Immune cells in milk have a hematopoietic origin and enter the mammary gland in response to an injury or infection (Boutinaud and Jammes, 2002b; Sharma et al., 2011). Immune cells transferred to milk are part of milk cells known as somatic cells, a routine parameter used to evaluate inflammation and udder health in dairy cows (Pilla et al., 2012). However, bulk measurement of immune cells in milk does not portray the wide cell type identity of the immune cells present. To consider the possible contribution or response from each cell type, initially, microscope and cytology-based techniques were used to identify subpopulations. Using cytology and cytometric light comparison by cell size and granularity, a broad classification of immune cells identified in bovine milk was assessed. The main cell types identified with these techniques were leukocytes, lymphocytes, and polymorphonuclear leukocytes (Rivas et al., 2001). It is important to note that this technique bases cell identification only on the morphological characteristics and only a general classification of cell type can be displayed.

The appearance of new technologies such as flow cytometry using cell surface markers to reveal heterogeneous immune cell subpopulations in milk allowed for a more complete understanding of cell diversity within this cell group. In a study by Trend et al. (2015), human colostrum, transitional and mature milk analyzed by flow cytometry showed the presence of multiple immune cell subtypes including monocytes, cytotoxic T cells, natural killer cells, basophils, T cells, B cells, neutrophils, eosinophils, immature granulocytes, and B cell precursors. Similarly, in bovine milk, leukocytes, lymphocytes, granulocytes, and neutrophils are present and subpopulation proportions can be altered by environmental factors such as heat stress (Lengi et al., 2022a).

Although cytology and flow cytometry offer the possibility to partially describe cell profiles, only a few cell surface markers can be used, limiting the cell information and profile

characterization of heterogenous immune subpopulations present in milk. Recent studies using scRNA sequencing have extended the profile of the types of immune cells present in human and bovine milk samples. The presence of small populations of macrophages and T cells in milk has been found in maternal breast milk from healthy subjects (Gleeson et al., 2022). Similarly, Martin Carli et al. (2020) reported a wider immune cell profile that includes T cells, natural killer cells, dendritic cells, monocytes, and macrophages in small proportions present in cells derived from human milk from 2 individuals with gestational diabetes mellitus. Additionally, an alteration of immune cell profile has been reported in immune cells including an increase of leukocytes and a decrease in basophils when mastitis and other infections are present (Hassiotou et al., 2013; Trend et al., 2015). On the other hand, the immune cell profile of bovine milk samples has been only explored in one study showing a similar profile to human milk cell populations. Most of the immune cells present in bovine milk in this study were macrophages with 5 different subclusters, followed by monocytes with 3 different subclusters and $CD8^+$ and $CD4^+$ T cells, and a smaller proportion of dendritic cells, natural killer cells, and B cells depicting one main subpopulation (Becker et al., 2021).

Epithelial cells

Although mammary gland development and function are the response to the combined function of the multiple cells previously discussed, most cells present in the mammary gland during lactation are mammary epithelial cells. Because of the tight relationship between MEC and lactation, many studies focus on MEC participation in mammary gland function and synthetic capacity in multiple species. Differentiated mammary epithelial cells form a bilayer structure composed of two cell types. The inner and outer layers are composed of a heterogenous apical monolayer of luminal cells and a basal monolayer of basal cells (Visvader, 2009; Cristea and

Polyak, 2018). Luminal MEC, also known as secretory MEC, are the chief cells synthesizing milk and are sub-classified according to hormone and growth factor receptor expression (i.e., estrogen receptor). Basal MEC include a subgroup of cells named myoepithelial cells that surround the luminal cells, and their main function is to express synthesized milk from the alveoli. Within the basal MEC, multiple putative progenitors and stem cells with specific lineages for both luminal secretory and basal myoepithelial cells have been identified (Visvader and Stingl, 2014; Seldin et al., 2017).

The capability to characterize and identify individual cells through cell surface markers and transcriptional signatures has been supported by the development of advanced single-cell technologies like FACS and scRNA-seq. Bach et al. (2017) mapped the cellular dynamics of sorted MEC from mammary gland samples obtained from two mice at each of four developmental time points (n = 8; nulliparous, 14.5 d of gestation, 6 d of lactation, and 11 d of involution). In this study, fifteen different subpopulations were identified with the following putative identities: hormone-sensing progenitors, hormone-sensing differentiated cells, luminal progenitors, alveolar differentiated cells, alveolar progenitor cells, basal cells, myoepithelial cells, and $Procr^{+}$ basal cells, with the last five groups present in greater proportion during lactation. Similarly, an integrative analysis using three different datasets from previous scRNA-seq analysis from murine MEC identified 10 main subpopulations within sorted MEC listed according to cell proportion as follows: luminal progenitors, basal myoepithelial, basal, luminal hormone sensing progenitor, luminal hormone sensing differentiated, luminal alveolar progenitor, luminal alveolar secretory, stem cell, luminal hormone sensing intermediate differentiated cells, and immune cells (García Solá et al., 2021).

The standard technique to study the mammary gland and MEC is through mammary gland biopsies. Nevertheless, the use of non-invasive "milk liquid biopsies" that can be regularly obtained in a safe and approachable manner offers the elimination of related surgical biopsy pitfalls and mimics the results obtained from mammary gland biopsies (Martin Carli et al., 2020). The collection of somatic cells present in milk is composed of secretory mammary epithelial cells and immune cells. It is known that secretory mammary epithelial cells result from the desquamation of the mammary epithelium of alveoli and ducts (Alhussien and Dang, 2018), and bovine milk-derived cells represent the secretory MEC present in the gland tissue responsible for milk synthesis (Boutinaud et al., 2013a, 2015).

Contrary to what was observed in tissue samples from murine species, the use of viable cells in milk samples depict a different profile for the MEC. Human milk samples from 2 subjects at two weeks postpartum revealed the presence of 8 different subpopulations within MEC. Most cells resemble the transcriptome profile of secretory alveolar MEC. This putative characterization included 6 out of the 8 observed subpopulations and suggest a potential functional relationship between the observed 6 subclusters present or different cell states within the secretory alveolar MEC. The 2 remaining subpopulations were characterized as immune cells. Myoepithelial cells, other basal MECs, and pluripotency transcripts were not detected in this study (Martin Carli et al., 2020). Only one study has explored the different cell profiles existing in milk-purified MEC from a cow at 52 weeks of lactation and also included primary bovine mammary epithelial cells isolated from mammary tissue sampled from a primiparous cow at 5 weeks of lactation and cultured in vitro (Becker et al., 2021). Similar to what was observed in human milk samples, the presence of MEC in bovine milk is minimal, with only a count of 176 cells. These cells represent a single homogeneous subcluster out of the 14 observed in this study, accounting for 2.47% of the total

cell count. On the other hand, as expected, cultured primary bovine MEC depicted 13 subclusters of epithelial cells and 1 subcluster of fibroblasts. The different expression of transcripts in the 13 subclusters from cultured primary bovine MEC is explained in the report mainly due to the sampling protocol of the cells, which were mainly isolated from ductal non-secreting regions of the gland.

MILK PRODUCTION AND LACTATION PERSISTENCY

The highest milk production in dairy cows is reached 4 to 8 weeks postpartum followed by a daily decline in milk production until lactation conclusion at the standardized lactation length of 305 d (Keown et al., 1986). The rate of decline in milk production after peak milk is defined as lactation persistency which is mainly determined by the number and activity of the secretory epithelial cells of the bovine mammary gland (Capuco et al., 2001, 2003; Stefanon et al., 2002). Understanding the mechanisms controlling secretory MEC number and activity and ultimate milk production is crucial to manipulate lactation persistence and promote efficiency in dairy cows.

The quantity and synthetic capacity of secretory MEC in the bovine mammary gland might decrease throughout lactation shaping the lactation curve (Capuco et al., 2001). However, if these two factors can regulate milk production and lactation persistency individually or collectively has not been concluded. This review will analyze how secretory MEC number and synthetic capacity can potentially participate in regulating milk production and the potential effectors that can regulate these two factors.

MEC number

Mammary cell number fluctuations mainly occur during gestation but are also present during lactation. The first insights about the relationship between mammary gland size, an indirect measure of mammary cell number, and milk production were evaluated by Linzell (1966) who

reported a positive correlation between mammary gland size and milk yield in goats. Concordantly, recent studies have shown that the number of mammary epithelial cells is a superior determinant factor for milk production (Capuco et al., 1997, 2001). Quantitative evaluations of mammary epithelial cell dynamics from nonpregnant multiparous cows attribute increasing milk yield during early lactation to increased proliferation and differentiation of secretory MEC with the subsequent decline through the lactation due to a reduction in the number of these cells. A 23% reduction in milk production is observed when DNA amount, an indirect measurement of cell number, is reduced by 17%, and as a percentage of total cells, the epithelial component declined from 79% to 73% from 90 to 240 days in milk (Capuco et al., 2001; Boutinaud et al., 2004b). In a different study with goats, milk production and DNA levels decreased by 20% and 19%, between the 8th and 36th weeks of lactation (Knight and Peaker, 1984). Consistent trends were found by Capuco et al. (1997) who reported that around the time of calving, 83% of mammary cells were epithelial, but during late lactation, the percentage decreased to 74%.

During lactation, cell number is determined by the relationship between secretory MEC proliferation or differentiation and cell death or loss. Capuco and Choudhary (2020) suggest that cell turnover provides for the replacement of senescent or damaged cells. During the onset of lactation, milk secretion increases to the highest point due to a continuing change in morphology and function of the cells that secrete milk. Nevertheless, after peak lactation, cell renewal during lactation is minimal, while the mammary gland undergoes gradual deterioration through apoptosis, exceeding mammary cell proliferation and regulating the gradual decrease in milk production (Capuco et al., 2001). Furthermore, the standard MEC loss type reported in the literature is cell death by apoptosis. However, cell loss mechanisms including secretory MEC extrusion and

exfoliation have been observed during lactation and might play a partial role in controlling cell number in the ruminant mammary gland (Herve et al., 2016).

Mammary epithelial cells loss – Cell death: Apoptosis

Apoptosis plays a fundamental role in mammary gland development and function, where it contributes to remodeling the tissue architecture before future lactations. The molecular processes regulating apoptotic events are not completely understood; however, a number of studies have been carried out generating a substantial amount of information in the last few years (Green and Streuli, 2004). Apoptosis is an active process of programmed autonomous cell death, which involves the genetically determined elimination of cells without producing inflammation (Fink and Cookson, 2005). Apoptosis usually occurs during normal development and aging as a homeostatic mechanism that helps to maintain cell populations in tissues. However, apoptosis can also occur as a defense mechanism that targets damaged cells (Elmore, 2007). Both physiological and pathological stimuli can trigger apoptosis, nevertheless, the response to these stimuli may differ between cells.

To date, two main apoptotic pathways have been defined: the extrinsic pathway and the intrinsic pathway. In the intrinsic or mitochondrial pathway, the cell destroys itself in response to internal cell stress signaling, while the extrinsic or death receptor pathway triggers apoptosis in response to external stimuli, where the cell is destroyed through signal transduction from other cells (Elmore, 2007). Both extrinsic and intrinsic apoptosis pathways lead to an end-point execution phase, considered the final phase of apoptosis.

Although apoptosis is characteristic of post-lactation mammary gland regression, at present, it is known that apoptosis is not limited to this period. Capuco and collaborators (2001) quantified cell apoptotic index across lactation (14, 90, 120, and 240 DIM) in multiparous Holstein cows. Interestingly, their results indicated that the apoptotic index peaks during early lactation and

decreases on d 90 with no difference in apoptosis rate after the peak of lactation. The authors suggest that the proportion of secretory epithelial cells in mammary tissue is influenced by lactation stage. Although the idea that the lactation curve is determined by changes in cell number which is influenced apoptosis, the assessment of cell death by apoptosis in the literature across lactation shows remarkably small proportions of secretory MEC being lost and a considerable disparity between reports using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (**TUNEL**) assay in mammary gland tissue sections (Table 2.1).

Table 2.1. In situ detection of apoptosis in bovine mammary gland tissue sections evaluated in the different studies using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Reference	Lactation day	Average of positive MEC death evaluated using TUNEL assay
Capuco et al. (2001)	116	0.07%
Dessauge et al. (2011)	87.5	0.22 %
Montazer-Torbati et al. (2016)	67	0.30%
Boutinaud et al. (2013)	162	0.50 %
Bernier-Dodier et al. (2010)	195	0.07%
Wall et al. (2013)	28	0.8%
Hale et al. (2003)	7 and 14	D7 = 1.1% D14 = 0.8%

Mammary epithelial cells loss – Cell extrusion

Cell extrusion occurs by the breaking of apical or basal spatial arrangement and relationships of tissue which leads to cell expulsion into the external environment through the lumen or towards entering the body (Nanavati et al., 2020). This phenomenon is characteristic of cell monolayers present in epithelial and endothelial tissues and its theoretically driven by mechanical and biochemical alterations related to apoptotic and oncogenic changes occurring in

the cells to be extruded (Hogan et al., 2009; Kuipers et al., 2014). Although there are no in vivo nor in vitro models demonstrating that MEC cell extrusion occurs in the bovine mammary gland, the presence of viable and non-viable MEC in milk suggests that this process might be occurring commonly during lactation. The amount of MEC present in milk is affected by multiple factors including lactation stage (Boutinaud et al., 2013b), endocrine status (Lollivier et al., 2015), management (Ben Chedly et al., 2013), and environmental factors. Heat stress can increase the quantity of MEC present in bovine milk by 82% (Lengi et al., 2022a), while feed-restricted lactating cows increased the rate of MEC exfoliation in milk by 65% (Herve et al., 2019).

Mammary epithelial cell proliferation and differentiation

The cycle of pregnancy, lactation, and involution can repeat itself multiple times during the reproductive life span of a cow exhibiting the remarkable potential to proliferate and differentiate mammary epithelial tissue. The proliferative capacity of this tissue encompasses a massive expansion in cell number and the formation of milk-producing alveoli. Mammary epithelial cell proliferation, and subsequent differentiation, is a progressive process, where the cell develops the essential components needed for milk synthesis, such as cellular organelles and enzymes (McFadden et al., 1987).

The average daily MEC proliferation rate estimated in multiparous non-pregnant dairy cows during lactation is 0.3% (Capuco et al., 2001). These researchers measured cell proliferation by labeling cells that synthesize DNA with bromodeoxyuridine, a common synthetic nucleotide used in the detection of proliferating cells in living tissues. Additionally, using the estimated apoptotic and proliferation rates, the cumulative cell loss, cumulative cell proliferation, and net cell number were calculated in a different experiment. The results showed that on day 252 of lactation, the MEC present in the mammary gland matched the number of cells formed during

lactation. This led to the authors to conclude that although the rate of cell proliferation is slow, by the end of lactation most cells in the mammary gland had proliferated post-partum (Capuco et al., 2001; Capuco and Choudhary, 2020).

Mammary epithelial cell activity

Mammary epithelial cell activity can be assessed in multiple ways including cytological analysis and measurements of transcript or protein abundance. In general, fully differentiated active secretory mammary epithelial cells secrete milk into the alveolar lumen. Three levels of mammary epithelial cell differentiation for histological evaluation of mammary epithelial cell activity have been described in the literature. Poorly differentiated cells lack polarity, show significant nuclei:cytoplasm ratio, and have large lipid droplets and a small number of cytoplasmic vacuoles. Intermediate differentiated cells have reduced nuclei:cytoplasm ratio, few apical vacuoles, and a medially to basally displaced nucleus. Finally, completely differentiated cells are characterized by exhibiting abundant supranuclear vacuoles, apical lipid droplets, basally located spherical nuclei, and large cytoplasmic: nuclear ratio (Akers et al., 1981; Akers, 2016).

Additionally, the secretory activity of MEC can be analyzed in terms of enzyme activity and target-gene expression using global transcriptomic or proteomic approaches (Boutinaud et al., 2019). In dairy cows and other ruminants, the metabolic and secretory activity of the MEC increases during the onset of lactation. Particularly, milk synthesis at peak lactation is largely determined by secretory MEC activity which is characterized by an elevation of total RNA per unit of DNA in mammary gland tissue. RNA increases occur as a response to the substantial synthesis of RNA needed for translation of milk proteins and milk component synthesis enzymes including acetyl-CoA carboxylase, fatty acid synthase, and galactosyl transferase (Knight and Peaker, 1984; Boutinaud et al., 2004a).

FACTORS INFLUENCING MEC NUMBER AND ACTIVITY

Management – Increased Milking Frequency

Increased milking frequency (**IMF**) is a management practice used to increase milk production by augmenting the number of times that dairy cows are milked in a period of 24 h. In a conventional dairy farm, cows are traditionally milked twice per day. The immediate and persistent increase in milk production when one or two milkings per day are added to the standard established milking frequency on the farm has been extensively reviewed (Lush and Shrode, 1950; Wall and McFadden, 2007a; Sorensen et al., 2008). On average, early lactation cows milked 4× in a day produce around 5 kg/d more than cows milked 2× (Wright et al., 2013).

The unilateral milking of the udder at different frequencies, known as the unilateral frequent milking model, suggests that increased milking frequency locally regulates milk yield (**MY**) and milk component enhancement at the mammary gland level. In two different studies, udder halves milked 4× for 21 d during the onset of lactation increased daily milk production by 2.8 kg/d and 6.0 kg/d on the last day of IMF (Wright et al., 2013; Hanling et al., 2021). In both studies, milk yield enhancement of 0.8 and 1.56 kg/d persisted until 270 and 300 DIM. Potential mechanisms driving increased milk production with IMF have been examined in previous studies with inconsistent results (Hale et al., 2003; Wall and McFadden, 2010). However, cell number and activity may be positive regulators increasing milk production when IMF is implemented.

A central hypothesis is that MY enhancement by IMF is mainly regulated by MEC number and activity. Specifically, it has been proposed that IMF stimulates cell proliferation and decreases cell death by apoptosis. The effects of IMF on MEC number to date are not conclusive, while some studies report no significant differences in cell proliferation or apoptosis rate in glands milked 4× compared to glands milked 2× (Hale et al., 2003; Nørgaard et al., 2005a), when udder half IMF is

incremented from 1× to 4×, MY increased by 80% and the relative cell rate proliferation, measured by Ki-67, increased by 32% in udder halves milked 4× with no differences in apoptosis rate (Murney et al., 2015). Similarly, in udder halves milked 3×, cell proliferation rate increased by 2.4 and 2.8 on weeks 4 and 8 of differential milking frequency compared to udder halves milked 1×. In this study, cell apoptosis rate, measured by TUNEL assay increased by 2.2 fold in udder halves milked 1× at week 4 of IMF (Bernier-Dodier et al., 2010).

Effects on cell activity by IMF have been evaluated by gene and protein abundance of multiple targets directly involved in milk synthesis. Milk synthesis and milk component related genes expression including β-casein, alpha-lactalbumin, acetyl-CoA carboxylase, and prolactin receptor increased in quarters milked 3× compared to quarters milked 1× (Toledo et al., 2020). Nevertheless, the increased expression of long and short isoforms of prolactin receptors was not related specifically to IMF treatment. The prolactin receptor (**PRLR**), a member of class I cytokine receptor, involved in casein synthesis, is part of the Janus kinase/signal transducer and activator of the transcription pathway activating the Janus tyrosine kinase 2 (**JAK2**) by signal transducer and activator of transcription 5 (**STAT5**) mediation downstream hormone signaling. When prolactin binds to its receptor on MEC it initiates a cascade of intracellular events essential for milk production. Upon binding, tyrosine residues in the intracellular portion of the receptor are phosphorylated, triggering a series of signaling events. One of the key outcomes of this signaling pathway is the phosphorylation and translocation of STAT5 to the nucleus of MEC. Once in the nucleus, STAT5 acts as a transcription factor by binding to specific DNA sequences within the regulatory regions of milk- protein encoding genes (Watson and Burdon, 1996). This process induced by prolactin and STAT5 activation leads to the initiation of gene expression programs that are responsible for the differentiation of mammary epithelial cells and the synthesis of milk

proteins. These milk protein-encoding genes include those encoding caseins, which are the major proteins found in milk and play a crucial role in milk synthesis and secretion (Chen et al., 2012). When IMF is increased by 3× (4× vs. 1×), STAT5 gene and protein expression increases suggesting that IMF might increase MEC activity increasing casein synthesis and ultimate milk production (Murney et al., 2015).

Environmental factors – Heat Stress

Environmental factors like increased temperature can negatively affect the mammary gland milk synthetic capacity by reducing cell activity and cell number. The gradual increase in global temperature in the last 50 years and the persistent temperature increase predicted globally have increased researchers' efforts to understand the mechanisms driving detrimental effects of heat on animal welfare, health, and production performance. Dairy cows express their maximum genetic merit and milk production capacity within a defined thermoneutral range which is intrinsically regulated to be maintained within the animal and despite alterations occurring in the environment (i.e., exposure to high temperatures). This process is known as thermoregulation, a neural process that interconnects the external environment and cellular metabolism-regulating directly and indirectly transcription factors and endocrine system responses (Nakamura and Morrison, 2008; Seebacher, 2009).

Thermoneutral regulation is a complex process, but in broad terms, thermo-transient receptors present on somatosensory-free nerves of the dermis and epidermis are activated by environmental temperature changes. Sensory information travels to the central nervous system, from the thalamus to the cerebral cortex, and triggers the activation of the sympathetic preganglionic neurons controlling thermoregulatory responses including cardiovascular responses, vessel vasodilation or constriction, metabolic changes, and hormone release (Figure 2.2) (Collier

and Gebremedhin, 2015; Collier et al., 2017). These responses modify transcription factors and regulate gene and protein expression, enabling adaptation to significant alterations in environmental heat conditions.

Due to the local regulation of milk synthesis within the mammary gland, the idea that heat stress (**HS**) affects the bovine mammary gland and MEC has been proposed. Heat exposure does appear to affect the ability of the mammary gland to synthesize milk by decreasing cell number and cell activity (Tao et al., 2018). Cell number has been evaluated in mammary gland tissue samples and milk samples by histology and flow cytometry methodologies. MEC present in milk are assumed to come from functional secretory cells in the gland. In particular, exfoliated epithelial cells present in milk are viable and exhibit characteristics of fully differentiated alveolar cells (Boutinaud and Jammes, 2002a). MEC loss increased by 82% in bovine milk when cows were exposed to HS (Lengi et al., 2022a). MEC number during lactation after HS has not been evaluated, however, in vitro studies demonstrated a 50% reduction in MEC number when cultured mouse cells were exposed to high temperatures (41°C) for 48 h (Wakasa et al., 2022). Additionally, when HS was present without cooling abatement during the entire dry period of cows, alveoli number during lactation decreased by 15% (Dado-Senn et al., 2019). For cows exposed to HS with non-cooled conditions during fetal development (intra-uterine exposure), alveolar area was reduced by 46% in their first lactation (Dado-Senn et al., 2018).

Heat stress has been found to affect the secretory capacity of MEC in various ways, leading to alterations in milk synthesis and milk component production. Studies have shown that HS induces mitochondrial dysfunction through oxidative stress, impacting mammary gland functionality (Guo et al., 2021). Additionally, expression of important milk component genes such as β-casein and butyrophilin is downregulated under HS conditions (Hu et al., 2016). Moreover,

there is a transcriptional downregulation of genes involved in milk protein synthesis pathways, including those related to late endosomal/lysosomal adaptor, mitogen-activated protein kinase, and mammalian target of rapamycin activator 2 (Gao et al., 2019). These findings highlight the complex and multifaceted effects of heat stress on milk synthesis and the regulation of key genes involved in this process. Increased environmental temperatures pose a significant challenge to the milk synthetic capacity of the mammary gland. The global rise in temperature over the past decades and the projected future temperature increases have generated extensive research efforts to understand the detrimental effects of heat on milk production performance. However, despite the progress made, many questions remain unanswered, underscoring the pressing need for further research in this field.

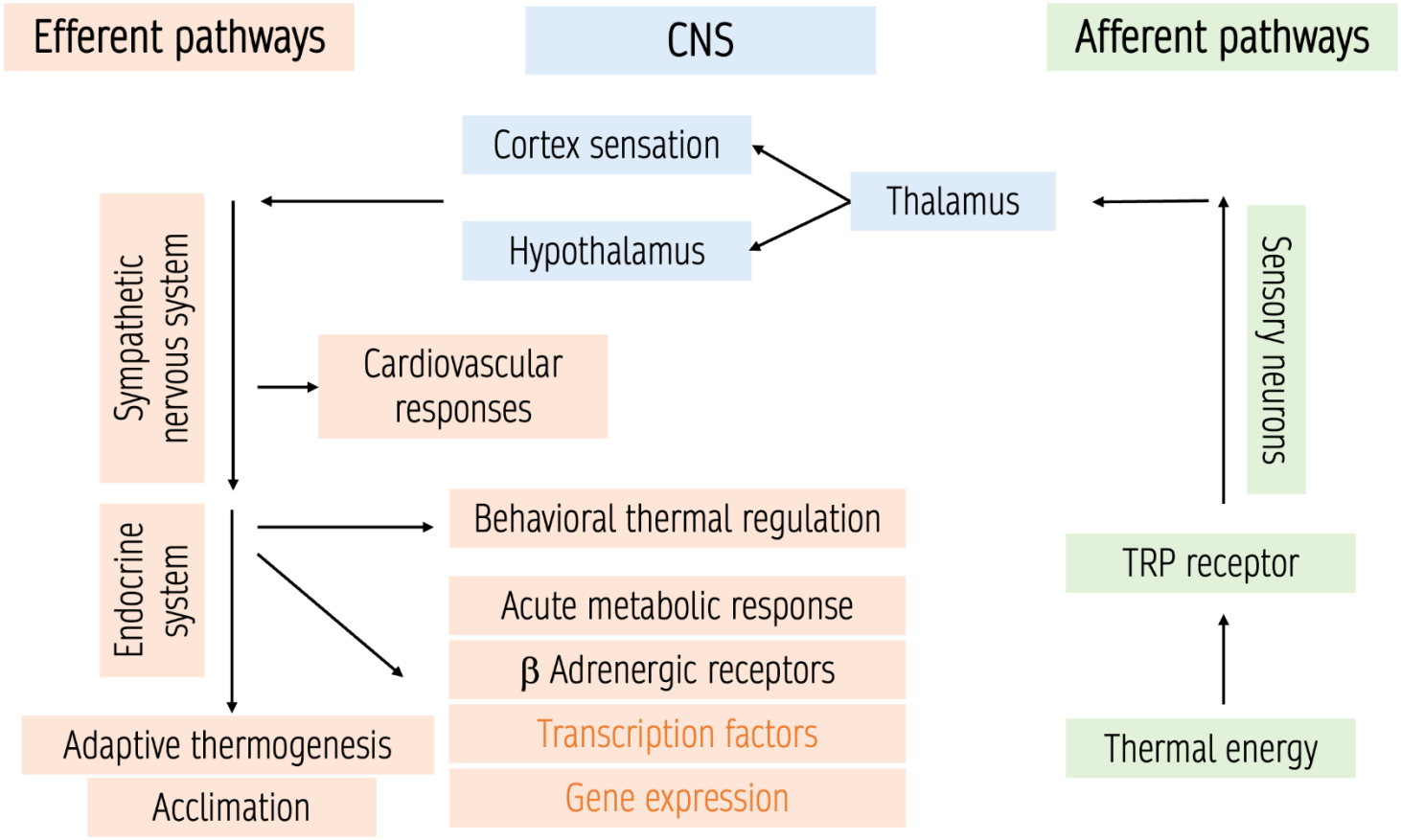

Figure 2.2. Physiological responses to heat stress. Abbreviations: TRP, transient receptor potential; CNS, central nervous system

Adapted from: Collier and Gebremedhin, 2015; Collier et al., 2017

MILK PRODUCTION AND ANATOMICAL UDDER CAPACITY CHANGES ON UDDER HALVES SUBJECTED TO INCREASED MILKING FREQUENCY AT TWO STAGES OF LACTATION[1]

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INTRODUCTION

Milking frequency is a tool used by dairy farmers to increase milk production efficiency and farm profitability. Since the end of the 20th century, the significance of milking frequency and its influence on the mammary gland as a positive local regulator of milk secretion has been well known. Several studies have demonstrated that increasing milking occurrences produce a local and abrupt increase in milk yield (**MY**) (Hale et al., 2003; Wall and McFadden, 2007a, 2012). Increased milking frequency (**IMF**) implementation for 21 d after calving results in a rise in milk production of 5.1 kg/d in udder halves milked 4× compared to 2× in multiparous cows. An improvement of 2.7 kg/d produced by the 4× udder halves remained throughout 180 DIM (Wall et al., 2013).

Since milk yield is modulated by milking frequency, it has a long-term effect on lactation persistency (Sorensen et al., 2008). Udder halves milked 4× from d 5 to 25 of lactation had an increased MY carryover effect of 1.56 kg/d compared to udder halves milked 2× through 300 DIM (Hanling et al., 2021). Potential mechanisms driving increased milk production with IMF have been examined in previous studies with inconsistent results (Hale et al., 2003; Wall and McFadden, 2010). Milk removal from the udder is a determining factor to stimulate milk secretion, consequently, milk removal enhances milk yield (Bruckmaier and Blum, 1996). Combined with this, the persistent response in milk yield across lactation suggests that IMF improves the productive capacity of the udder (Wall et al., 2013). In the bovine mammary gland, milk is distributed between two compartments, the gland cistern and the alveoli. It has been proposed that non-total removal of the cistern milk increases intramammary pressure, and consequently, milk secretion is compromised (Bruckmaier and Blum, 1996). As the interval between milkings increases, milk accumulation in the udder also increases. In this way, milking frequency reduction

negatively influences milk production and milk secretion rate. This has been demonstrated to occur as a function of reduced milking frequency and incomplete milking (Bernier-Dodier et al., 2010; Penry et al., 2017).

Milk yield response due to IMF implementation declines with time. The application of IMF more than once during lactation could possibly reestablish increased milk yield and enhance lactation persistency. Although the effects of IMF on MY at mid-lactation have been investigated (Bernier-Dodier et al., 2010; Toledo et al., 2020), to our knowledge, the combined effects of early and mid-lactation IMF implementation on milk, protein, and fat yield, and udder compartment capacities have not been explored. Therefore, the objective of this study was to characterize the effects of increased milking frequency at early and mid-lactation on milk, protein and fat yields and its association with changes in cistern and alveolar capacity in multiparous Holstein cows.

MATERIALS AND METHODS

Experimental design

The use of animals and all procedures for this investigation were approved by the Virginia Tech Institutional Animal Care and Use Committee (19-241). Fourteen mid-lactation, multiparous (2.5 ± 0.76 lactations) Holstein cows were used in the study. A planned sample size of 14 cows per treatment combination was based on the availability of cows at the Virginia Tech Dairy Center and supported by power calculations to identify significant differences for cistern and alveolar capacity using a 0.05 P-value with a power of 0.8 (SD = 1.19 kg for cistern and 1.80 kg for alveolar). Means used for power calculation were derived from MY records from the cows used in the study during previous lactations and the cistern and alveolar capacity proportions in Holstein cows reported in the literature. All cows enrolled in the experiment were healthy and without signs of clinical mastitis. At the start of the mid-lactation experiment the average milk yield was 22.5 kg/milking, fat concentration 3.3%, protein concentration 3.1 %, and somatic cell score (**SCS**) 2.28. The trial started at 139 DIM and finished at 290 DIM. Cows were housed in a free-stall barn at Virginia Tech's Kentland Farm throughout the experiment. Cows were fed ad libitum a complete total mixed ration once every morning and had free access to clean water. The trial was conducted from January to September 2021.

Increased milking frequency – Treatments

In this study, cows were subjected to IMF at the beginning of lactation for 21 days. Three days postpartum the udder half milk yield was measured in order to ensure even production between left and right udder halves. The milk yield difference threshold between udder halves used to enroll a cow in the experiment was set at 0.75 kg. The average MY difference between left and right udder halves at enrollment was 0.01 kg with MY average per milking of 12.9 kg. Cows selected to be part of the experiment were subjected to IMF, using the unilateral frequent milking

method, from d 3 to 24 of lactation. Left udder halves were milked 2×, and right halves were milked 4× on a 12 and 6 h interval at 0100 and 1300 h, and 0100, 0700, 1300, and 1900 h, respectively. After treatment completion, on d 25 of lactation, right udder halves were milked 2× until mid-lactation. Cows were randomly assigned to one of two treatments, Control and Repeated at 139 DIM. On day 150 of lactation, IMF treatment for the Repeated group was reimposed for 20 days, from 150 to 170 DIM. The Repeated group's (n = 7) left udder halves were milked 2× at a 12 h interval and right udder halves were milked 4× using an equidistant interval of 6 h. For the Control group (n = 7), left and right udder halves were milked at a 12 h interval 2×.

In order to compare yield differences between groups during the differential milking period and across lactation, milk samples and milk production from individual udder halves were collected on d 150, 170, 200, 230, 260, and 290 of lactation. For milk sample collection and milk weights, a Surge quarter milking claw was used to collect left and right udder half milk into two separate buckets. Milk samples (35 mL) were sent to the Lancaster Dairy Herd Improvement Association (Manheim, PA) and analyzed for milk fat and protein (Foss Milkoscan FT+, Foss North America). One cow was removed from the experiment at 250 DIM due to clinical mastitis. Samples at 260 and 290 DIM were not collected from this cow and data until this time were included in analyses.

Udder compartment capacity

In order to evaluate the effects of IMF on alveolar and cistern capacity from separate udder halves, the udder was completely filled, and capacity of each compartment was quantified ten days before and immediately after IMF treatment imposition (140 and 172 DIM). Davis et al. (1998) estimated that the udder reaches maximum capacity 25±3 h post milking. Based on this observation, and previous studies (Toledo et al., 2020), cows were not milked for 26 h to maximize

the milk volume in the alveoli and cisterns. Cows were milked at 1300 h on d 139 and 171 of lactation, and the compartment capacity measurements were completed at 1500 h the next day.

To facilitate and ensure effective intravenous drug administration, a temporary jugular catheter was placed one day before capacity assessment. The procedure involved the localization of the jugular vein, clipping and scrubbing the surgical area followed by the application of local anesthesia to the catheter site. An incision was made over the jugular vein, and a polyurethane catheter (MILA International, Inc., Florence, KY) was placed, sutured to the skin, and flushed with heparinized sterile saline solution. After catheterization, cows were moved to an individual pen with unrestricted access to water and feed.

To separate milk collection from the alveolar and cistern milk compartments, an oxytocin (**OT**) inhibitor (Atosiban; Adooq Bioscience, Irvine, California) was used at a previously reported effective dose of 50 µg/kg of BW (Wellnitz et al., 1999). Atosiban (**ATO**) was dissolved according to the manufacturer protocol in 10% v/v dimethyl sulfoxide (**DMSO**), 40% v/v polyethylene glycol 300 (**PEG300**), 5% v/v polyoxyethylene sorbitan monooleate (**Tween-80**), and 45% v/v saline solution to obtain a concentration of 1.7 mg/mL. Cows were weighed three times from January to May 2021. To ensure the amount of ATO administrated was adequate, body weight was measured within one month of udder capacity assessment.

To collect cistern milk, ATO was administrated in the jugular vein via the catheter. Directly after ATO administration (≤ 15 min), cows were moved to the milking parlor and milked until milk flow ceased using an individual quarter milking claw. Wellnitz et al. (1999) demonstrated that a dosage of 10 IU of oxytocin fully displaced ATO (50 µg/kg of BW) from the oxytocin receptors. To ensure complete ATO displacement to collect alveolar milk, 20 IU of oxytocin (OxoJect, Henry Schein Animal Health, Dublin, OH) was administered in the jugular catheter, and

cows were milked again. To separate individual milk yields of udder halves, separate buckets were used to collect the cistern and alveolar compartments. Cistern and alveolar milk yield after 26 h were assumed to reflect cistern and alveolar capacity. A catheter obstruction occurred in one cow prior to drug administration on day 140 DIM of udder compartment capacity evaluation; compartment capacity measurement was not assessed in this animal on that specific day.

Statistical Analysis

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, NC). Descriptive statistics were obtained using the UNIVARIATE procedure to test normality using the Shapiro-Wilk test. Statistical analysis for milk, protein, and fat yields, cumulative yields, and udder volume compartment capacity was performed using the GLIMMIX procedure of SAS. Significance was established at $P \leq 0.05$, and trends were established at $P \leq 0.10$. Data are expressed as least squares means ± standard error of the means.

Udder half difference yields were calculated by subtracting left half yield (2×) from right half yield (4×) for individual cows. The difference between udder halves from zero was statistically analyzed as the statistical conjecture. The model to estimate yield differences considered the effect of treatment (Control and Repeated), sampling day (150, 170, 200, 230, 260, and 290), and treatment by sampling day interaction as fixed effects. Cow nested within treatment was considered a random effect. Milk, protein, and fat yield differences between udder halves at day 150 were used as a covariate. The SLICE function was used to detect differences in least squares means between treatments on each sampling day. Cumulative yields of milk, fat, and protein were calculated using the trapezoidal rule, estimating the area under the curve for each response variable. Total cumulative udder half difference included d 150 to 290 of lactation, while cumulative carryover included d 200 to 290 of lactation. The model for cumulative yields included the effect of treatment as a fixed effect and cow nested within treatment as a random effect.

Volume and proportion of the alveolar and cistern capacity were evaluated using an individual model for each sampling day (140 and 172 DIM) and each udder compartment (alveolar and cistern). The model for the response variables at 140 DIM considered only the treatment imposed at the beginning of lactation (udder half milking frequency), while the model for the response variables at 172 DIM considered treatment imposition at the beginning of lactation as well as mid-lactation. The model for 140 DIM data included the fixed effect of milking frequency (2× and 4×). The model for the 172 DIM data included the fixed effects of treatment (Control and Repeated), milking frequency (2× and 4×), and treatment by milking frequency interaction. Cow was considered as a random effect in all models.

RESULTS

Milk and component yields

Udder half difference was calculated to evaluate the effects of IMF on milk and milk component yields (Figure 3.1). On day 150 of lactation, 126 d after early lactation IMF ended but before mid-lactation IMF treatment imposition, the Control and Repeat treatment group udder halves produced similar milk (0.57±0.43 vs. 0.45±0.43 kg; P = 0.20), protein (16.30±16.4 vs 11.70±16.4 g; P = 0.50), and fat yields (21.64± 24.19 vs. 48.77±24.19 g; P = 0.05). At 150 DIM milk, protein and fat yields for the left and right udder halves for the Control treatment were L: 10.9±0.8 kg and R: 11.5±0.9 kg, L: 331±29 g and R: 347±31 g, and L: 368±45 g and R: 390±52 g, respectively. For the Repeated group milk, protein and fat yields were L: 10.9±0.79 kg and R: 11.3±0.88 kg, L: 332±29 g and R: 344±31.2 g, and L: 360±45 g and R: 408±52 g, respectively. There was no significant milk yield difference across the experiment from 150 to 290 DIM between udder halves in the Control treatment which received IMF only at the beginning of lactation (0.65±0.28 kg), and the Repeated treatment udder halves that received IMF at early and mid-lactation (1.22±0.28 kg; P = 0.18). Milk yield difference between right and left udder halves tended to change across time from mid to late lactation (P = 0.07). The greatest differences between udder halves were observed on d 170 (1.60±0.30 kg) and lowest on d 150 (0.51±0.30 kg) regardless of treatment. Even though no significant differences were detected for the main effects of treatment and sampling day on udder half milk yield difference, a significant difference for treatment by DIM interaction was observed (Figure 3.1a; P = 0.02). After 20 d of mid-lactation IMF, the difference between udder halves milked with IMF at the beginning of lactation and at mid-lactation was 2.27 kg greater than the difference of udder halves milked 4× only during early lactation (0.46±0.43 vs. 2.73±0.43 kg). Immediately after IMF imposition, at 170 DIM milk yield in left and right udder halves for Control were L: 10.4±0.8 kg and R: 10.9±0.9 kg, while for the repeated

Repeated group were L: 10.6±0.8 kg and R: 13.3±0.9 kg. After IMF imposition from 150 to 170 DIM, the Repeated group udder halves milked 4× produced 0.49 kg, 0.33 kg, and 0.38 kg more milk than 2× udder halves at d 200, 230 and 260 of lactation (Figure 3.1a; $P < 0.05$). A trend was observed at d 290 of lactation, where udder halves milked 4× at mid-lactation produced 0.11±0.43 kg more milk compared to udder halves milked 2× per day ($P = 0.08$). As shown in Figure 3.1a, milk yield increased rapidly with IMF imposition at d 170 of lactation and decreased progressively after d 200 of lactation for Repeated treatment cows milked with IMF from d 3 to 24 and d 150 to 170 of lactation.

Udder half difference protein yield followed the same pattern as milk yield (Figure 3.1b). Protein yield difference between right and left udder halves was similar between Control (18.0±12.7 g) and Repeated treatments (39.6±12.7 g; $P = 0.44$) across the experiment from 150 to 290 DIM. However, a significant main effect of day ($P = 0.03$) was observed. Regardless of mid-lactation treatment, udder half protein yield differences across time were 14.0 g, 54.0 g, 27.6 g, 30.4 g, 21.7 g, and 25.0±11.8 g at 150, 170, 200, 230, 260 and 290 DIM, respectively ($P = 0.03$). Likewise, a significant interaction between treatment and DIM ($P = 0.01$) was observed. As presented in Figure 3.1b, the greatest differences between udder halves of Repeated and Control treatments were observed on d 170 (14.9±16.4 g for Control and 93.1±16.4 g for Repeated; $P < 0.05$). Protein yields for left and right udder halves for Control were 309±28 g and 324±31 g, while for Repeated were 320±29 g and 413±31 g. Fat yield was not affected by treatment ($P = 0.89$), sampling day ($P = 0.85$) or their interaction ($P = 0.69$). Fat yield difference between udder halves was comparable between Control (29.3±16.5 g) and Repeated (25.9±16.5 g) treatments (Figure 3.1c).

The effects of IMF at mid-lactation did not impact milk yield improvement continuously. Milk yield improved during the differential milking frequency period, from d 150 to 170 of lactation, for the Repeated group and declined gradually after mid-lactation IMF imposition till d 290 of lactation (Figure 3.1a). In order to quantify the average milk, protein, and fat yield per treatment across the experimental period, area under the curve was calculated using the trapezoidal rule for cumulative (150 to 290 DIM) and carryover yield (200 to 290 DIM) and is presented in Table 3.1. Total udder half milk yield difference from udder halves milked at IMF at two stages of lactation was not different from udder halves milked at IMF only at the beginning of the lactation ($P = 0.66$). Cumulative protein yield differences ($P = 0.64$) and fat yield difference ($P = 0.90$) were not different between treatments across the entire experimental period. To quantify the effect of mid-lactation IMF on lactation persistency, milk, protein, and fat yield difference from d 200 till 290 of lactation were used to evaluate treatment differences. Milk, fat, and protein yield carryover differences between Control and Repeated udder halves did not differ significantly from 200 to 290 DIM ($P > 0.80$).

Cistern and alveolar capacity

Cows were subjected to IMF at the onset of lactation with left udder halves milked 2× and right udder halves milked 4× from d 3 to 24 of lactation. On day 25, cows were returned to the standard farm milking routine with a 12 h interval. Subsequently, the cistern and alveolar milk yield capacity was assessed on day 140 of lactation to evaluate the effect of IMF treatment on compartment capacity (Figure 3.2). Alveolar milk yield did not differ between udder halves milked 2× (8.5±0.75 kg) or 4× (8.7±0.75 kg; Figure 3.2a; $P = 0.33$). Contrastingly, cistern milk yield was greater (8.3±1.31 kg) for udder halves milked 4× compared to udder halves milked 2× per day (7.3±1.31 kg; Figure 3.2b; $P < 0.01$). Totaling the cistern and alveolar yields from each udder half

at d 140 revealed that udder halves milked 2× held less milk (15.8±1.25 kg) compared to the udder halves milked 4× at the beginning of the lactation (16.9±1.25 kg; P < 0.01; Figure 3.2c).

On day 172 of the lactation, after a 20 d period of IMF imposition at mid-lactation for the Repeated treatment, individual udder compartment capacity was assessed once more in both groups. Alveolar capacity was not affected by treatment (10.3±1.11 vs. 8.2±1.11 kg; $P = 0.19$), udder half (9.1±0.81 vs. 9.3±0.81 kg; $P = 0.60$) or its interaction (Figure 3.3a; $P = 0.99$). Unlike the findings for the alveolar compartment, cistern milk yield differed between treatments (Figure 3.3b; $P = 0.04$). Cisterns in the Repeated group (8.2±1.02 kg) held 4.8 kg more milk compared to the Control group (3.3±1.02 kg) after mid-lactation IMF imposition for 20 days. However, the effect of milking frequency reimposition between udder halves was not significant for cistern capacity ($P = 0.49$). Whole udder capacity (Figure 3.3c) at 172 DIM showed no differences between treatment ($P = 0.50$), udder half ($P = 0.46$) or its interaction ($P = 0.95$).

To estimate the relative amount of milk produced for each udder compartment despite milk yield differences by udder halves, the proportional volume of the alveolar and cistern compartments were evaluated. Within each individual udder half, alveolar or cistern milk yield was divided by the total udder half milk yield (alveolar + cistern). At 140 DIM, before mid-lactation IMF, udder halves milked 2× in early lactation tended to have greater alveolar proportion (0.57±0.05 vs. 0.54±0.05; Figure 3.4a) and smaller cistern proportion (0.43±0.05 vs. 0.46±0.05; Figure 3.4b) than udder halves milked 4× ($P = 0.09$). To delineate the effects of IMF treatment at mid-lactation, udder half alveolar (Figure 3.4c) and cistern (Figure 3.4d) proportion at d 172 of lactation were analyzed. Treatment had a significant effect on alveolar and cistern proportions ($P = 0.03$). Alveolar proportion for the Control group (0.68±0.05) was greater than the Repeated group (0.50±0.05) regardless of the udder half (Figure 3.4c). As expected, the cistern proportion

was smaller in the Control group cows (0.32±0.05) and larger in the Repeated group cows regardless of udder half (0.50±0.05; Figure 3.4d). Neither udder half (P = 0.37) nor the treatment by udder half interaction (P = 0.65) had a significant effect on the alveolar and cistern proportion.

As an alternative data presentation for clarity and visualization, the ratio of cistern to alveolar volume was assessed before and after IMF imposition for 20 d at mid-lactation (Figure 3.5). On day 140 of lactation, the cistern to alveolar ratio was similar between udder halves milked 2× (1.00±0.2) and 4× (1.08±0.2) at the beginning of the lactation (Figure 3.5a). Although no statistical differences were observed between udder halves, the range of the ratio was dispersed equally in both udder halves. After IMF imposition at mid-lactation till 170 DIM, treatment had a significant effect on capacity at 172 DIM (P = 0.03). Cows in the Repeated group (1.24±0.22) had a greater cistern to alveolar volume ratio compared to the cows in the Control group (0.49±0.22). We did not observe an effect of udder half (P = 0.72) or treatment by half udder interaction (P = 0.82; Figure 3.5b).

DISCUSSION

This study evaluated the effects of IMF at two stages of lactation on milk, fat, and protein yields and its association with udder anatomical compartment capacity in multiparous Holstein cows. The effects of IMF on milk yield and lactation persistency have been studied since the 1950s (Lush and Shrode, 1950; Pearson et al., 1979; Allen et al., 1986). Dahl et al. (2004) reported that cows milked 6× during the first 21 d of lactation produce 8.4 kg more summit milk than cows milked 3×. Also, IMF enhanced MY after IMF treatment withdraw, with cows milked 6× at the beginning of the lactation, producing 1,118 kg more MY than cows milked 3× over 305 d lactation. Similarly, Hale and collaborators (2003) evaluated the effects of 2×, 4×, and the combination of 2× (d 1 to 3) and 4× (d 4 to 21) IMF during the first 21 d postpartum in multiparous Holstein cows and found that cows milked 4× and 2×4× produced 8.8 and 4.8 kg more MY than cows milked 2× alone. Milk yield increase response in this study persisted 70 d and tended to remain up to 40 weeks after IMF imposition with cows milked 4× producing 4.6 kg and 3.6 kg more MY than cows milked 2×, respectively.

In the last decades, the use of the unilateral frequent milking model has improved IMF studies by removing the variation between cows and exposing udder halves to the same systemic factors (Wall and McFadden, 2007b; Weaver and Hernandez, 2016). Previous IMF studies focused on exploring its impact only during early lactation. To the best knowledge of the authors, only a few recent studies have specifically evaluated the effect of IMF at mid-lactation. Bernier-Dodier et al. (2010) compared fore and hind left and right quarters milked one and three times per day during eight weeks using Holstein cows averaging 169 DIM. The authors observed a 2.5 fold increase in milk yield in quarters milked 3× compared to 1× quarters in the last week of IMF imposition. After the differential milking period, MY enhancement continued for 6 weeks with udder quarters milked three times per day producing 1.6 fold more milk than quarters milked once

per day. The use of once daily milking frequency during a short period, after a standard 2× milking frequency, stands out in this study and might alter the effects exclusively produced by IMF. Multiple studies have demonstrated that an immediate reduction in milking frequency produces a swift milk yield reduction regardless of duration (Stelwagen et al., 2013).

When IMF is imposed during early lactation, an increase in milk production of 2.80 kg/d and 4.78 kg/d was observed in udder halves milked 4× compared to 2× in primiparous and multiparous Holstein cows (Wright et al., 2013; Hanling et al., 2021). The results of the aforementioned studies agree with the findings reported in the present investigation where MY increased by 2.27 kg/milking in udder halves milked at IMF at early and at mid-lactation. This estimate considers only the yield from one-half udder at a single milking. In a traditional farm setting, where cows are milked 2×, an increase of 9.1 kg/d would be expected from the whole udder when milking frequency is doubled at early and mid-lactation. Combined with the increase in milk yield observed during the IMF phase treatment, a milk yield enhancement of 1.52 kg/d (0.38 kg per udder half per milking) in udder halves milked 4 times per day at early and mid-lactation remained through 260 DIM, which agrees with previous reports for Holstein cows and udder halves that maintained a milk production enhancement of 0.8 kg/d through 270 DIM (Wright et al., 2013) and 1.56 kg/ through 300 DIM (Hanling et al., 2021) after IMF implementation at early lactation. The estimates obtained in this study reflect the productive and potentially profitable benefits of IMF implementation at early and mid-lactation in multiparous Holstein cows which are enhanced when a carryover effect prevails. The possibility that less milk removal will induce an increase in somatic cells that will produce a decrease in MY in the 2× udder halves was considered. However, there were no significant differences ($P = 0.9$) in SCS between the left and right udder halves of the Control (2.4±0.7 vs 2.02±0.7) and Repeated (2.4±0.7 vs. 2.9±0.7) groups at d 150.

The same was observed after IMF implementation for 20 d on the left and right udder halves for Control (2.9±0.7 vs. 2.05±0.7) and Repeated (1.9±0.7 vs. 1.5±0.7; $P = 0.8$).

In this study, the yield of milk components fluctuated across the experimental period. As expected, protein yield increased 78.2 g per udder half per milking after 20 days of 4× IMF. However, no differences in fat yield were observed. The change in protein and fat yields and percentages during and after increased milking frequency treatment varies between studies. Bernier-Dodier et al. (2010) reported that protein percentage was unaffected by IMF implementation, while fat concentrations were higher in udder quarters milked 1× compared to 3× udder quarters after 8 weeks of IMF treatment. Opposing this, in the same study, the authors observed that protein and fat yields were enhanced during and after 3× differential milking. However, other studies report no differences in protein percentage due to IMF implementation (Toledo et al., 2020). Fat concentration, in agreement with other studies, can be negatively affected by IMF. Fat content declines in udder halves milked at 3× increased milking frequency at mid-lactation compared to udder halves milked once per day (Toledo et al., 2020).

The persistent effect of milk and milk component yield enhancement by IMF can remain for a period past the increased milking period and even throughout the entire lactation (Hale et al., 2003; Connor et al., 2008). However, this effect does not consistently persist. Contrasting with previous reports, in this study milk, protein, and fat yields were significantly improved by IMF treatment only during the differential milking frequency period. Milk, protein, and fat yield udder half difference during the cumulative (days 150-290) and cumulative carryover periods (days 200-290) did not differ between treatments. However, the comparison tested in our study only evaluated the carryover and cumulative udder half difference between udder halves subjected to IMF at the beginning of the lactation vs. udder halves subjected to IMF at early and mid-lactation.

In this study, there were no udder half differences before early or mid-lactation treatment imposition for any of the response variables evaluated preventing confounding treatment effects. The lack of difference between cumulative and carryover milk yield difference between treatments suggests that IMF implementation solely for 20 d at the beginning of lactation has a greater impact on lactation persistency than when implemented at mid-lactation. The more noticeable increase in milk yield at the beginning of the lactation perhaps occurred by the stimulation of secretory mammary epithelial cell (**MEC**) number and activity.

Mammary epithelial cell number and activity are established factors defining milk synthesis capacity of the bovine mammary gland and lactation persistency (Capuco et al., 2001, 2003). A different response of the factors defining milk synthesis to IMF at different lactation stages can be the basis shaping lactation persistency. As proposed by Wall and McFadden (2012), the mammary gland is especially sensitive during early lactation, which influences the shape of the lactation curve. A foundational study by Capuco et al. (2001) proposed that the increase in MY at the beginning of the lactation occurs due to an increase in the synthetic capacity of the MEC. It has been demonstrated in rodents and ruminants that frequency of milk removal and suckling stimuli are major factors influencing MEC number and activity (Hadsell et al., 2007; Wall and McFadden, 2008). The effects of IMF on MEC number and cell activity have been evaluated by various researchers with variable results (Hale et al., 2003; Nørgaard et al., 2005b; Wall et al., 2006; Alex et al., 2015). The dearth of definite results has opened the window to consideration of other factors affecting milk production. Although cell number and cell activity were not evaluated in this study, udder capacity might be intrinsically related with these MEC features.

Within the udder, milk is distributed between two anatomical compartments, the gland cistern and alveoli. In the present study, the alveolar and cistern capacity varied greatly between

individuals. In general, the alveolar and cistern proportion of cows used in this experiment were minimally 26% and 17% for alveolar and cistern volumes and maximally 83% and 74% for alveolar and cistern capacity. The alveolar proportion averaged 57% and the cistern proportion averaged 43% regardless of treatment. The averaged proportions observed in our study, concurs with previous reports. It has been estimated that before milking 60% of the milk synthesized by the udder is held in the secretory tissue and 40% in the cistern (Nickerson and Akers, 2011). Likewise, Davis et al., (1998) estimated 64% alveolar and 46% cistern milk proportion after a 24 h milking interval. Other authors have reported percentages ranging from 74% to 80% and 26 to 20% for alveolar and cistern compartments, respectively (Bruckmaier et al., 1994; Knight et al., 1994; Ayadi et al., 2003a; Caja et al., 2004). Compared with these studies, cistern estimates observed in the present study are greater than previously reported. However, the studies which report smaller cistern proportion were completed in Europe. The existence of anatomical differences between Holstein populations from different hemispheres due to genetic or management differences is plausible. Also, the differences observed between cistern and alveolar percentages can be explained due to the use of cows at different lactation stages. Alveolar and cistern capacity decrease throughout the lactation. It has been estimated, using ultrasonography, that alveolar milk volume is reduced by 68% between mid and late lactation, and cistern milk volume is reduced by 49% between early and mid-lactation (Caja et al., 2004).

Between milking sessions, uninterrupted milk synthesis occurs in the mammary epithelial cells. Synthesized milk is distributed in each udder compartment in a distinct manner. Shortly after milking, a small volume of the cistern compartment is filled with residual milk. As time passes, milk synthesis continues within the secretory tissue. As milk accumulates in the alveolar luminal space, the pressure on the epithelial lining causes the secretory cells to compress (Nickerson and

Akers, 2011). Between 1 and 4 hours after milking, a significant pressure upsurge occurs increasing flow of milk from the alveoli into the teat and gland cisterns which increases mammary gland pressure (Schmidt, 1971; Knight et al., 1994). Maximum alveoli fill occurs 25±3 h post milking (Davis et al., 1998) while cisternal milk volume plateaus 20 h after last milking (Ayadi et al., 2003b). Milk secretion rate depends on the pressure accumulation of milk within the mammary gland. When milk accumulates within the mammary gland for a long enough period of time, pressure builds to a sufficient level to inhibit secretion and milk can be resorbed into the blood (Schmidt, 1971). Frequent milking appears to relieve the pressure within the mammary gland. Consequently, milk removal is a critical factor to increase milk production (Knight et al., 1998) with potential effects on MEC activity and number (Wall and McFadden, 2008).Based on studies indicating the potential IMF effects on MEC number (Hale et al., 2003), we hypothesized that an increase in MEC number would be reflected in an increased alveolar volume explaining the MY enhancement observed during IMF imposition. In opposition to this, we observed that IMF imposition for 20 d at the beginning and middle of lactation did not alter the alveolar volume independent of udder half milking frequency. However, the Repeated group held 4.9 kg more cistern milk than the Control group even though IMF was imposed exclusively in the half udder of the cows. Likewise, udder halves milked 4× at the beginning of lactation held 0.97 kg more milk at 140 DIM. These results together indicate that IMF might impact cistern capacity for an extended period after its implementation. In small ruminants, differences between udder compartments indicate greater daily milk yields. It has been reported that Lacaune sheep had greater milk yields, doubled cistern milk volume, and similar alveolar milk volume compared with Manchega sheep (Rovai et al., 1999) suggesting that morphological characteristics, in particular cistern capacity, substantially influence lactation performance (Nickerson and Akers, 2011).

Likewise, morphological traits such as udder width and height have been positively correlated with milk yield, suggesting the importance of udder volume to milk yield (Nickerson and Akers, 2011). However, in this study we did not find a significant correlation between compartment capacity and milk yield (not shown).

Notably in this study, left and right udder halves had increased cistern capacity in the Repeated treatment group at 172 DIM. This was unexpected as the 4× IMF udder halves produced more milk than 2× at the end of the mid-lactation IMF period. Also, at 140 DIM, prior to mid-lactation IMF, the effect of IMF on cistern volume was apparent. This response suggests that the single act of going to the parlor at a higher frequency influenced udder capacity perhaps by a systemic effect in the gland rather than a localized effect in each half udder. The milking routine triggers the production and systemic secretion of multiple hormones including oxytocin. Oxytocin is released into the bloodstream by a neuroendocrine reflex and acts directly on the myoepithelial cells that surround the secretory MEC of the alveoli and the finer ducts. Oxytocin binding to myoepithelial OT receptors causes the contraction of these cells surrounding the alveoli and milk ducts releasing alveolar milk to the ducts and progressively to the cisternal space (Soloff, 1982; Bruckmaier and Blum, 1996). An oxytocin receptor antagonist, such as ATO, blocks this process allowing for exclusive cistern and alveolar milk harvesting. Using this approach, we evaluated changes in cistern and alveolar capacity after shortening milking interval in udder halves milked 2× and 4× during early and mid-lactation. Despite that, the possibility that ATO effects were overcome, reflected by a greater cistern capacity independent of treatment, prevails. Due to the systemic nature of OT synthesis and action, possibly stimulated during the IMF period, OT acts on both udder halves independent of treatment causing milk ejection to the cistern and causing maximum pressure within the cistern which could produce an enlargement of the cisternal volume

as a response. Bruckmaier and Blum, (1992) demonstrated that OT injection ($2.0xl0^{-3}$ i.u./kg BW) induced spontaneous enlargement of the gland cistern volume and pressure of cows, goats, and sheep.

So far, it has not been investigated if an acoustic or habituation response producing an increase in OT occurs in cows milked at an increased milking frequency as these cows go to the parlor more frequently. Additionally, OT release has been associated with the habituation of physiological responses (Kéri and Kiss, 2011), but little is known about the association between OT and habituation in animals (Sutherland and Tops, 2014). Alternative explanations can be based on the fact that the increase in OT level in the dairy cow and consequent milk ejection is an innate reflex that occurs in response to a neuroendocrine loop produced by tactile stimulation of the neural receptors located in the tip of the teat (Crowley and Armstrong, 1992; Bruckmaier and Blum, 1998). The tactile stimulus of pre-milking and udder cleaning is sufficient to induce alveolar milk ejection (Bruckmaier and Blum, 1998). However, other stimuli like visual and olfactory stimulations, present before or during milking time, are suitable to produce milk ejection due to OT release contributing to the OT concentration at systemic level. Cleverley and Folley (1970) observed that conditional stimuli including auditory and visual stimuli associated with the milking routine caused oxytocin release by conditional reflex in dairy cows. Coupled with this, it has been reported that OT administration in rabbit mammary gland tissue accelerates intracellular transit of caseins in mammary epithelial cells, increasing the secretory process by stimulating the intracellular transport of newly synthesized proteins. The emptying of the mammary epithelial cells might avoid negative feedback of accumulation of the milk constituents stimulating the synthesis of new milk (Lollivier et al., 2006b). Based on this premise, we propose that IMF stimulates systemic OT production through conditional stimuli associated with the milking routine.

This stimulation leads to a more thorough emptying of the alveoli that might have increased the volume or the elasticity of the cistern compartment, particularly in the Repeated group. However, in this study, the oxytocin levels before and after increased milking frequency treatment were not evaluated, and little is known about changes in udder compartment elasticity.

CONCLUSION

In this study, we demonstrated that implementing IMF at early and mid-lactation for 20 and 21 d in multiparous Holstein cows influenced the cistern capacity of the mammary gland while the alveolar capacity remained unaffected. The effect on udder compartment observed in this study occurred possibly due to a systemic effect induced by IMF. We acknowledge the possible systemic effect, which may have altered the efficacy of quantifying alveolar and cistern capacity. The absence of studies focused on udder compartment capacity, udder elasticity, and behavioral and stimulatory oxytocin demonstrates the necessity for future studies focused on changes occurring in the udder when IMF is implemented. Specifically, studies focused on how removing milk within the secretory or storage tissue of the bovine mammary gland can stimulate MEC number and activity enhancing milk yield as ultimate response.

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TABLES AND FIGURES

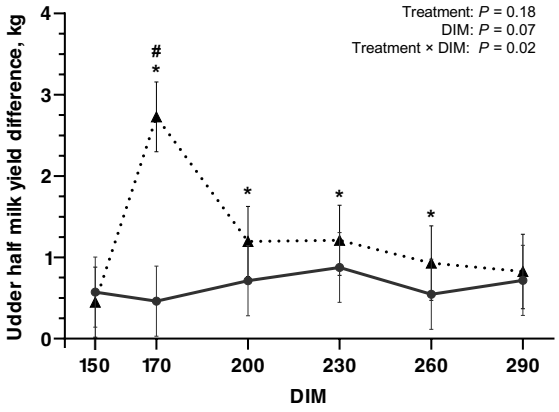
Table 3.1. Milk, protein, and fat yield udder half differences1 between left and right udder halves milked 2× and 4× during early and mid-lactation for 20 d after treatment imposition during cumulative (150 to 290 DIM) and carryover period (200 to 290 DIM)

	Cumulative yield			Carryover yield		
	Control[2]	**Repeated[3]**	***P***	**Control**	**Repeated**	***P***
Milk, kg	130±54	163±54	0.66	79±52	74±52	0.94
Protein, kg	3.5±2.3	5.0±2.3	0.64	2.3±1.6	2.3±1.6	1.0
Fat, kg	3.5±2.5	3.9±2.5	0.90	2.9±1.7	2.4±1.7	0.83

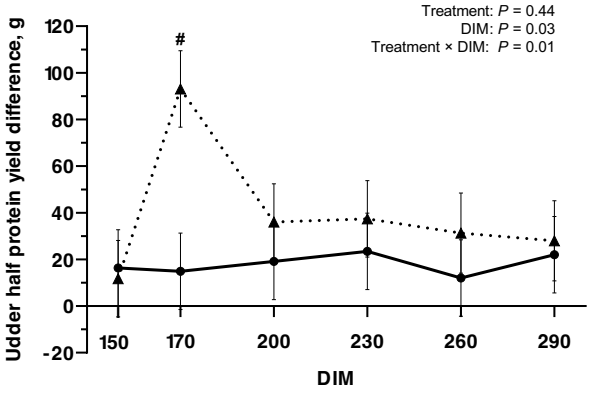
[1]Udder half differences were calculated by subtracting left half yield from right half yield.
[2]Control group right udder halves milked 4× at the beginning of the lactation and 2× milked at mid-lactation, left udder halves were milked 2× in both lactation stages.
[3]Repeated group right udder halves were milked 4× at the beginning of lactation and 4× milked at mid-lactation, left udder halves were milked 2× at both lactation stages.

Figure 3.1. Milk (a), protein (b), and fat (c) yield difference from left and right udder halves milked at 12 and 6 h milking interval. The dotted line represents the Repeated group (right udder halves milked 4× at the beginning of lactation and milked 4× at mid-lactation, left udder halves were milked 2× at both lactation stages). The solid line represents the Control group (right udder halves milked 4× at the beginning of lactation and milked 2× at mid-lactation, left udder halves were milked 2× in both lactation stages). Number sign represents statistical differences for the treatment by day interaction ($P < 0.05$); asterisk represents the differences between udders halves from zero that was tested as the statistical conjecture ($P < 0.05$).

a.


Treatment: P = 0.18
DIM: P = 0.07
Treatment × DIM: P = 0.02
Udder half milk yield difference, kg
DIM
#
*
*
*
*
0
1
2
3
4
150
170
200
230
260
290

b.


Treatment: P = 0.44
DIM: P = 0.03
Treatment × DIM: P = 0.01
Udder half protein yield difference, g
DIM
#
-20
0
20
40
60
80
100
120
150
170
200
230
260
290

c.

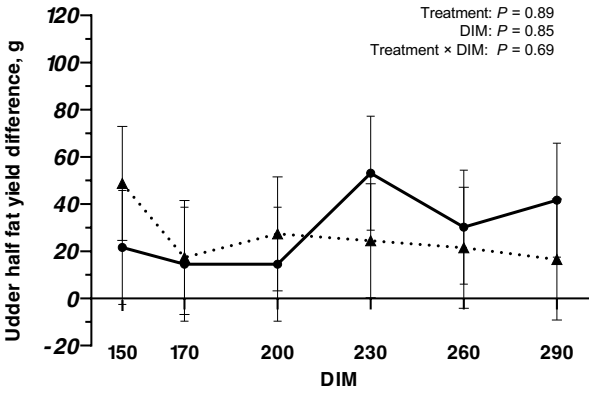

Treatment: P = 0.89
DIM: P = 0.85
Treatment × DIM: P = 0.69
Udder half fat yield difference, g
DIM
-20
0
20
40
60
80
100
120
150
170
200
230
260
290

Figure 3.2. Alveolar (a), cistern (b), and whole udder (c) milk holding capacity of udder halves milked 2× and 4× times from 3 to 24 DIM. Measurements were completed on day 140 of lactation. Asterisk represents statistical difference between treatment effects ($P < 0.05$).

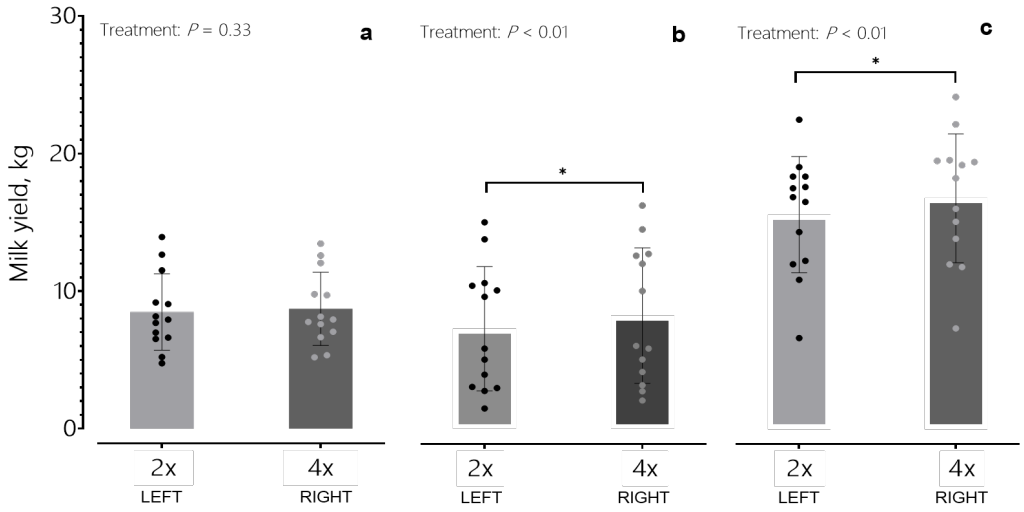


Figure 3.3. Alveolar (a), cistern (b), and total udder (c) milk yield capacity of left and right udder halves milked 2× and 4× times per day for 21 days at the beginning of the lactation. Left (2×2×) and right udder halves (4×2×) in the Control treatment were milked 2× from d 150 to 170 of the lactation. In the Repeated treatment, left udder halves (2×2×) were milked 2×, while right udder halves (4×4×) were milked 4×, from d 150 to d 170 of the lactation. Measurements were completed at 172 DIM. Asterisk represents statistical difference between treatment effects ($P < 0.05$).

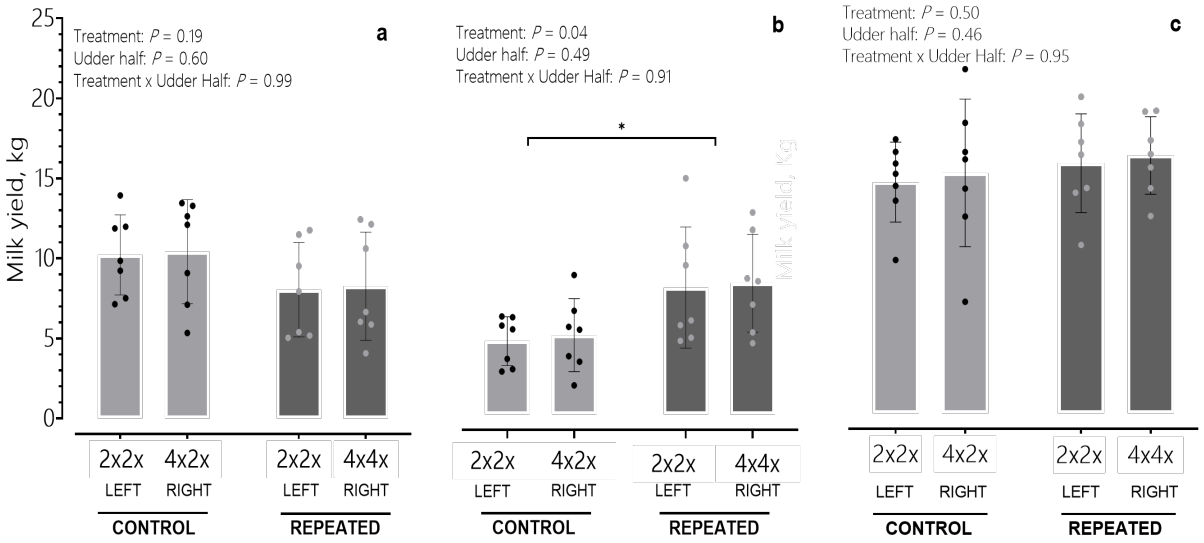


Figure 3.4. Alveolar (a and c) and cistern (b and d) proportion of udder halves subjected to IMF for 21 days at the beginning of the lactation assessed at 140 (a and b) and 172 DIM (c and d). Left and right udder halves in the Control group were milked 2× from d 150 to 170 of the lactation. In the Repeated group, left udder halves were milked 2× and right udder halves were milked 4×, from d 150 to d 170 of the lactation. The proportion was calculated by dividing the milk yield from each compartment within each half udder by the total milk yield (cistern and alveolar) from each half udder. Asterisk represents the statistical difference between treatment effects ($P < 0.05$).

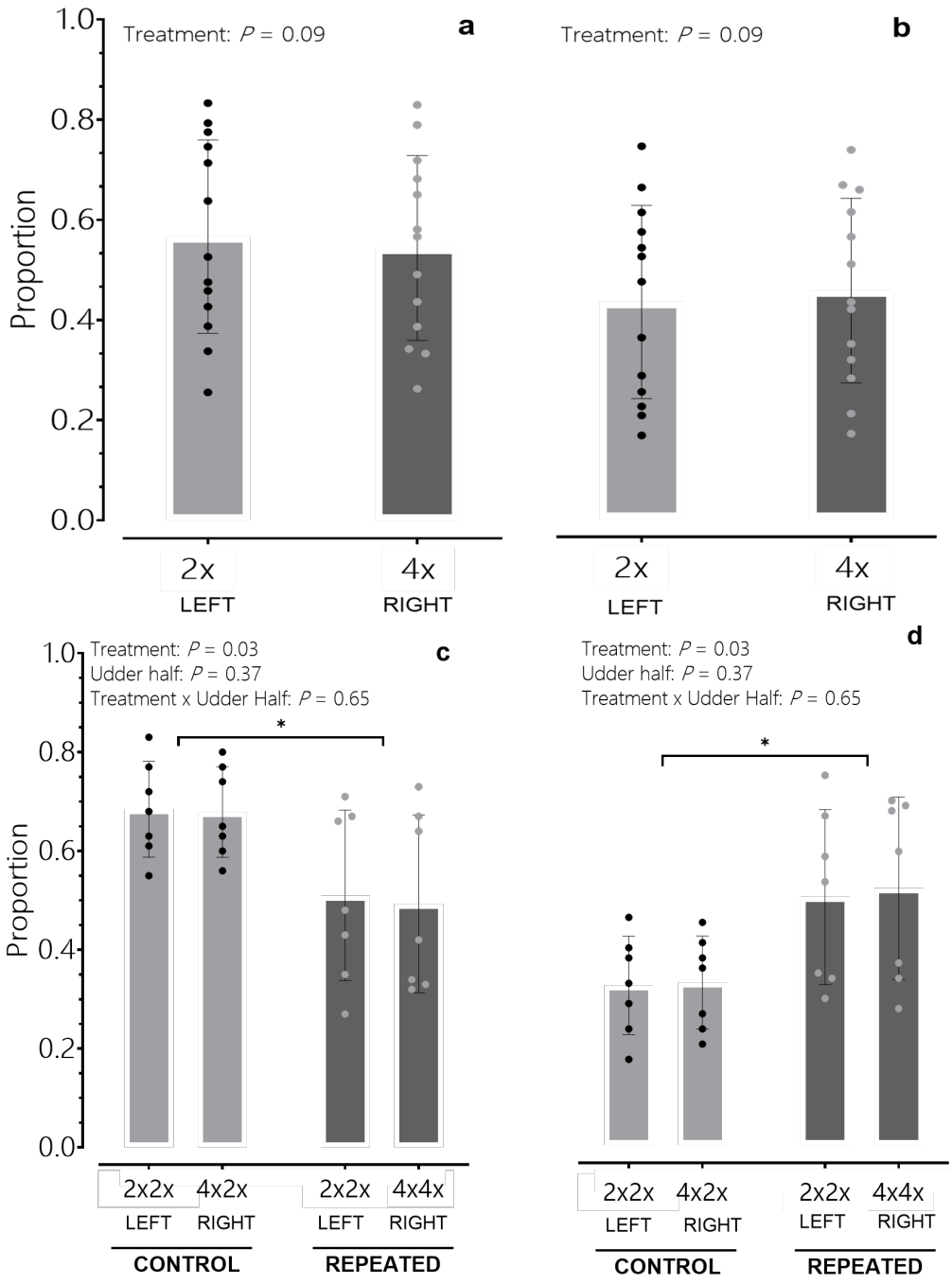
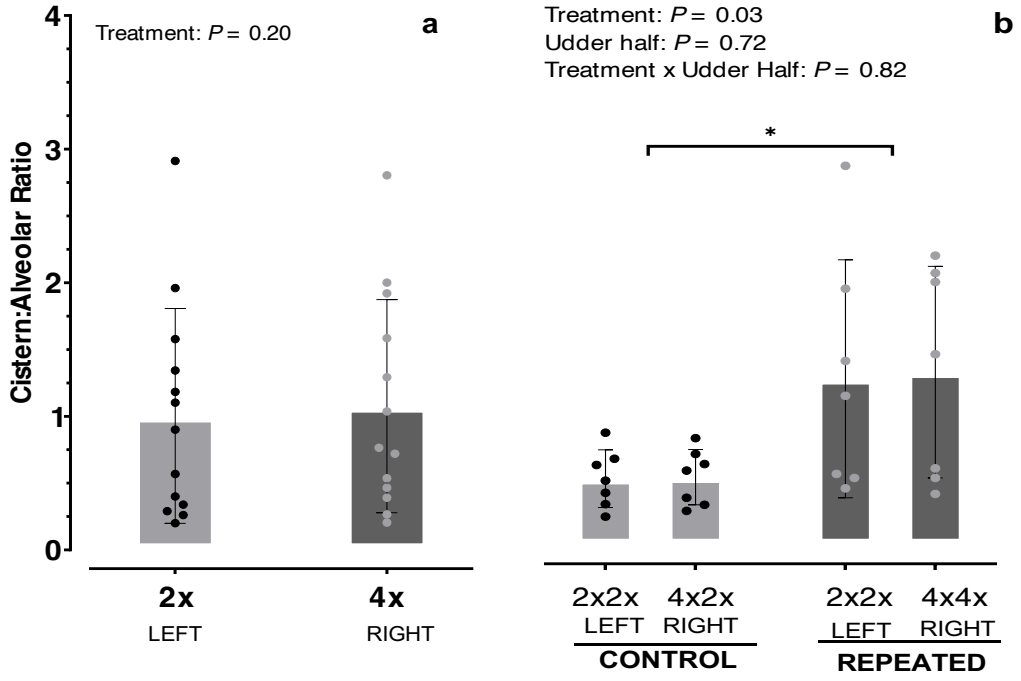

a
Treatment: P = 0.09
Proportion
1.0
0.8
0.6
0.4
0.2
0.0
2x
4x
LEFT
RIGHT
b
Treatment: P = 0.09
2x
4x
LEFT
RIGHT
c
Treatment: P = 0.03
Udder half: P = 0.37
Treatment x Udder Half: P = 0.65
*
2x2x
4x2x
2x2x
4x4x
LEFT
RIGHT
LEFT
RIGHT
CONTROL
REPEATED
d
Treatment: P = 0.03
Udder half: P = 0.37
Treatment x Udder Half: P = 0.65
*
2x2x
4x2x
2x2x
4x4x
LEFT
RIGHT
LEFT
RIGHT
CONTROL
REPEATED

Figure 3.5. Cistern to Alveolar ratio at 140 (a) and 172 (b) DIM. The ratio was calculated by dividing the milk yields from the cisternal compartment between the alveolar milk yields for each half udder. Left udder halves were milked 2× and right udder halves were milked 4× from 3 to 24 DIM, and evaluated at 140 DIM. Left (2×2×) and right udder halves (4×2×) in the Control treatment were milked 2× from d 150 to 170 of the lactation. In the Repeated treatment, left udder halves (2×2×) were milked 2×, while right udder halves (4×4×) were milked 4×, from d 150 to d 170 of the lactation, and evaluated at 172 DIM. Asterisk represents the statistical difference between treatment effects ($P < 0.05$).



CYCLICAL HEAT STRESS DURING LACTATION INFLUENCES THE MICROSTRUCTURE OF THE MAMMARY GLAND

INTRODUCTION

Heat is an environmental threat to dairy farms. Dairy cows are homeothermic animals that express their maximum genetic merit and production capacity within a demarcated thermoneutral zone. The thermoneutral zone is the range in ambient temperature where temperature regulation is achieved by the organism to maintain physiological homeostasis through dry heat loss without regulatory changes in metabolic heat production (Blatteis et al., 2001). However, temperature is not the only factor determining the apparent or perceived temperature. Perceived temperature results from combined meteorological factors such as air temperature, relative humidity, and wind speed, and is reflected by the temperature and humidity index **(THI)** with an inherent THI limit that high-producing dairy cows can tolerate. In dairy cows, the THI threshold is about 68 (Ravagnolo et al., 2000; Zimbelman et al., 2011; Fabris et al., 2019), and the initiation of a heat stress **(HS)** response in the cow is triggered when this THI threshold is exceeded.

The remarkable economic (St-Pierre et al., 2003; Key et al., 2014) and physiological effects of heat stress in livestock and dairy cattle have been broadly studied in the last decade (Zimbelman et al., 2009; Wheelock et al., 2010; Baumgard and Rhoads, 2013). One of the major HS responses described in dairy cattle is milk yield reduction and milk component alteration. The use of pair-feeding studies has shown that around 50% of the milk production reduction during HS occurs due to the associated decrease in daily dry matter intake (**DMI**; Cowley et al., 2015; Gao et al., 2017; Wheelock et al., 2010). Nevertheless, the remaining causative mechanisms of milk yield deterioration during HS independent of DMI reduction have not been fully elucidated. The mammary gland produces milk through secretory mammary epithelial cells. The number and activity level of epithelial cells are determining factors of synthetic and secretory abilities within each mammary gland, consequently, milk production (Capuco et al., 2001, 2003). Potential effects

on metabolic and physiological alterations of MEC that impact intracellular signaling pathways involved in productivity have been proposed. These effects have been evaluated using in vitro (Collier et al., 2008) and in vivo models (Silanikove et al., 2009; Baumgard and Rhoads, 2012). Several studies have explored HS effects on the mammary gland during the dry period (Tao et al., 2011; Dado-Senn et al., 2019; Fabris et al., 2020), however, the effects of HS on the bovine mammary gland during lactation, beyond noting lowered milk production and alterations to composition, have not been extensively characterized. We hypothesized that HS specifically alters mammary epithelial cell numbers and cell losses into milk and, more generally, affects mammary cellular activity. Therefore, using a pair-feeding model the objective of this study was to evaluate the impact of HS on the mammary gland related to cell number and activity and cell losses in milk of lactating Holstein cows.

MATERIALS AND METHODS

Animals, experimental design, and treatments

The use of animals and all procedures for this investigation were approved by the Virginia Tech Institutional Animal Care and Use Committee (19-247). Sixteen lactating multiparous Holstein cows (100 ± 14 DIM; 632±12 kg BW) were used in the study. All cows enrolled in the experiment were apparently healthy. Cows were housed in individual climate-controlled rooms at the Metabolic Research Laboratory at the Virginia Tech Dairy Farm (Blacksburg, VA) throughout the experiment. Upon arrival at the climate-controlled facilities, cows were given an adaptation period where no treatments were applied of 4 days for HS cows and 5 days for TN cows. During the adaptation period, one cow from the HS treatment had to be removed from the experiment due to health problems unrelated to the trial. During this time, the THI was set to 64 for both groups, and the cows had ad libitum access to water and feed. MY and DMI were recorded during this time to monitor health status and cow adaptation response, but no data are included in the experiment analyses. The trial was conducted from May to August 2022 with two cohorts of 8 cows. Each cohort was divided into two periods which lasted for four days each. Cows were stratified by body weight and randomly allocated to one of two treatments, heat stress (**HS**, n = 7) and pair-fed thermoneutral (**PFTN**, n = 8). During period 1, both treatments had ad libitum access to a common total mixed ration (**TMR**) and were exposed to a daily THI of 64. In period 2, HS cows were exposed to cyclical heat stress with daily THI from 74 (8 h daily) to 80 (16 h daily). During period 2, TN cows remained at 64 THI. Cows were fed twice per day a complete TMR formulated to meet all nutrient requirements (NRC, 2001). Cows had free access to clean water at all times. Feed intake from both groups was paired using as reference the day-before intake from the HS group during period 2. Individual feed intake, cow vital signs and milk yield were recorded

daily. Temperature and humidity data loggers were used throughout the experiment to calculate the daily THI (Collier et al., 2012). For a complete description of the experimental design see Ellett et al. (2022).

Milk sample collection and flow cytometry analysis

Cows were milked twice daily at 0700 and 1900 h. Milk yield was recorded at each milking and compiled daily. We used flow cytometry to quantify proportion of living and dead epithelial and immune cells present in milk. For this, milk samples representative of the entire morning milking (3.8 L) were collected on the last day of each period from each cow and processed according to the protocol of Lengi et al. (2021). Briefly, milk samples containing a final concentration of 0.5 mM ethylenediaminetetraacetic acid (**EDTA**) were centrifuged (850 g for 10 minutes) to pellet the total cells present in milk. The resulting pellet was washed once with Dulbecco's (**DPBS**) and EDTA (0.5 mM final concentration), centrifuged, re-suspended for 15 min in red blood cell lysis buffer, and filtered sequentially through 100- and 40-µm sterile cell strainers (Genesee Scientific) to remove noncellular debris. Cell number was assessed using a hemocytometer and samples were adjusted to 2×10^6 cells for flow cytometry analysis. Primary cell surface antibodies previously reported and tested for hematopoietic cells (**CD45**; clone CACTB51A, Kingfisher Biotech, 3.1 ng/µL), macrophages (**CD14**; clone CAM 36A, Kingfisher Biotech, 1.25 ng/µL), and mammary epithelial cells (Butyrophilin 1A1 (**BTN**); clone MAB8467 conjugated to APC, NOVUS Biologicals, 7 ng/µL) were used to label individual cells. Secondary antibodies used were rat anti-mouse IgG2a-phycoerythrin (clone SB84a, Southern Biotech Associates, 1.0 ng/µL) and goat anti-mouse IgG1-AlexaFluor 488 (Southern Biotech Associates, 1.25 ng/µL). Cell viability was determined using propidium iodide (**PI**; BD Biosciences, 5 µg/mL) dye, and Hoechst 33342 (Invitrogen, 10 µg/mL) was used as a nucleic acid stain.

Flow cytometry was performed on a BD FACSAria Fusion (BD Biosciences) using FACSDiva software (BD Biosciences). Side and forward scatter (**SSC** and **FSC**) thresholds were defined to eliminate cellular debris and aggregates. Single stains of each fluor and dye were used to determine flow cytometry compensation settings. Gates were used to discriminate positive and negative staining cells and were applied consistently to all samples allowing for minor adjustments for SSC variability. Live and nucleated cells were selected by gating on PI and Hoechst, and different subpopulations were selected by gating on cells triple-labeled with CD45-PE, CD14-AF488, and BTN-APC.

Mammary gland tissue collection and processing

On the fourth day of the second period, animals were euthanized via penetrating captive bolt and exsanguinated immediately. Mammary tissue samples from right rear glands (~100 g total, collected from mid-gland region) were collected within 30 minutes of slaughter and rinsed with cold PBS. Mammary tissue was then cut into smaller pieces and subsamples intended for histology were stored in room temperature 10% neutral buffered formalin for 24h for fixation, whereas those for later gene and protein analyses were homogenized and snap-frozen for storage.

Histological analysis

After fixation, mammary tissue samples were subsequently stored in 70% ethanol at 4°C. Later, samples were processed and embedded in paraffin. For this, tissue samples were processed starting with 70% ethanol for one-hour (2×), 80% ethanol for one hour (2×), 95% ethanol for one hour (2×), 100% ethanol for one hour (2×), xylene for one hour (2×) using a Leica TP-1020 Tissue Processor (Leica Microsystems, USA), and embedded in paraffin. Embedded paraffin blocks were sliced using an HM-340E Microm GmbH microtome (Walldorf, Germany) in 5-micron thick

sections, incubated in a water bath for 5 min, and placed on positively charged microscope slides (Fisher Scientific; Pittsburgh, PA). Three tissue sections were placed on each slide with one slide per cow. Slides were placed on a slide warmer after draining to thoroughly dry. For staining, slides were deparaffinized and rehydrated by submerging them in a series of solutions: xylene - twice for 5 min each, 100% ethanol - twice for 3 min each, 95% ethanol - once for 3 min, 70% ethanol - once for 3 min, and distilled water twice for 5 min each. Slides were taken directly from the distilled water bath for hematoxylin and eosin (**H&E**) tissue staining using a standard protocol.

Alveoli number, alveoli area, and MEC number per alveolus were quantified from H&E stained slides. Histologic sections were randomly photographed using OCULAR Imaging Acquisition Software (Teledyne Photometrics, Tucson, AZ) avoiding tissue edges. Seven images from each tissue section were captured using a 20× objective (200× magnification) for alveoli area, alveoli number, and cell number. For histological analysis, one tissue section was randomly selected and the seven images were evaluated for alveoli number and alveoli area. For cell number analyses, five images, randomly selected, were evaluated. Slide evaluation was carried out using ImageJ software (National Institutes of Health, Bethesda, MD). The ImageJ Point Picker plugin was utilized to count alveoli number and nucleated cells, while the alveoli area was traced using the freehand tool. The image was calibrated according to the corresponding magnification (pixels to μm). Total tissue area for photomicrographs taken at 20x is 304,821 μm^2. The percentage area was calculated as the portion of the alveolar area compared to the total image area. Ducts were not measured or considered in the analysis. Alveoli, cell number, and alveoli area were averaged across images per section for each cow for statistical analysis.

Reverse-Transcription quantitative PCR

Tissue samples used for transcript analysis were sectioned into small pieces and frozen in liquid nitrogen and stored at -80°C. For total RNA extraction, approximately ~100 mg of tissue were homogenized in 1 mL RNAzol RT Reagent (Molecular Research Center Inc., Cincinnati, OH) using a PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT) following the manufacturer's instructions. Homogenates were centrifuged, and RNA was isolated and resuspended in nuclease-free water. Concentrations were determined at 260 nm using a Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). For complementary DNA synthesis, RNA (2 μg per reaction) was reverse transcribed using the Omniscript Reverse Transcription kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Oligo-dT (Qiagen, Valencia, CA) was used as the primer for reverse transcription. After cDNA synthesis, samples were stored at -20°C until PCR analysis. Genes of interest were selected for analysis due to known roles in: nutrient transport across MEC membranes, milk component synthesis, and cellular response to heat (Table 4.1).

Real-time quantitative PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) in an Applied Biosystems 7300 real-time PCR machine (Applied Biosystems, Foster City, CA). Amplification reactions were as follows: enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation for 72°C for 1 min. Each reaction was performed in duplicate wells.

To define the most reliable endogenous control genes for normalization, primers for six different transcripts (*B2M*, *EIF3K*, *ACTIN*, *MRLP39*, *RLP0*, *PPIA*) were tested to identify the most stable. The stability of candidate control transcripts was analyzed and ranked using RefFinder (Xie

et al., 2012) and the two most stable, actin, and *EIF3K*, were used as the endogenous controls. Fold change data were analyzed using the common base method (Ganger et al., 2017). All primer pairs were designed to span at least one intron and generated a single product on dissociation curve analysis. Gene-specific primers for the transcripts used in the study and their efficiencies are shown in Table 4.1.

Immunoblotting

Tissue samples used for immunoblotting were removed from storage at -80°C and homogenized in lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA pH 8.0, 1 mM sodium orthovanadate, and protease inhibitor cocktail) using a homogenizer (PRO 200; Scientific Inc., Oxford, CT). Samples were kept on ice at all times. For protein quantification, tissue homogenates were centrifuged at 14,000 x g for 10 min at 4°C, pellets were discarded, and protein concentration was assessed (Bradford Reagent; BioRad, Hercules, CA). Homogenates were standardized using Laemmli Sample Buffer (Sigma Chemical Co., St. Louis, MO) and incubated at 95°C on a heat block for 10 min. Aliquoted homogenates were stored at -20°C until analysis. For immunoblot analysis, the precision Plus Protein Kaleidoscope (BioRad) was used as a molecular weight ladder, and 42.5 μg of protein was loaded into each polyacrylamide gel well (10, 12, or 15%; Mini-PROTEAN TGX Stain-Free Precast; Bio-Rad) for electrophoresis according to the target protein molecular weight. Stain free gel activation was completed using UV light and the ChemiDoc MP Imagen System (BioRad) and transferred onto a mini polyvinylidene difluoride (**PVDF**) membrane (Trans-Blot Turbo RTA Transfer Kit, BioRad) for 7 min using a Bio-Rad Trans-Blot Turbo semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 h at room temperature under slight agitation using 2% bovine serum albumin (**BSA**) in tris-buffered saline solution (**TBST**; 0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween) for phosphorylated target proteins

or 5% non-fat dry milk (**NFDM**) in TBST for total quantification. Membranes were incubated with primary antibodies overnight at 4°C on a rocker. The following day membranes were washed with TBS and incubated in the corresponding HRP-conjugated secondary antibody for one hour on a rocker at room temperature. Enhanced chemiluminescence protein detection reagent was used for protein detection according to the manufacturer's protocol (Amersham ECL Prime, Cytiva, Washington, DC). The ChemiDoc MP system was used for imaging and ImageLab software (BioRad) was used for densitometric analysis. Total protein quantification was assessed by quantifying the intensity of all protein bands in the entire lane as well as the intensity of the target protein band. Target protein band intensity was normalized by dividing the intensity of the target protein band by the intensity of the corresponding total protein in the entire lane.

To evaluate cell activity, proteins of interested were selected due to known roles in: cell survival, cell differentiation, and protein synthesis **(Table 4.2).** Antibodies used for immunoblotting were Atg7 (anti rabbit monoclonal, Cell Signaling Technology, Danvers, MA, cat# 8558), CD14 (anti mouse, BioRad, cat# MAC5940GA), LC3 I (anti rabbit polyclonal, Invitrogen Thermo Fisher Scientific, cat# PA5 – 22990), LC3 II (anti rabbit polyclonal, Invitrogen Thermo Fisher Scientific, cat# PA1– 16930), phosphorylated S6K1 (anti rabbit monoclonal, LS Bioscience, Seattle, WA, cat#C368518-30), S6K1 (anti rabbit polyclonal, biorbyt, cat# orb 30457), phosphorylated ERK1/2 (anti rabbit polyclonal, Invitrogen Thermo Fisher Scientific, cat# 44-680G), ERK1/2 (anti rabbit monoclonal, Invitrogen Thermo Fisher Scientific, cat# MA5-15134), phosphorylated AKT (anti rabbit polyclonal, Cell Signaling Technology, cat# 9271), AKT (anti rabbit polyclonal, Cell Signaling Technology, cat# 9272), phosphorylated STAT5 (anti rabbit polyclonal, Cell Signaling Technology, cat# 9351), STAT5 (anti rabbit monoclonal, Cell Signaling Technology, cat# 94205), phosphorylated p38 MAPK (anti rabbit monoclonal, Cell Signaling

Technology, cat# 4511), p38 MAPK (anti rabbit polyclonal, Cell Signaling Technology, cat# 9212), phosphorylated mTOR (anti rabbit monoclonal, Cell Signaling Technology, cat# S2448), mTOR (anti rabbit polyclonal, Cell Signaling Technology, cat# 2972), prolactin receptor (anti rabbit polyclonal, MyBiosource.com, cat# 2026485), prolactin (anti rabbit polyclonal, Invitrogen, cat# MA1-10597), and 4EBP1 (anti rabbit, MyBiosource.com, cat# MBS8211698). Corresponding secondary antibodies used were (mouse anti-rabbit IgG-HPR cat# sc-2357 and goat anti-mouse IgG_1-HPR cat# sc-2060, Santa Cruz Biotechnology). Antibodies against CD14, LC3 I, prolactin, mTOR, pmTOR and pMAPK did not produce detectable bands with these samples. Dilutions for primary and secondary antibodies can be found in Table 4.2.

RNA to DNA ratio

Genomic DNA and total RNA quantities were determined using the AllPrep DNA/RNA Mini Kit (QIAGEN). Briefly, frozen tissue samples were weighed to ensure no more than 30 mg were used and homogenized by hand using a 5 mL glass tissue grinder. The homogenized lysate was centrifuged using individual spin columns at 8,000 x g for 30 seconds. Flow-through was used to purify total RNA and nucleic acids bound to the silica membrane were used to purify genomic DNA following the manufacturer's protocol. Purified RNA and DNA concentration were determined using a Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Total RNA and DNA were calculated by multiplying RNA or DNA concentration by the total volume. RNA to DNA ratio was calculated by dividing total RNA by total genomic DNA; this is an acknowledged measurement of metabolic activity.

Statistical Analysis

All statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, NC). Descriptive statistics were obtained using the UNIVARIATE procedure to test normality

using the Shapiro-Wilk test. Data not following a normal distribution was log10, log, square root, or Box-Cox transformed in order to fulfill the normality assumption. Statistical analysis was performed using the GLIMMIX procedure of SAS. The evaluation of all independent responses considered the fixed effect of treatment, replicate, and treatment*replicate interaction. Treatment nested within cow was considered as the random effect. For cell loss analyses, period one was used as baseline and included as covariate in the model. Model selection was completed using a backward selection method, where no statistically significant predictor variables were removed and the model with the best fit, based on Akaike information criteria, was used. Significance was established at $P \leq 0.05$, and trends were established at $P \leq 0.10$. Data are expressed as least squares means ± standard error of the means.

RESULTS

The values for respiration rate, rectal temperature, milk yield, and yield of milk components at the end of treatment are presented in Table 4.3. More complete production data can be found in Ellett et al. (2022). Respiration rates and rectal temperatures were increased for cows during HS indicative of treatment effectiveness ($P \leq 0.01$). Cows exposed to a 4 d period of HS produced 4.3 less kg milk on the last day compared to PFTN ($P = 0.01$). Fat yield and protein yield decreased for HS cows by 13% and 17% ($P \leq 0.05$).

To determine if heat stress directly influences cell shedding from the bovine mammary gland, flow cytometry and cell surface markers were used to evaluate cell losses of MEC and hematopoietic cells present in milk. The combined labeling of the three cell surface markers used in this study (Butyrophilin 1A1, CD45, and CD14) resulted in the identification of eight different subpopulations. Cells in milk were assessed on a yield and concentration basis with similar results. Results are presented on a yield basis and the data were transformed to achieve normal distribution for analysis (Table 4.4). There were no changes observed in live or dead MEC (BTN^+), macrophages ($CD45^+CD14^+$), or hematopoietic ($CD45^+$) cells in milk between treatments. Live and dead putative progenitor cells ($CD45^-CD14^+$) present in milk increased in cows under HS conditions by 3.3 times and 3.9 times compared to PFTN cows, respectively (estimates based on back-transformed data; $P \leq 0.02$). Live triple-negative cells, with an unknown identity, tended to increase two fold in cows under cyclical HS compared to PFTN (estimates based on back-transformed data; $P = 0.08$). Conversely, yield of dead triple-negative cells decreased slightly in milk samples from HS cows ($P = 0.04$). Dead BTN^+CD45^+ cells, with an unknown identity in milk tended to decrease by 43% under HS conditions (estimates based on back-transformed data; $P = 0.06$).

To evaluate key changes occurring in the mammary epithelium due to the direct effect of HS, bovine mammary gland tissue was evaluated histologically. Perceptible differences were observed in the microstructure of the mammary gland after 4 days of cyclical HS (Figure 4.1 – A and B). The quantitative evaluation revealed that alveolar area, presented in Figure 4.1-C, was reduced by 25% (10953 vs. 8175 μm^2; $P = 0.002$) in cows exposed to HS. In agreement with this, alveoli number per area was greater in tissue from HS cows compared to PFTN (Figure 4.1-D; 22.3 vs. 26.4 alveoli; $P = 0.03$). The total nucleated MEC per area was 21% greater in mammary gland tissue samples from cows under HS conditions (Figure 4.1-E; 389 vs. 321 cells; $P = 0.01$). However, the number of nucleated MEC per individual alveolus did not differ between treatments (Figure 4.1-F; 24.9 vs. 26.3). Additionally, the correlation between alveolar area and milk yield was evaluated and found to be weak (<0.25) and non-significant.

Transcript expression of previously reported genes involved in: nutrient transport (glucose transporter 1 (***GLUT1***), glucose transporter 8 (***GLUT8***), amino acid transporter (***SLC7A5***)), milk component synthesis (β-casein (***CSN2***), κ-casein (***CSN3***), α- lactalbumin (***LALBA***), fatty acid synthase (***FASN***)), and heat response (heat shock protein family A (***HSP70***) 5 (***HSPA5***), heat shock protein family A 8 (***HSPA8***) were evaluated (Figure 4.2). There was no statistical difference in the relative fold expression between treatments for any of the evaluated transcripts. To further evaluate cell activity, the relative abundance of proteins involved in cell survival, cell differentiation, and protein synthesis were evaluated by immunoblotting (Figure 4.3). From targets involved in the JAK-STAT signaling pathway (Figure 4.3-A), phosphorylated signal transducer and activator of transcription 5 (**pSTAT5**) abundance decreased in mammary gland tissue samples from cows exposed to HS compared to PFTN. Total STAT5 and prolactin receptor abundance were not affected by treatment. Within the mitogen-activated protein kinase (**MAPK**) signaling

cascade (Figure 4.3-B), a tendency to decrease the signal-regulated kinase 1 and 2 (**ERK**) abundance and to increase the MAPK abundance was observed in mammary gland tissue samples from HS cows. For the mTOR pathway (Figure 4.3-C and D), phosphorylated protein kinase B (**pAKT**), AKT, ribosomal protein S6 kinase beta-1 (**S6K1**), and the eukaryotic translation initiation factor 4E binding protein 1 (**4EBP1**) protein abundance did not differ between treatments. However, phosphorylated S6K1 abundance decreased in tissue samples from HS cows. For the proteins involved in autophagy (Figure 4.3-E), the lipidated microtubule-associated protein light chain 3 (**LC3 II**) abundance increased considerably in tissue samples from HS cows compared to PFTN cows. However, autophagy-related protein 7 (**ATG7**) abundance did not change between treatments. The ratio of phosphorylated and total proteins is presented in Figure 4.4 and there were no significant differences for any of the proteins evaluated.

Finally, as a companion measurement for metabolic activity, the RNA to DNA ratio was evaluated on mammary tissue homogenates and did not differ between HS and PFTN cows (Figure 4.5; 1.32 vs 1.46).

DISCUSSION

Physiological responses such as increased respiration rate and rectal temperature are main criteria for determining thermal stress in dairy cows (Do Amaral et al., 2011; Tao et al., 2012; Fabris et al., 2017). Respiration rate and rectal temperature observed in heat stressed cows are a response to insufficient evaporative heat loss from the skin surface. Elevations in both measurements support the effectiveness of temperature regulation and HS treatment imposition in this study. Additionally, the use of a pair-feeding model removed the effects of impaired nutrition between the treatments caused by DMI reduction after HS imposition, revealing the definite effects of HS in the mammary gland microstructure, cell activity, and cell loss.

Milk yield reductions due to HS are the most tangible and widely reported effects directly associated with revenue losses on a dairy farm. As expected, MY decreased 4.3 kg on the fourth and last day of treatment for cows under cyclical HS compared to PFTN cows. This result is similar to a previous studies that reported around 7.5 kg/d less milk in mid-lactation multiparous Holstein cows exposed to HS (THI from 73 to 82) for 9 d compared to pair-fed thermoneutral cows (THI = 64) (Rhoads et al., 2009). The increased loss in milk compared to the present investigation might have occurred due to the extended HS period. However, it has been reported that the highest effect of HS on milk yield occurs after 4 d under non-controlled conditions (Spiers et al., 2004; Bernabucci et al., 2014). The results from this study also agree with MY decrease estimations reported by West et al. (2003) who found a linear reduction of 0.88 kg/d of MY per unit increase of THI in Holstein cows. Here, the THI difference between treatments was 13 units (HS = 77 weighted average vs. PFTN = 64), but considering that HS is triggered at THI of 68, an increase of 9 THI units would account for a MY reduction of 7.9 kg per d. Coupled with this, it has been assessed that MY is reduced by 2.1 kg/d per temperature degree increase in rectal temperature

(Zimbelman et al., 2009). In this study, the difference between rectal temperatures was 1.4 degrees, which would account for a decrease in milk yield of 2.9 kg, 1.4 kg less than the actual reduction. Combined with the effects on MY, milk composition was similarly affected by HS. Protein and fat yield reduction were mainly driven by the reduction in MY. There were no differences in milk protein and fat concentrations between treatments (data not shown) agreeing with previous studies reporting no differences for fat (Smith et al., 2013) and protein (Hammami et al., 2015; Weng et al., 2018) concentration in milk from cows exposed to HS conditions.

Based on MY reduction observed in this study, we hypothesized that a short period of HS might trigger a reduction in the chief factors controlling milk synthesis capacity of the secretory epithelium (i.e., measures of MEC number and activity) of the bovine mammary gland. We propose that these are potential mechanisms, independent of dry matter intake reduction, for the rapid decrease in MY observed under HS during lactation in high-producing Holstein cows. To investigate the effects of HS on both cell number and cell activity, we evaluated a range of variables related to these cell features. Mammary epithelial cell number was evaluated for losses into milk and histologically in mammary gland tissue, while cell activity was evaluated by gene transcription and protein abundance measures in mammary gland tissue samples.

Quantity and metabolic activity of secretory mammary epithelial cells determine the mammary gland milk synthesis capacity (Stefanon et al., 2002; Akers, 2016). The number of secretory MEC in the gland fluctuates across lactation and is determined by proliferation and cell death rate (Capuco et al., 2001). Most literature describes changes in MEC death by apoptosis. However, other mechanisms of cell loss occur in the mammary gland including MEC exfoliation during lactation. Herve et al. (2016) propose that MEC exfoliation can play a partial role in controlling cell number in the mammary gland. MEC exfoliation can be modified by factors such

as lactation stage (Boutinaud et al., 2013b), endocrine status (Lollivier et al., 2015), management (Ben Chedly et al., 2013), and environmental factors (Lengi et al., 2022b). We evaluated MEC cell loss into milk, our measure of MEC exfoliation, to examine this mechanism of potential reductions in cell number based on the assumption that MEC present in milk, identified by BTN – the major protein in bovine milk fat globules – were functional secretory cells in the gland. It has been shown that most of the exfoliated epithelial cells present in milk are viable and exhibit characteristics of fully differentiated alveolar cells (Boutinaud and Jammes, 2002a). Live and dead BTN^+ cells in milk were not affected by treatment in this study. These results are divergent from a previous study that reported an 82% and 78% increase in total and live BTN^+ cells in milk from cows exposed to HS for 9 days (Lengi et al., 2022b). Although the methods used in both studies were the same, other factors might explain differences. Lengi and collaborators (2022b) evaluated the effects of HS without using a pair-feeding model, and it is possible that the effects on BTN^+ cell loss are mainly an effect of DMI reduction and impaired nutrient availability for the mammary gland and the MEC leading to a lack of support for secretory MEC maintenance. The last has been demonstrated in previous studies, where animals in thermoneutral conditions that were feed-restricted by 20% of ad libitum DMI had 65% greater rate of MEC in milk (Herve et al., 2019). Another crucial factor that differentiates our study and the study by Lengi et al. (2022b) is the exposure time of HS, which was greater by 2.25 fold (4 vs. 9 d). Exposure to HS might play a significant role in the mammary gland response and cell shedding pattern. The results observed in this study suggest that cows exposed to HS longer might produce a chronic response in the secretory mammary epithelial cells that cannot be achieved under 4 days of cyclical HS. Additionally, Lengi et al. (2022b) compare BTN^+ cells in milk within the same cow, contrary to our investigation where comparisons were completed between different cows, and substantial

variability across samples from animals allocated in different treatments was observed decreasing the ability to detect significant differences between treatments. However, due to the experimental design involving slaughter for sample collection, repeated sampling was not possible in this study.

To further analyze cell number using a direct approach, we evaluated secretory MEC number through histology analysis. Cell number per alveolus was not affected by heat stress aligning with the lack of HS effect on the yield of MEC in milk. There are no reports in the literature evaluating the effects of heat stress during lactation on bovine MEC number. However, in vitro studies using isolated MEC from mouse mammary gland have demonstrated that MEC number is reduced by 55% when cultured at 41°C for 2 d compared to 37 °C (Wakasa et al., 2022). The lack of differences in MEC number in this study might be explained by the moderate HS conditions used as indicated by the low differences in rectal temperature among cows exposed to PFTN and HS conditions (1.4°C). Combined with this, the average rectal temperature reached in HS cows was 40°C. West et al. (2003) proposed that an increase in ambient temperature will increase mammary gland inner temperature indirectly. Nevertheless, an increase greater than 41°C for a substantial period occurring in the gland seems unlikely. Although cell number per alveolus was not affected, cell number per area was greater in tissue samples from HS cows compared to PFTN. This was mainly caused by the evident structural changes in the mammary gland secretory tissue. Individual alveolus area was reduced by 25% and consequently, alveoli number per area increased by 18% in tissue samples from cows exposed to cyclical HS for four days. The possibility that the differences observed in alveolus area and alveoli number occurred due to differences in milking and slaughtering time between HS and PFTN cows was considered. However, this was unlikely given similar milking and slaughtering times (PFTN – 2h 54m vs. HS – 3h 21m).

Previous results from mammary gland tissue samples from cows exposed to intrauterine HS during fetal development have reported a 46% reduction in alveoli area in the first lactation compared to tissue samples from animals in cooled conditions during fetal development (Skibiel et al., 2018). However, in this study, alveoli number did not differ between treatments. Contrary to these results, Dado-Senn et al. (2019) observed a 15% decrease in alveoli number in mammary gland samples obtained during lactation from cows exposed to HS during the entire dry period. The lack of consistent results of the effect of HS on the bovine gland structure from these studies might be explained by the differences between HS time exposure (4 d vs. 46 d) and physiological state (lactation vs. dry period). Heat stress might influence the mammary gland differently during the dry period vs. lactation. The dry period is a non-lactating phase between lactations where senescent mammary epithelial cells are replaced with active cells for the next lactation, and HS might directly affect MEC number (proliferation vs. cell death) during this period (Dado-Senn et al., 2018). In contrast, it is known that HS can impact intracellular signaling pathways responsible for productivity (Collier et al., 2008). During lactation in the mammary gland, a remarkable and dynamic process occurs, leading to changes in the size and structure of individual alveoli, the functional units responsible for milk production. Still, the specific cellular mechanisms responsible for the area decrease in individual alveoli during lactation remain largely unknown and represent an area of active research and scientific inquiry. However, the sole reduction of the MEC capacity to synthesize milk could decrease luminal area, and thus drive individual alveolus area reduction observed in mammary gland tissue samples obtained from cows under HS.

To evaluate the synthetic capacity, or cell activity of the bovine mammary gland, we measured the transcript abundance of genes related to signaling pathways involved in cell activity, heat response, solute transport, and the expression of proteins involved with cell survival, cell

differentiation, and protein synthesis. Protein and fat encoding gene expression were not affected by treatment (*CSN2*, *CSN3*, *LALBA*, and *FASN*). This agrees with the results of Cowley et al. (2015) who reported no differences for β- and κ-casein in milk samples from mid-lactation Holstein cows exposed to heat stress for 7 days compared to milk samples from ad libitum and pair-fed thermoneutral cows. On the other hand, it has been observed that HS decreased *CSN2* and *CSN3* gene expression in mammary gland tissue from lactating Holstein cows exposed to a non-controlled ambient HS for 21 d (Yue et al., 2020). Similarly, in a controlled crossover study using lactating multiparous cows exposed to HS during 9 d, the expression of milk protein-encoding genes (*CSN3* and *LALBA*) in mammary gland tissue samples were downregulated compared to samples from the PFTN group (Gao et al., 2019). Fatty acid synthase (*FASN*), unaffected by HS in our study, is the major enzyme involved in fatty acid synthesis in the ruminant mammary gland (Smith, 1994). In vitro studies have shown a decrease in protein expression of *FASN* in cultured MEC exposed to HS for 1 hour at 40°C (Li et al., 2017).

Multiple nutrients are essential for milk synthesis including glucose and amino acids, and most nutrients are taken up by MEC through specific transporters. Here we evaluated the transcript expression of glucose transporters 1 and 8, and the amino acid transporter *SLC7A5*; gene expression levels were unaffected by HS. Glucose transport in the lactating mammary gland is essential for multiple processes such as lactose synthesis, *NADPH* generation, milk lipid synthesis, energy production, and nucleic acid and amino acid synthesis (Zhao, 2014). In the bovine mammary gland, the expression of *GLUT1* and *GLUT8* has been reported (Zhao et al., 2004). Particularly, in bovine mammary gland biopsies obtained from lactating cows exposed to HS for 9 days, a decrease in *GLUT1* and *GLUT8* gene expression was observed compared to tissue samples from pair-fed thermoneutral cows (Gao et al., 2019). Opposing what we observed in the

present investigation, in the same study, the expression of *SLC7A5* was downregulated. The results obtained herein, are similar to the ones reported by Dado-Senn et al. (2021), where *SLC7A5* gene expression was not significantly changed in bovine mammary tissue samples at 14, 42, and 82 DIM from cows exposed to ambient HS during the dry period.

To evaluate the effect of HS on heat shock response in mammary tissue, we evaluated gene expression of two orthologs of the HSP70 family, *HSPA5* and *HSPA8*. Heat shock proteins are highly conserved and their production is characterized as a cellular response to HS (Tao et al., 2018). HSP70 protects the cell from detrimental HS effects by functioning as a chaperone and stabilizing proteins in a folding-competent state. Contrary to what was observed in previous in vitro studies, neither of the two HSP70-related genes evaluated were affected by HS. It has been shown that HSP70 gene and protein expression was upregulated in two different in vitro studies where MEC were incubated at 42°C for more than 30 min (Collier et al., 2008; Hu et al., 2016).

Differential gene expression observed in our study might have occurred due to the sample type used. Although the use of mammary tissue homogenates in our study introduces an inherent complexity to the analysis of gene expression. While cell culture experiments can provide valuable insights into specific cellular behaviors, studying gene expression in whole tissue homogenates allows us to capture the collective response of various cell types within the mammary gland. However, the heterogeneity of mammary tissue homogenates presents both opportunities and challenges in interpreting the differential gene expression results. On one hand, the complexity of the tissue allows us to investigate the regulatory interactions between different cell types and understand how they collaborate to achieve a specific biological function, such as milk production in lactation. On the other hand, the presence of multiple cell types can introduce noise and variability to the data, potentially masking subtle gene expression changes specific to MEC.

The connection between gene expression and protein expression is crucial, as the information contained in the gene's DNA sequence determines the sequence of amino acids in the resulting protein. However, it is essential to recognize that not all transcribed genes will be translated into proteins. In general terms, the lack of significant changes in gene expression of the targets evaluated here might be explained due to the shorter period of heat stress exposure compared to the aforementioned studies and temperatures used in the in vitro studies. Gene transcription changes produced by environmental alterations allow for a physiological adaptive response (Feugeas et al., 2016), however, the evaluation of gene expression only reflects the steady-state abundance of mRNA, a function determined by mRNA production and turnover rate (Schwanhäusser et al., 2011). Based on this, the evaluation of transcript translation and protein synthesis could show different results because proteins are the main regulators of cell function. Heat stress impacts numerous intracellular signaling pathways responsible for cell maintenance, productivity, and survival (Collier et al., 2008). To further evaluate these effects, proteins involved in cell survival, cell differentiation, and protein synthesis pathways were evaluated by immunoblotting. From all target proteins evaluated, only pSTAT5 and pS6K1 decreased and LC3 II increased significantly in the HS cows.

The Jak/Stat pathway is crucial in the regulation of milk and milk protein synthesis in the mammary gland. This pathway is activated by prolactin signaling that works in conjunction with other lactogenic hormones to induce cell differentiation and promote the expression of mRNA encoding milk proteins. One of the key components involved in this process is the transcription factor STAT5. Through its activation by prolactin, STAT5 plays a significant role in regulating the genes responsible for milk protein synthesis (Watson and Burdon, 1996).

In vitro studies have shown that a 3 d exposure of cultured MEC to HS at 41°C leads to the inactivation of STAT5 and a decrease in milk synthesis capacity (Kobayashi et al., 2018). In vivo studies have demonstrated a decrease in gene and protein expression of PRLR, STAT5A, STAT5B, and JAK2 in bovine mammary gland tissue homogenates from cows exposed to 3 weeks of environmental HS (THI from 72.5 to 86.9; Yue et al., 2020). In the present investigation, only phosphorylated STAT5 was significantly reduced in HS and no effects of HS were observed for STAT5 and PRLR. This might indicate that a short period of HS does not alter the synthesis of STAT5, but its activation instead. A reduction of phosphorylated STAT5, an important regulator of milk synthesis, suggests a decrease in the synthetic capacity of the bovine secretory mammary epithelial cells under HS.

The MAPK intracellular signaling cascade with the extracellular regulated kinase (ERK1/2) pathway is implicated in mammary epithelial cell function (Krishna and Narang, 2008). In this study, a tendency for increased and decreased protein abundance of MAPK and ERK (1/2) was observed, respectively. There are no reports available in the literature evaluating the effects of HS on the MAPK signaling cascade, and its effectors in the bovine mammary gland. Despite this, microRNA sequencing analysis of mammary gland tissue samples from cows exposed to environmental HS for a month showed 72 differentially expressed microRNAs enriched in the MAPK pathway suggesting that specific microRNAs influence the MAPK signal cascade in the mammary gland (Fan et al., 2021). The increased MAPK, an essential regulator of the immune response suggests the amplification of cellular responses to guarantee cell survival and inflammatory responses. Additionally, one of the most recognized biological functions of ERK1/2 is cell proliferation, differentiation, and survival (Pearson et al., 2001). Although there are no reports evaluating ERK1/2 in the bovine mammary gland under HS conditions, immunoblot

analysis in mammary gland human cells incubated at 37°C in an HSP90 inhibitor (SL-145) markedly downregulated ERK expression and reduced its phosphorylation suggesting the simultaneous depletion of multiple cellular signaling pathways in the absence of HSP90, a chaperone protein activated under HS (Kim et al., 2022). Similarly, relative expression of phosphorylated and total ERK1/2 increased in murine mammary organoids cultured at 41°C for three days compared to mammary organoids cultured at 37°C (Wakasa et al., 2022). What was reported in these studies does not align with what was observed in our study, where total ERK1/2 protein abundance tended to decrease in mammary tissue samples obtained from cows exposed to HS for four days. The downregulation of ERK1/2 might suggest a reduction in cell proliferation and differentiation instead of a pro-survival response in the bovine mammary gland under acute HS.

Another protein involved in cell survival and growth is the mammalian Target of Rapamycin (mTOR). This kinase is predominantly activated by the PI3K/Akt pathway, where Akt directly phosphorylates and activates mTOR. Other proteins involved in this pathway are 4EBP1, a translation inhibitor, and S6K1, a protein kinase, that participate in protein synthesis (Berchtold and Walther, 2009; Kakumoto et al., 2015). In this study, of the PI3K/AKT/mTOR proteins evaluated only phosphorylated S6K1 protein abundance was reduced in mammary gland samples from HS cows. In agreement with what was observed in our study, previous studies found that pS6K1 protein abundance decreased at 42 and 84 d of lactation in mammary gland samples from cows exposed to HS during the entire dry period (46 d) compared to TN cows (Dado-Senn et al., 2021). Furthermore, in the same study, contrary to what we observed in this study, a decrease in protein abundance for AKT at 84 d of the lactation was observed. Agreeing with our results, no changes for 4EBP1 protein abundance were observed in mammary gland samples from cows

exposed to environmental HS during the dry period (Dado-Senn et al., 2021). The differences observed between these studies might originate from the time exposure to HS difference (46 vs 4 d) and the physiological state (dry period vs lactation) when HS was imposed.

Of the two autophagy-related proteins evaluated in this study, only LC3 II protein relative abundance increased considerably with no changes observed in ATG7 in mammary gland samples from lactating cows exposed to cyclical HS for 4 days. Mammary epithelial cell death in the mammary gland occurs across lactation and can be altered by HS. In vitro studies have shown that programmed cell death increases at 42°C in incubated mammary tissue explants of HS cows and bovine mammary epithelial cells (Cai et al., 2018; Chen et al., 2020; Ouellet et al., 2021). Programmed cell death includes three major cell death types: apoptosis, autophagy, and necrosis (Andón and Fadeel, 2013). The most described programmed cell death type in the bovine mammary gland is apoptosis. Nevertheless, it has been shown that autophagy plays a crucial role during the dry period to support post-lactation bovine mammary gland regression (Motyl et al., 2007). Multiple papers have shown an alteration in autophagy-related proteins and transcripts during the dry period after HS (as reviewed by Tao et al., 2018). However, the incidence of autophagy in bovine mammary gland tissue exposed to HS during lactation has not been described. The increase in LC3 II, a protein that participates in engulfment and autophagosome formation, observed in the present investigation contrasts with previous reports. Wohlgemuth et al. (2016) observed decreased protein abundance for LC3 I and II in mammary gland tissue samples from cows exposed to HS during the dry period. In agreement with the results observed in this study, previous reports have not observed changes in ATG 3, 5, and 7 at transcript and protein levels in mammary gland samples from cows exposed to HS during the dry period (Wohlgemuth et al., 2016; Ouellet et al., 2021). A recent study has shown increased LC3 II protein abundance in the

mammary gland of early lactating cows under hyperketonemia (Li et al., 2020). The increased level of LC3 shows that autophagy and phagosome formation is activated after a short period of HS suggesting an increase in damaged cell organelles that could decrease the synthetic capacity of MEC under HS. Furthermore, the lack of changes in MEC number in milk and tissue suggests that autophagy might be activated to remove dysfunctional cell components, to guarantee cell survival instead of cell death during short periods of HS.

Finally, to evaluate the overall synthetic capacity in the mammary gland tissue we evaluated the ratio between RNA and DNA. Most of the RNA measured in the sample is ribosomal RNA reflecting the amount of protein synthesis occurring at the individual cell level, whereas the DNA measured amount is a fixed value per cell. Therefore, higher ratios of RNA to DNA indicate a higher synthetic capacity in the cell. The RNA to DNA ratio estimates in this study were not different between the treatments. The ratio for the HS treatment was 1.32, while PFTN ratio was 1.46. These RNA to DNA ratios are slightly smaller than what has been reported in previous studies. For instance, in mammary tissue samples from lactating dairy cattle at 49 d of lactation, the ratio was 2.00 (Keys et al., 1989), and at 10 d of lactation, it was 1.86 (Akers et al., 1981). On the other hand, RNA to DNA values reported in mammary tissue samples from lactating beef cattle at 49 d postpartum were notably lower, at 1.07 (Keys et al., 1989). These variations in RNA to DNA ratios across different studies may be attributed to factors such as lactation stages, experimental conditions, or the heterogeneity of tissue samples. Previously reported values for lactating dairy cows might reflect the earlier stage of lactation compared to cows in this experiment and increased milk synthesis relative to beef cattle. Notably, the use of tissue homogenates might impede the exclusive analysis of MEC. The presence of other cell types in the homogenized sample could potentially complicate the interpretation of activity features specific to MEC when

evaluating RNA to DNA ratios. Additionally, the evaluation of mostly ribosomal RNA might overshadow any changes in the quantities of messenger RNA, which play a crucial role as the template for protein synthesis during translation.

Coupled with this, the lack of differences in RNA to DNA ratio between treatments in this study might be explained due to a different response to acute and chronic HS. Exposure to HS for four days might not reveal the changes to ribosomal RNA, which could keep in a steady state during an acute response to HS. However, it is plausible that at the end of HS exposure in this experiment, RNA cellular degradation or reduced synthesis have just started. This premise agrees with what was observed with the LC3 II abundance in this study, where LC3 II abundance – an adaptor protein involved in intracellular degradation – increased significantly in the HS cows suggesting an increase in phagosome degradation of cell organelles and components. It seems probable that after an extended time of HS exposure RNA degradation or a lack of synthesis as a response to reduced milk production will occur and a difference in RNA to DNA ratio could be observed.

In the present investigation, somatic cell count was not affected by HS, even though it has been reported in observational studies that environmental factors like an increase in temperature and humidity are positively correlated with an increases in somatic cell count at the farm level (Tao et al., 2018). The results observed in the present investigation agree with the findings reported by multiple studies which found no influence on milk somatic cell count (**SCC**) by the sole effect of HS in controlled experimental conditions (Wheelock et al., 2010; Weng et al., 2018; Lengi et al., 2022b) indicating that environmental conditions and bedding pathogen presence might be involved in increased SCC in bovine milk during warmer and more humid seasons.

Although no quantitative changes in SCC were observed in the study, the possibility of alterations in the immune population existed. Heat stress might impair the immune function of the bovine mammary gland at the cell or organ level (Tao et al., 2018). To further explore immune cell profile in milk from bovine mammary glands under HS, we evaluated the presence of immune cells in milk using cell surface markers. In general, HS affected neither live nor dead macrophages ($CD45^+CD14^+$) consistent with previous findings assessing the impact of HS on these specific cell populations (Lengi et al., 2022b). Similarly, live and dead immune cells ($CD45^+$) were not affected by heat stress in this study. These results contrast with the results reported by Lengi et al. (2022b) where live $CD45^+$ immune cell concentration decreased 71% in milk samples from multiparous Holstein cows under HS during early lactation. The use of only two immune markers in the present investigation prevents us from precise cell type identification. It is possible that due to the nonspecific identities of immune cells, we might overlook changes in specific immune cell subpopulations such as T cells or granulocytes. In vitro studies have shown impaired function in immune cell activity of mononuclear and polymorphonuclear bovine cells exposed to hyperthermia (Lacetera et al., 2006; Lecchi et al., 2016).

Additionally, alteration of other unidentified cells in milk by HS was observed including $CD45^-CD14^+$, BTN^+CD45^+, and $CD14^-BTN^-CD45^-$. Suggested identities for these populations are putative progenitors, unidentified dual origin cells, and other cell types not identified by the available markers used in this study. Live and dead $CD45^-CD14^+$ cells increased by 300-400% in milk samples obtained from cows under HS for four days. These results agree with previous reports from our group that have shown an increase of 60% in the same cell subpopulation ($CD45^-CD14^+$) in response to HS (Lengi et al., 2022b). The expression of the CD14 transcript is denoted as a progenitor cell marker in mammary epithelial cells from several studies in rodents and humans

(Bach et al., 2017b; García Solá et al., 2021; Martin Carli et al., 2021). Heat stress has adverse impacts on cellular processes like cell proliferation. It has been reported that cell proliferation is reduced during the transition period in mammary tissue samples from cows exposed to HS during the dry period (Tao et al., 2011). Heat stress might affect progenitor cells robustly within the mammary gland directly affecting cell proliferation instead of cell death. More studies about the effects of environmental factors like HS in specific cell identities like progenitor cells and the presence of progenitor cells across lactation in the bovine MEC are needed.

Cells that were BTN^{+} and $CD45^{+}$ were not identified in this study. These cells express markers for MEC and hematopoietic cells which have different organogenesis, ectoderm vs. mesoderm. Multipotent cells are undifferentiated cells with the capability to differentiate into various lineages (Sobhani et al., 2017), because of this, multipotent stem cells undergoing differentiation can express multiple cell markers. Coupled with this, the presence mesenchymal stem cells in human milk has been previously reported (Mane et al., 2022) but not extensively studied. The presence of multipotent cells in bovine milk exists, nevertheless, based on the results obtained in our study we cannot identify these dually labeled cells conclusively.

Live triple-negative cells, $CD14^{-}BTN^{-}CD45^{-}$, tended to increase with HS, but dead cells of the same type decreased significantly in milk samples from HS cows. These unidentified cells can be any other differentiated or undifferentiated cell type present in milk. Potential identities for these could include myoepithelial or ductal epithelial cells as both cell types would be negative for CD45 and BTN. Nevertheless, the possibility that myoepithelial cells can be shed into milk is less likely due to their basal position, outside the basement membrane. It has been reported in primary murine mammary organoids that even when luminal cells are induced to protrude into the extracellular matrix, the myoepithelial cell layer is not altered (Sirka et al., 2018). Therefore, the

presence of nonsecretory epithelial ductal cells that might be exfoliated into milk is more plausible. Each lobule in the gland has an individual lactiferous duct, where synthesized milk is collected and conducted to the sinus lactiferous between milkings to be secreted. It is feasible that HS can stimulate live, nonsecretory ductal epithelial cell shedding by altering the barrier integrity of ductal cells. Alternatively, the decrease of dead nonsecretory ductal epithelial cells in milk samples from cows exposed to HS might be a result of the decrease in milk yield production and concomitant passage across the lactiferous ducts. Heat-stressed cows producing less milk might have a reduction in the erosion effect of milk passage and drag of ductal epithelial cells.

The use of flow cytometry as the only methodology used in this study to identify cell type presents difficulties like the limited use of cell markers for identification, because of the wavelength overlap between fluorescent dyes needed for this technique. New technologies like single-cell RNA sequencing with the capacity to identify the specific identity of each cell within a heterogeneous sample might help to overcome this challenge. The identification of changes in cell sub-populations within bovine milk can contribute to delineation of specific mechanisms and the functionality of individual cells that constitute the mammary gland and regulate milk synthesis.

CONCLUSION

To delineate the mechanisms producing a reduction of 4.3 kg of MY after 4 d exposure to cyclical HS at the level of the bovine mammary gland, using a pair-feeding model, we evaluated HS direct effects on mammary characteristics related to cell number and activity and cell losses in milk in lactating Holstein cows. Evident acute effects of cyclical HS on the mammary gland structure were the reduction of alveolar area, increase of alveoli number per area, and increase in nucleated MEC per area. Further examination of cell number demonstrated no effects of HS on MEC number per alveolus and MEC shed in milk. The abundance of phosphorylated proteins associated with protein synthesis (pSTAT5) and cell survival (pS6K1) in mammary gland tissue were reduced by HS, indicating an impairment in these pathways. Conversely, HS upregulated an autophagosome protein abundance (LC3 II), suggesting an increase in the degradation of dysfunctional components in the mammary gland following acute HS. Based on our results, cell activity plays a significant role in regulating milk yield during the initial phase of heat stress. Additionally, live and dead $CD14^{+}$ cells shed into milk with a presumed progenitor identity increased considerably with HS exposure for 4 days. More studies focused on HS effects on specific cell identities and the functionality of heterogeneous subpopulations in the bovine mammary gland are needed to better understand their role in milk production across lactation.

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TABLES AND FIGURES

Table 4.1. Gene targets, primer sequences, and efficiencies for real-time qPCR

Target	Gene	Sequence (5′ to 3′)	Reaction Efficiency, %
Beta casein	***CSN2***	F: TGGCTCCTAAGCACAAAGAAA R: GGACTGAGAAAGGGACAGCA	102.4
Kappa casein	***CSN3***	F: AGCCCACCTGAGATCAACAC R: GCCGGATCTGTGAAAATCAT	106.4
Fatty acid synthase	***FASN***	F: CTGCAACTCAACGGGAACTT R: AGGCTGGTCATGTTCTCCAG	99.6
Glucose Transporter 1	***GLUT1***	F: TCCTGCTCATTAACCGCAAC R: GGCTCTCCTCCTTCATCTCC	88.5
Glucose Transporter 8	***GLUT8***	F: TGGCCCCGGTCTATATCTCT R: GGAGGATGCCTGTGACTACC	93.4
Heat Shock Protein 70 A5	***HSPA5***	F: TTCCCAACATCAAGCAAGAA R: AGAGCCACCAACAAGAACAA	99.7
Heat Shock Protein 70 A8	***HSPA8***	F: GCATTCCAGGTTGCTGACTCT R: AATGCCAACTGCAGGTCCTT	99.3
Alpha lacto albumin	***LALBA***	F: TACTGGTTGGCCCATAAAGC R: CAAGGGGGTACAAAGAAGCA	97.3
Solute Carrier 7 A5	***SLC7A5***	F: CCTGCACACCAAGTACCAGA R: CCCAGCATCAACAACAAAAA	88.2

Table 4.2. Antibodies, protein target function, and concentrations (primary and secondary) used for immunoblotting

Primary Antibody	Protein Abbreviation	Category	Primary Antibody Concentration	Secondary Antibody Concentration
Eukaryotic translation initiation factor 4E binding protein 1	4EBP1	mTOR pathway	1:1000	1:2000
Autophagy related 7	Atg7	Cell survival/ Autophagy	1:1000	1:2000
Microtubule-associated protein 1A/1B-light chain 3 - phosphatidylethanolamine conjugate	LC3 II	Cell survival /Autophagy	1:500	1:20000
Prolactin receptor	PRLR	JAK-STAT signaling pathway	1:1000	1:2000
Protein kinase B	AKT	mTOR pathway	1:1000	1:2000
Phosphorylated Protein kinase B	pAKT (Ser473)	mTOR pathway	1:1000	1:10000
Extracellular signal-regulated protein kinases 1 and 2	ERK1/2	MAPK signaling cascade	1:1000	1:2000
Phosphorylated extracellular signal-regulated protein kinases 1 and 2	pERK1/2 (Thr185, Thr187)	MAPK signaling cascade	1:1000	1:5000
p38 mitogen-activated protein kinase	p38 MAPK	MAPK signaling cascade	1:1000	1:2000
Signal transducer and activator of transcription 5	STAT5	JAK-STAT signaling pathway	1:1000	1:2000
Phosphorylated signal transducer and activator of transcription 5	pSTAT5 (Tyr694)	JAK-STAT signaling pathway	1:1000	1:5000
Ribosomal protein S6 kinase β-1	S6K1	mTOR pathway	1:1000	1:2000
Phosphorylated ribosomal protein S6 kinase β-1	pS6K1 (Thr412)	mTOR pathway	1:1000	1:2000

Antibodies were diluted using blocking buffer 2% BSA or 5% NFDM in 1XTBST

Table 4.3. Vital signs, milk yield and milk components of lactating multiparous Holstein cows under thermoneutral (pair-fed) or cyclical heat stress conditions for 4 days

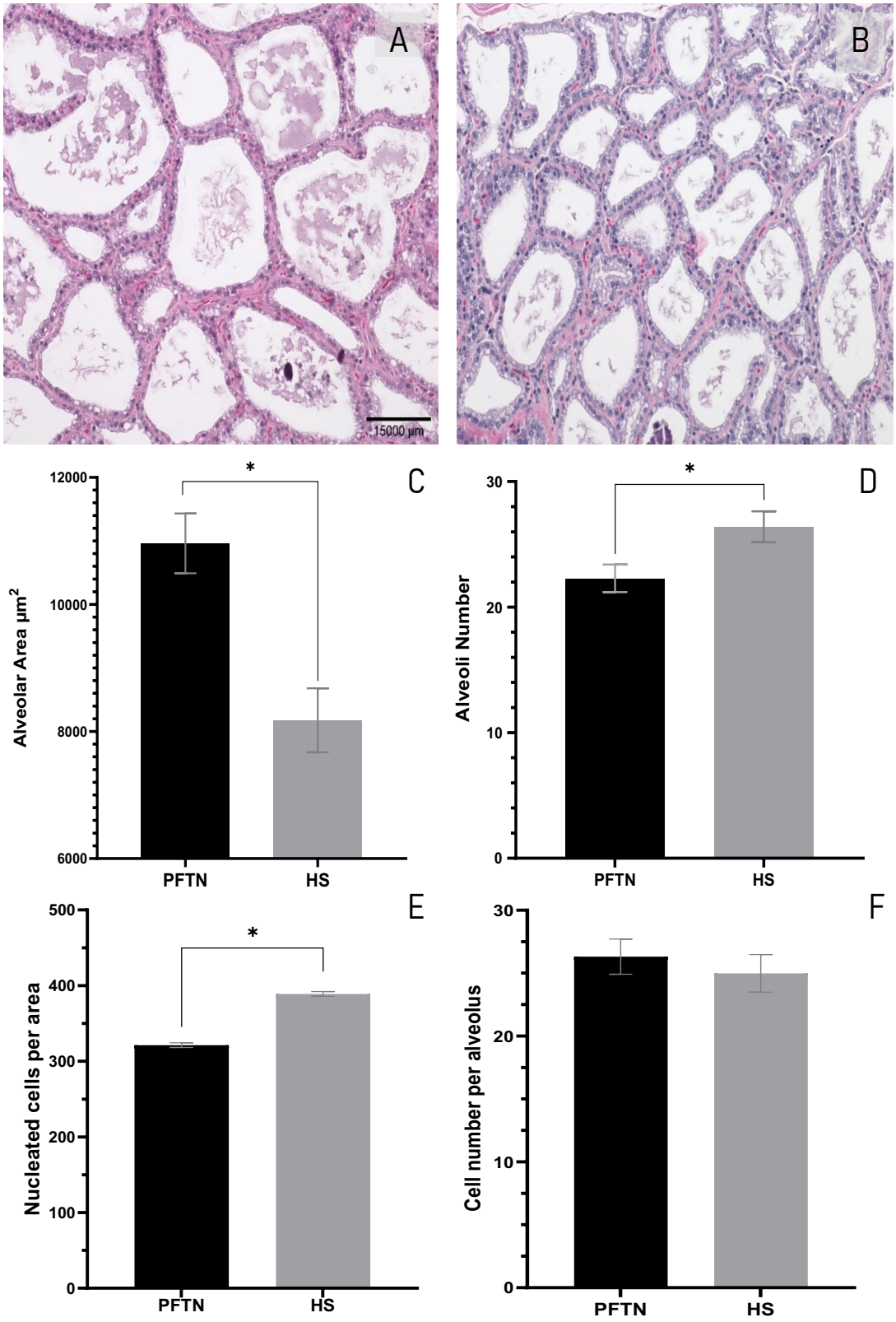
ITEM	Pair-fed Thermoneutral	Heat Stress	SEM	P Value
Respiratory Rate, breaths per minute	50.29	79.75	4.11	0.0001
Rectal temperature, °C	38.6	40.0	0.14	0.0001
Milk Yield, kg d^{-1}	37.54	33.20	1.62	0.01
Protein Yield, kg	1.05	0.87	0.063	0.02
Fat Yield, kg	1.42	1.24	0.075	0.03
SCCx10^3 cells/mL	19.6	26.9	7.0	0.40

Values are reported as least square means

Table 4.4. Log10 and BoxCox estimates for cell yield (103 cells milking kg-1) expressed of each cell subpopulation present in milk from cows exposed to thermoneutral or cyclical heat stress conditions for four days

Status	Cell labeling	Transformation	Pair-fed Thermoneutral			Heat Stress			*P* Value
			Estimate	CI		Estimate	CI		
				Lower	Upper		Lower	Upper	
Live	**BTN$^-$CD45$^-$CD14$^-$**	Log	1.15	-0.38	2.67	1.49	-0.16	3.15	0.08
	BTN$^-$CD45$^+$CD14$^-$	BoxCox	3.07	2.67	3.46	3.06	2.63	3.49	0.90
	BTN$^-$CD45$^+$CD14$^+$	BoxCox	2.79	2.40	3.17	2.80	2.38	3.22	0.76
	BTN$^-$CD45$^-$CD14$^+$	Log	1.04	0.78	1.30	1.56	1.28	1.84	0.02
	BTN$^+$CD45$^-$CD14$^-$	Log	0.01	-0.22	0.25	-0.22	-0.47	0.02	0.17
	BTN$^+$CD45$^+$CD14$^-$	Log	0.31	-0.15	0.77	0.10	-0.40	0.60	0.51
	BTN$^+$CD45$^+$CD14$^+$	Log	0.48	-1.02	1.98	0.37	-1.26	2.00	0.54
	BTN$^+$CD45$^-$CD14$^+$	Log	0.61	-0.89	2.12	0.36	-0.93	1.66	0.19
Dead	**BTN$^-$CD45$^-$CD14$^-$**	BoxCox	1.39	1.32	1.47	1.37	1.29	1.45	0.04
	BTN$^-$CD45$^+$CD14$^-$	BoxCox	2.41	2.38	2.44	2.42	2.39	2.44	0.64
	BTN$^-$CD45$^+$CD14$^+$	BoxCox	2.76	2.57	2.95	2.75	2.55	2.95	0.65
	BTN$^-$CD45$^-$CD14$^+$	Log	1.50	-0.15	3.15	2.09	0.30	3.87	0.01
	BTN$^+$CD45$^-$CD14$^-$	Log	0.54	-1.00	2.09	0.52	-1.08	2.12	0.90
	BTN$^+$CD45$^+$CD14$^-$	Log	0.10	-0.92	1.11	-0.15	-1.24	0.94	0.06
	BTN$^+$CD45$^+$CD14$^+$	BoxCox	-0.19	-0.77	0.39	-0.53	-1.16	0.10	0.41
	BTN$^+$CD45$^-$CD14$^+$	Log	0.92	0.13	1.71	0.85	0.06	1.63	0.42

Figure 4.1. Histology analysis of the lactating mammary gland from multiparous Holstein cows exposed to thermoneutral (PFTN; n = 8) or heat stress conditions (HS; n = 7) for four days. Panel A and B show representative images for hematoxylin and eosin (H&E) stained mammary tissue at a 200× magnification exposed to thermoneutral (PFTN; A) or heat stress conditions (HS; B). Alveolar area (C) and alveoli number (D) in mammary gland samples from lactating Holstein cows exposed to thermoneutral (PFTN; black bars; n = 8) or heat stress (HS; gray bars; n = 7) conditions for four days. Nucleated mammary epithelial cells per area (E) and cell number per alveolus (F) in mammary gland samples from lactating Holstein cows exposed to thermoneutral (PFTN; black bars; n = 8) or heat stress (HS; gray bars; n = 7) conditions for four days. Data presented are least-square means ± standard error of the mean. An asterisk indicates a statistically significant difference between treatments ($P < 0.05$).


A
15000 µm
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Alveolar Area µm²
12000
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8000
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*
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30
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HS
E
Nucleated cells per area
500
400
300
200
100
0
*
PFTN
HS
F
Cell number per alveolus
30
20
10
0
PFTN
HS

Figure 4.2. Gene expression in mammary gland tissue samples from lactating Holstein cows exposed to thermoneutral (PFTN; n = 8) or heat stress (HS; n = 7) conditions for four days. Gene expression is reported as fold change (2-ΔΔCt) relative to pair-fed cows and under thermoneutral conditions.

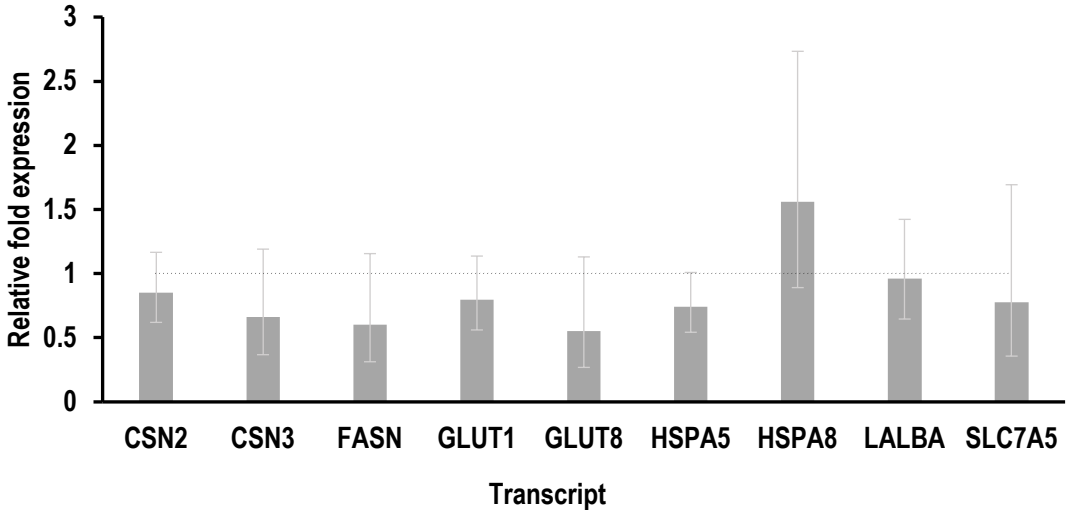
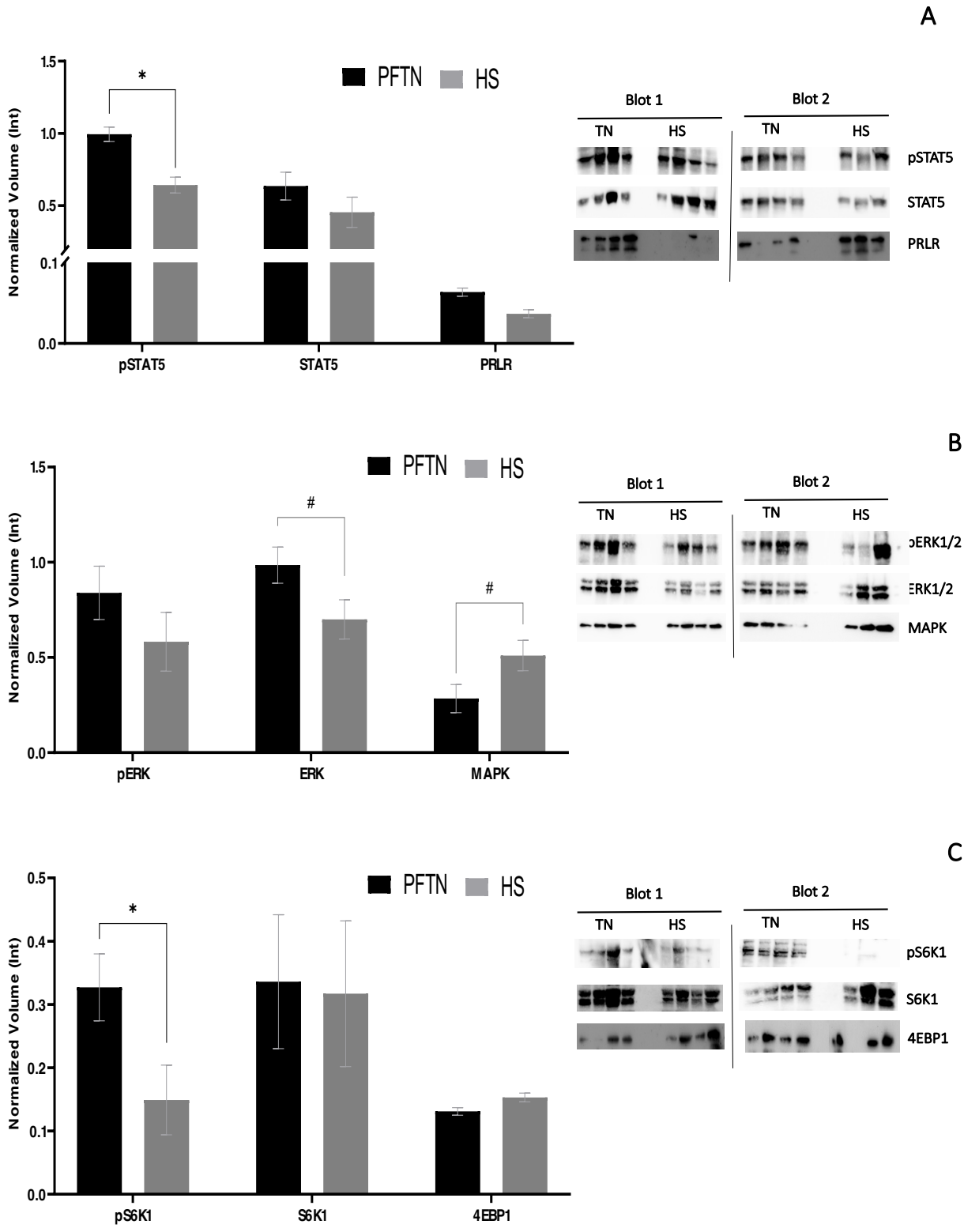
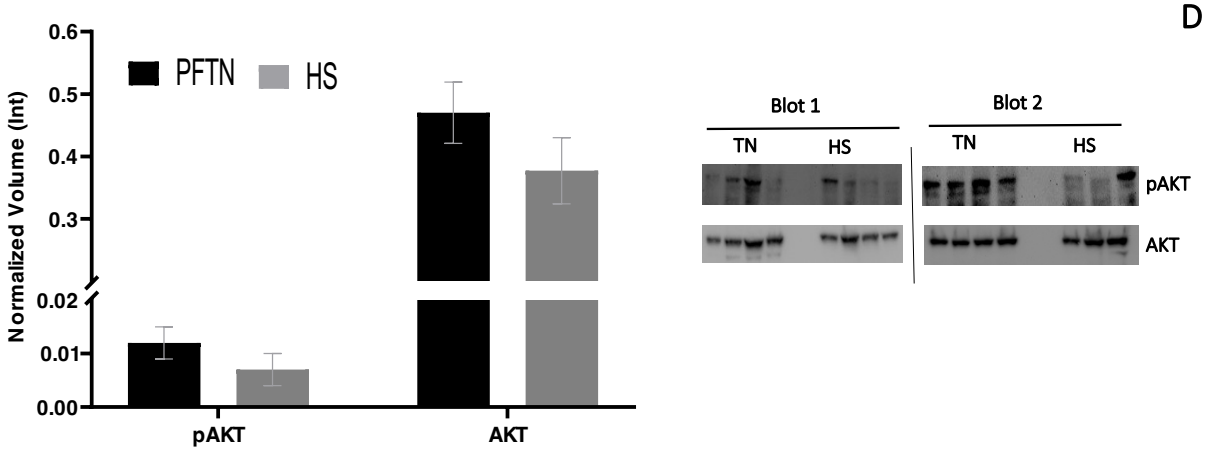


Figure 4.3. Protein relative abundance from targets involved in the JAK-STAT signaling pathway (A), MAPK signaling cascade (B), mTOR pathway (C & D), and autophagy (E) in mammary gland tissue samples from lactating Holstein cows exposed to thermoneutral (PFTN; black bars; n = 8) or heat stress (HS; gray bars; n = 7) conditions for four days. Data presented are least-square means ± standard error of the mean. An asterisk indicates a statistically significant difference ($P < 0.05$) and a pound sign indicates a tendency ($0.05 < P \leq 0.15$) between treatments.


A
PFTN
HS
Normalized Volume (Int)
1.5
1.0
0.5
0.1
0.0
*
pSTAT5
STAT5
PRLR
Blot 1
Blot 2
TN
HS
TN
HS
pSTAT5
STAT5
PRLR
B
PFTN
HS
Normalized Volume (Int)
1.5
1.0
0.5
0.0
#
#
pERK
ERK
MAPK
Blot 1
Blot 2
TN
HS
TN
HS
pERK1/2
ERK1/2
MAPK
C
PFTN
HS
Normalized Volume (Int)
0.5
0.4
0.3
0.2
0.1
0.0
*
pS6K1
S6K1
4EBP1
Blot 1
Blot 2
TN
HS
TN
HS
pS6K1
S6K1
4EBP1


D
Normalized Volume (Int)
0.6
0.5
0.4
0.3
0.02
0.01
0.00
PFTN
HS
pAKT
AKT
Blot 1
Blot 2
TN
HS
TN
HS
pAKT
AKT

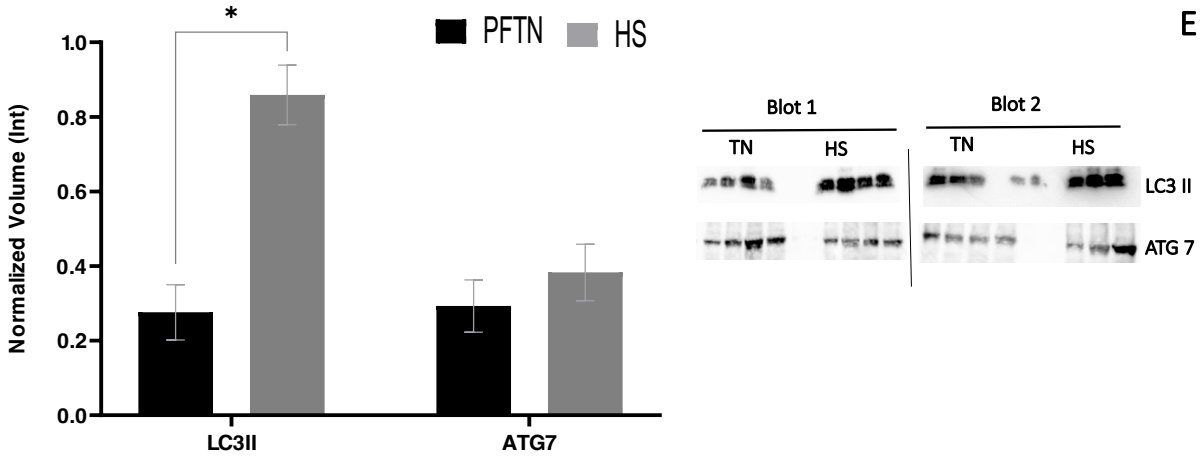

E
*
Normalized Volume (Int)
1.0
0.8
0.6
0.4
0.2
0.0
PFTN
HS
LC3II
ATG7
Blot 1
Blot 2
TN
HS
TN
HS
LC3 II
ATG 7

Figure 4.4. Ratio of phosphorylated to total protein from lactating Holstein cows exposed to thermoneutral (PFTN; black bars; n = 8) or heat stress (HS; gray bars; n = 7) conditions for four days. Data presented are least-square means ± standard error of the mean.

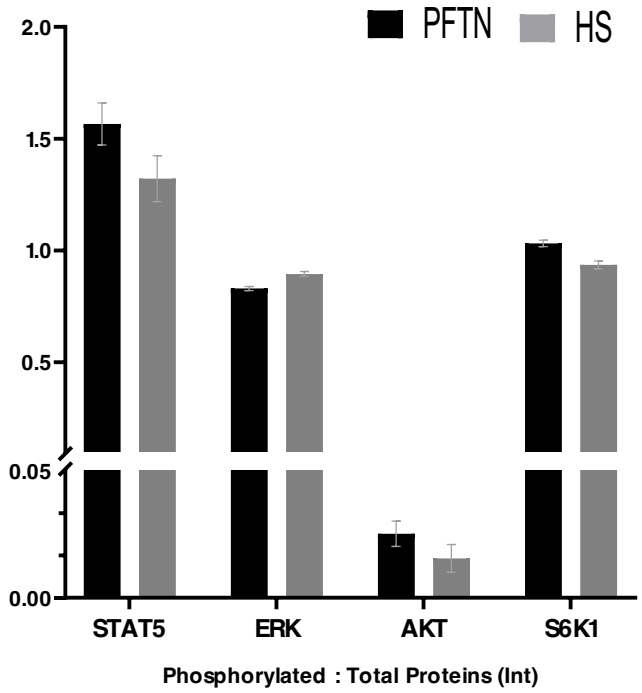
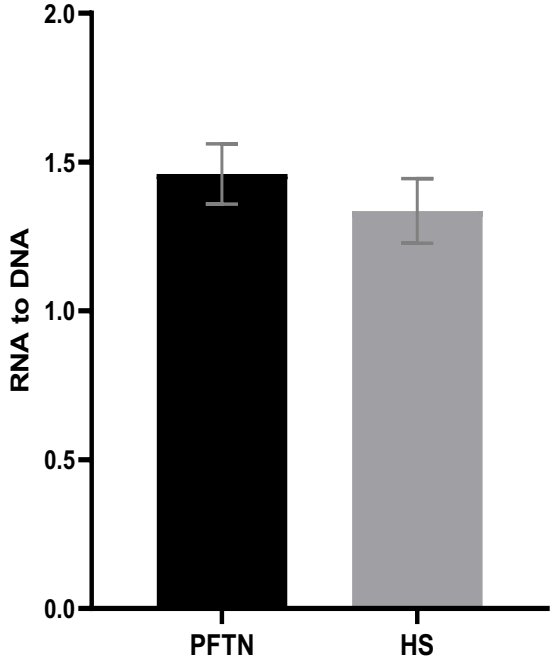
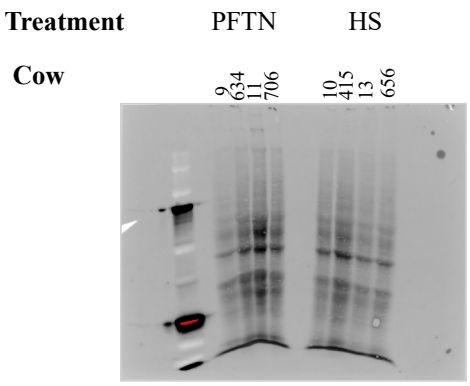


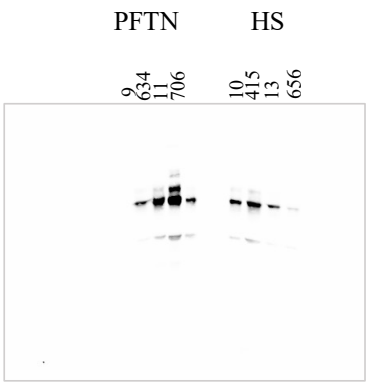
Figure 4.5. Ribonucleic acid to deoxyribonucleic acid abundance from lactating Holstein cows exposed to thermoneutral (PFTN; black bars; n = 8) or heat stress (HS; gray bars; n = 7) conditions for four days. Data presented are least-square means ± standard error of the mean.



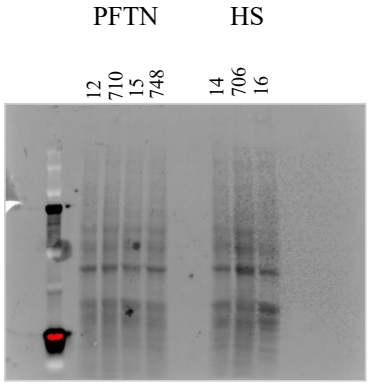
APPENDIX – Immunoblotting results



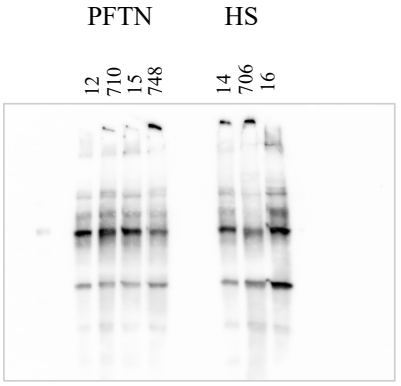
Phosphorylated STAT5
Stain free
Blot 1



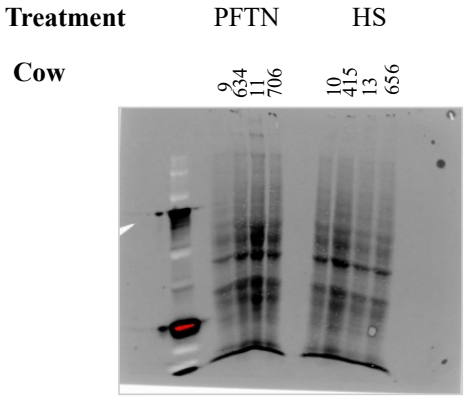
Phosphorylated STAT5
Chemiluminescence
Blot 1



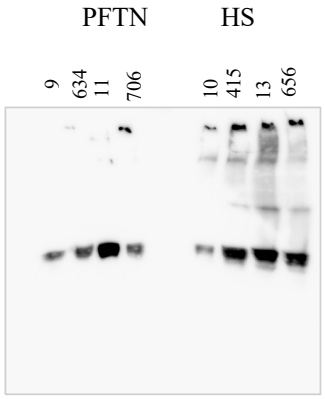
Phosphorylated STAT5
Stain free
Blot 2



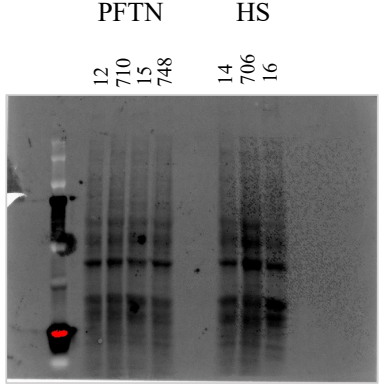
Phosphorylated STAT5
Chemiluminescence
Blot 2



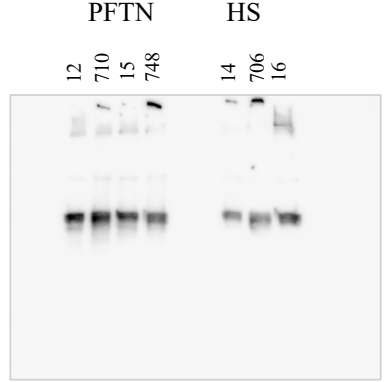
Total STAT5
Stain free
Blot 1



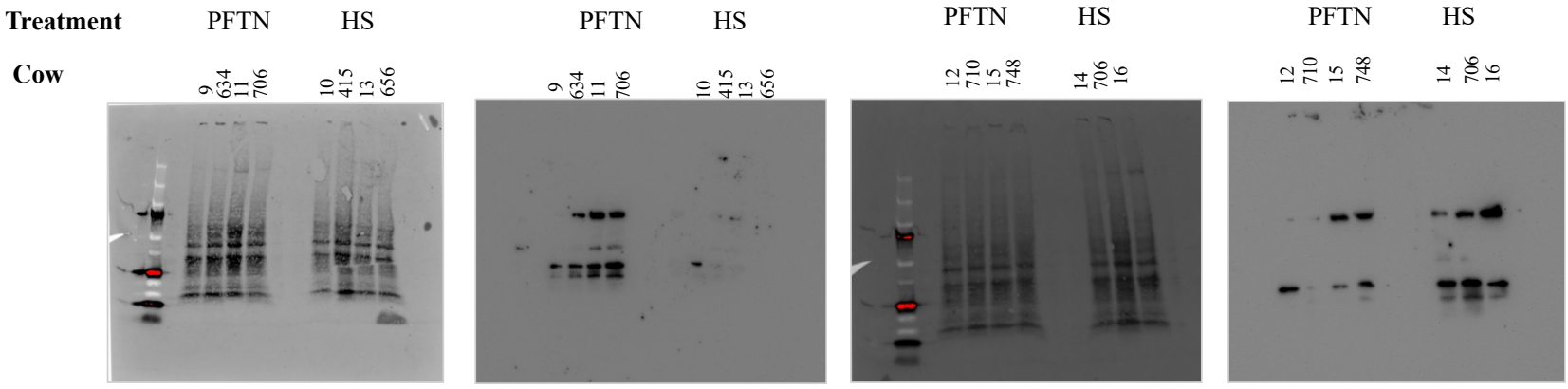
Total STAT5
Chemiluminescence
Blot 1



Total STAT5
Stain free
Blot 2



Total STAT5
Chemiluminescence
Blot 2

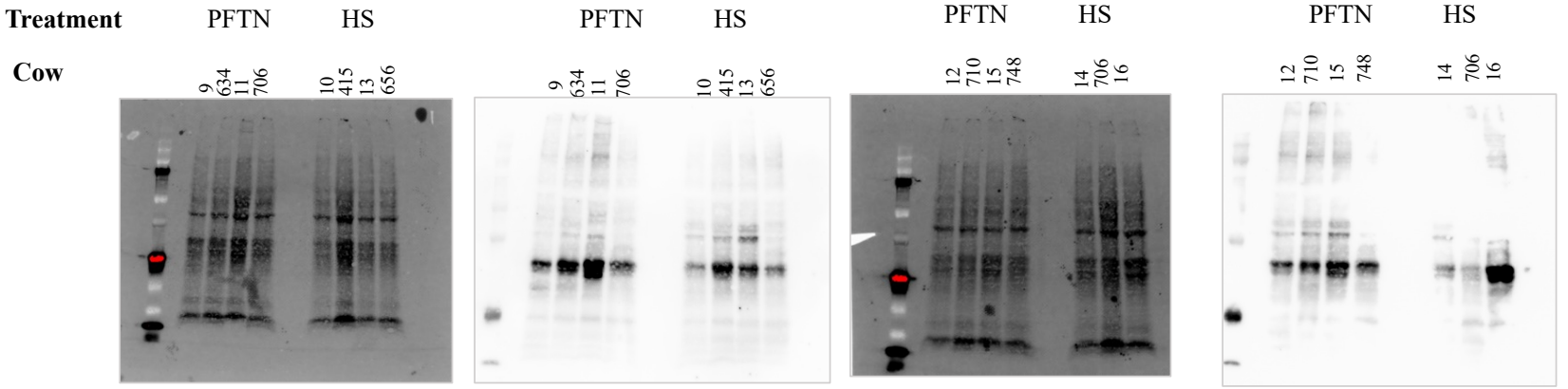


Total Prolactin Receptor
Stain free
Blot 1

Total Prolactin Receptor
Chemiluminescence
Blot 1

Total Prolactin Receptor
Stain free
Blot 2

Total Prolactin Receptor
Chemiluminescence
Blot 2

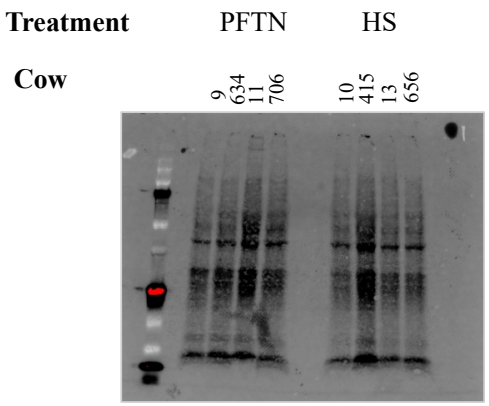


Phosphorylated ERK1/2
Stain free
Blot 1

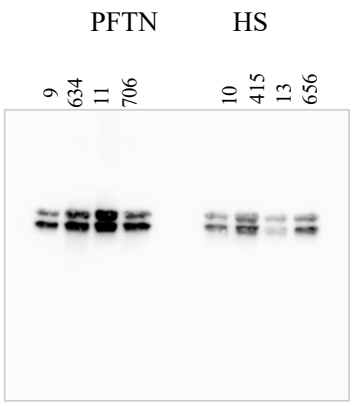
Phosphorylated ERK1/2
Chemiluminescence
Blot 1

Phosphorylated ERK1/2
Stain free
Blot 2

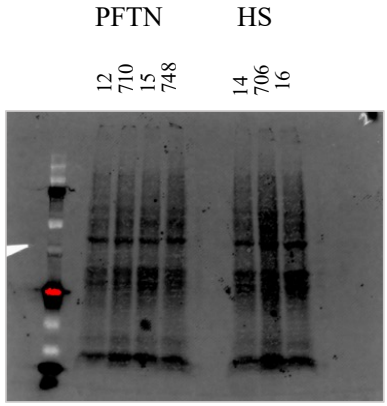
Phosphorylated ERK1/2
Chemiluminescence
Blot 2



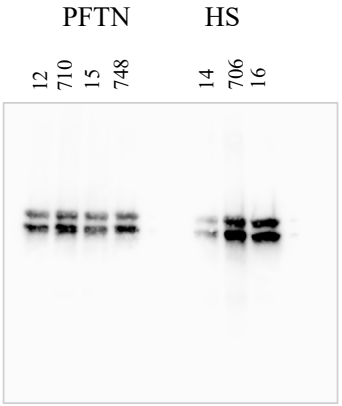
Total ERK1/2
Stain free
Blot 1



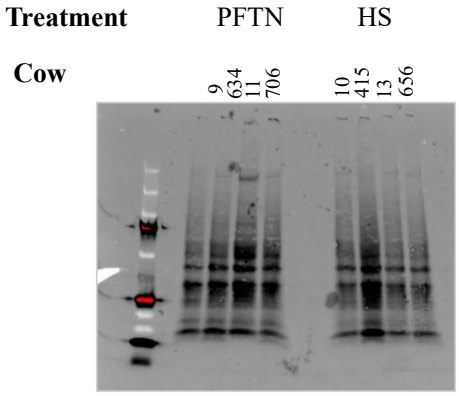
Total ERK1/2
Chemiluminescence
Blot 1



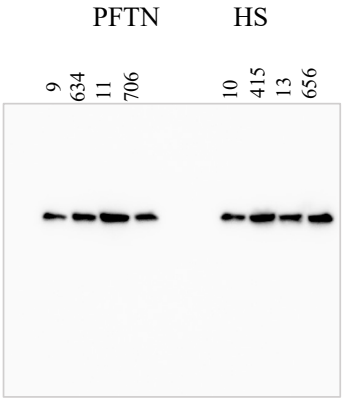
Total ERK1/2
Stain free
Blot 2



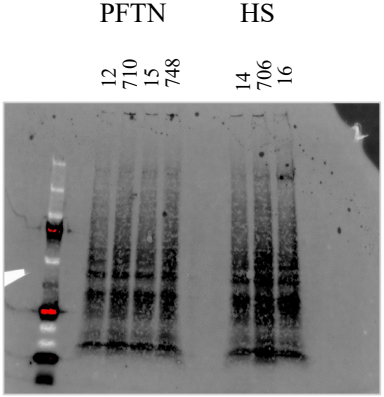
Total ERK1/2
Chemiluminescence
Blot 2



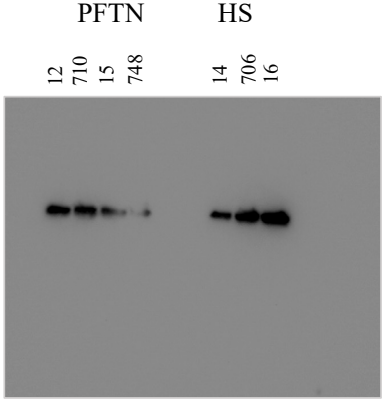
Total MAPK
Stain free
Blot 1



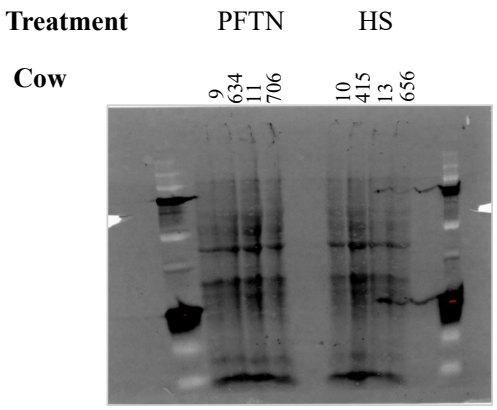
Total MAPK
Chemiluminescence
Blot 1



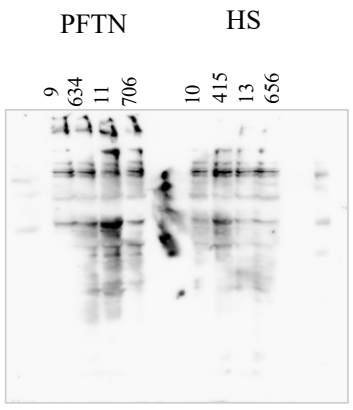
Total MAPK
Stain free
Blot 2



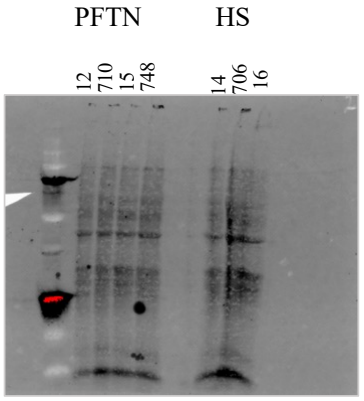
Total MAPK
Chemiluminescence
Blot 2



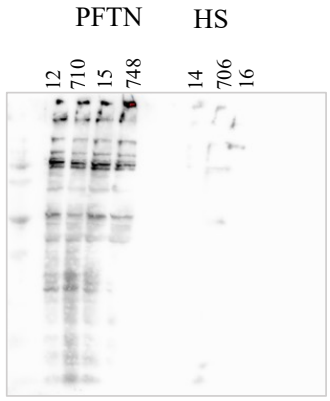
Phosphorylated S6K1
Stain free
Blot 1



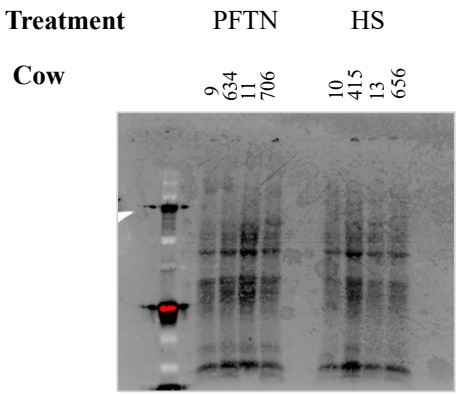
Phosphorylated S6K1
Chemiluminescence
Blot 1



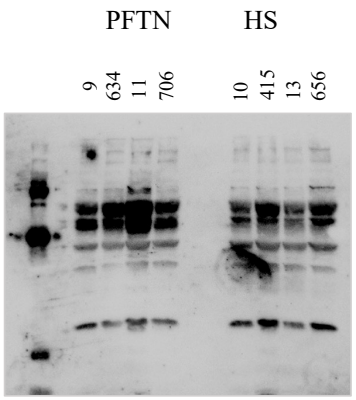
Phosphorylated S6K1
Stain free
Blot 2



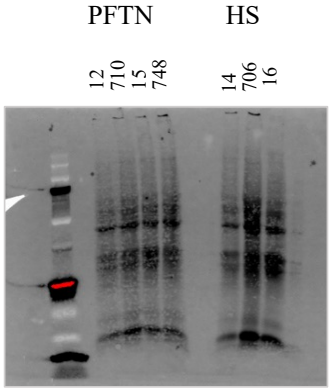
Phosphorylated S6K1
Chemiluminescence
Blot 2



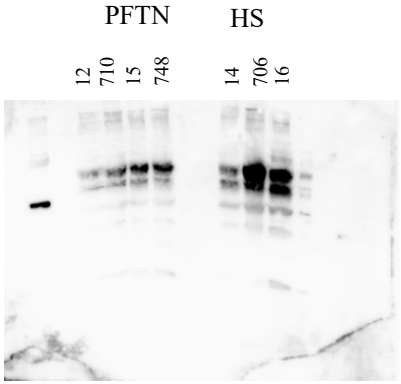
Total S6K1
Stain free
Blot 1



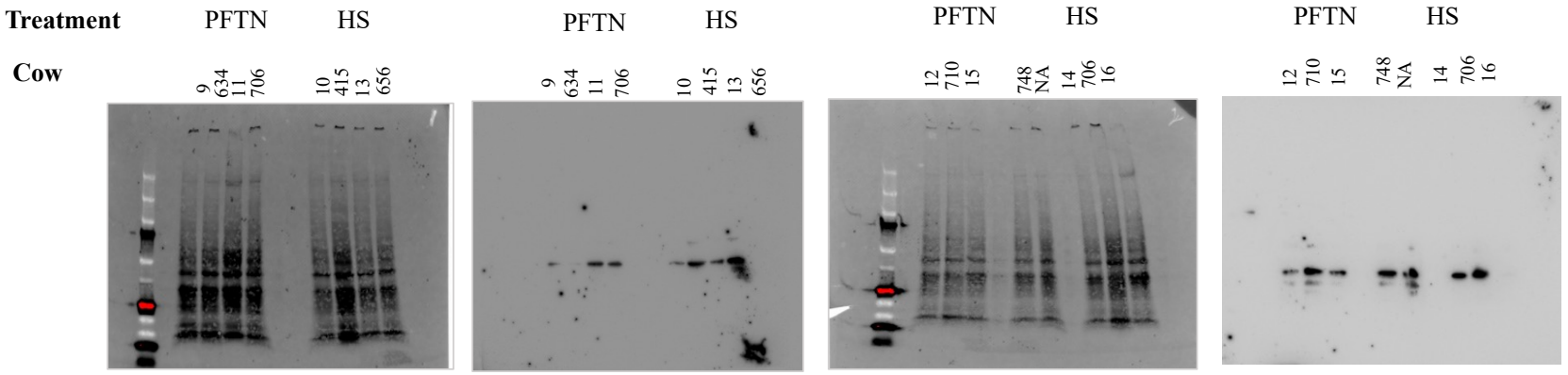
Total S6K1
Chemiluminescence
Blot 1



Total S6K1
Stain free
Blot 2



Total S6K1
Chemiluminescence
Blot 2

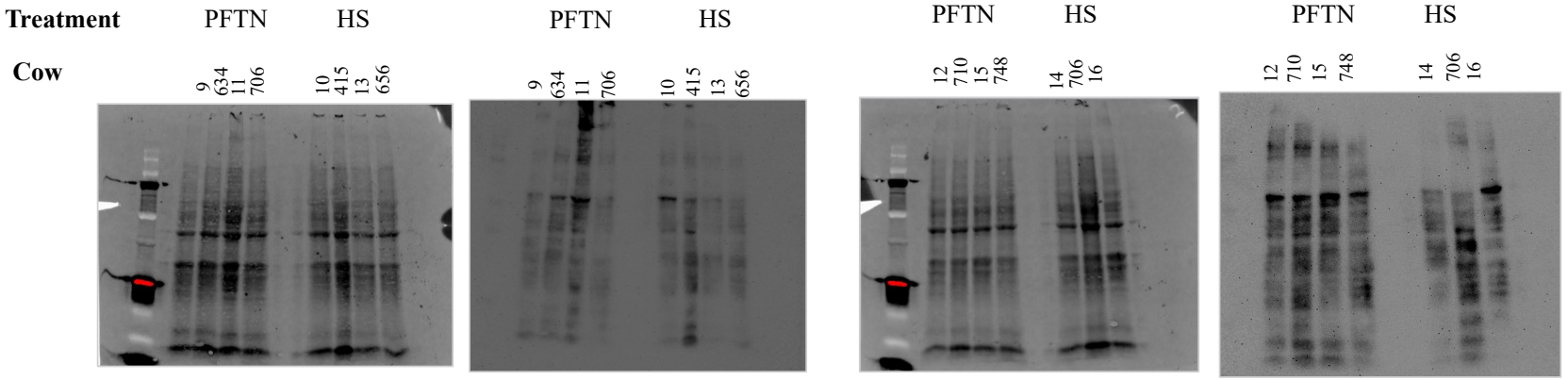


Total 4EBP1
Stain free
Blot 1

Total 4EBP1
Chemiluminescence
Blot 1

Total 4EBP1
Stain free
Blot 2

Total 4EBP1
Chemiluminescence
Blot 2

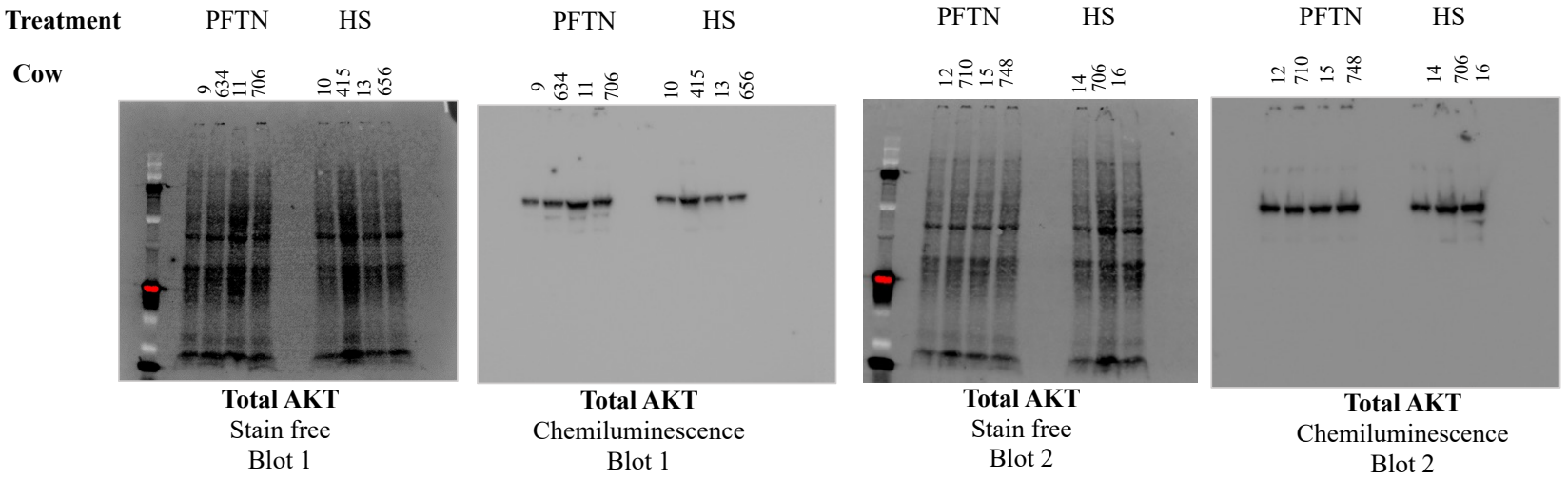


Phosphorylated AKT
Stain free
Blot 1

Phosphorylated AKT
Chemiluminescence
Blot 1

Phosphorylated AKT
Stain free
Blot 2

Phosphorylated AKT
Chemiluminescence
Blot 2

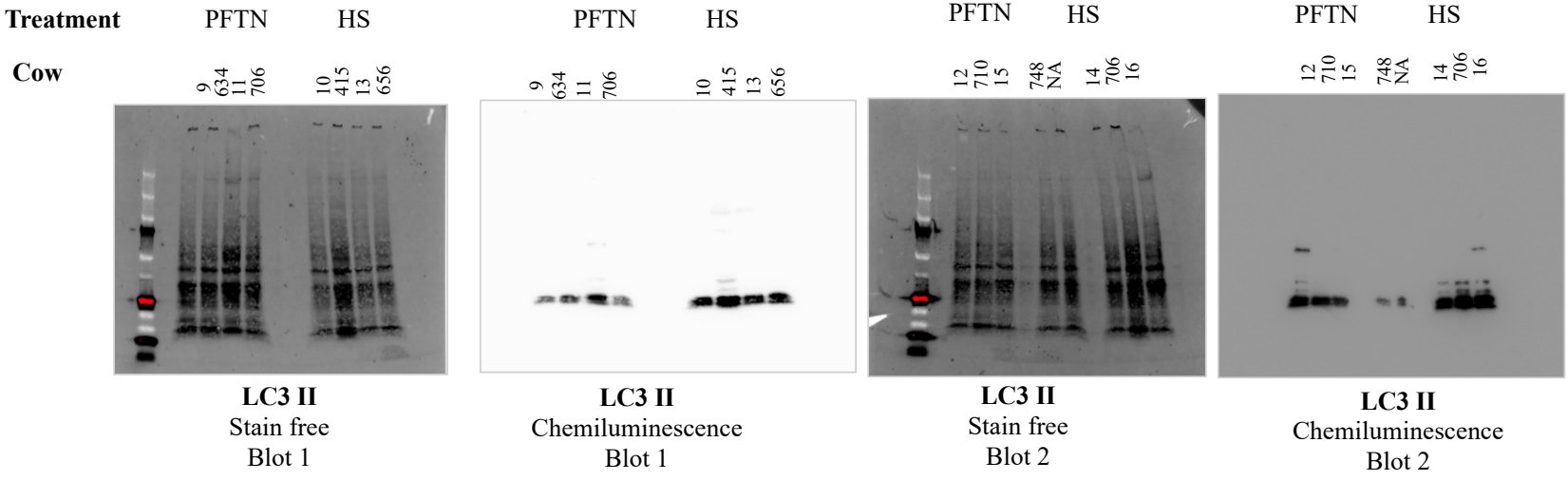


Total AKT
Stain free
Blot 1

Total AKT
Chemiluminescence
Blot 1

Total AKT
Stain free
Blot 2

Total AKT
Chemiluminescence
Blot 2

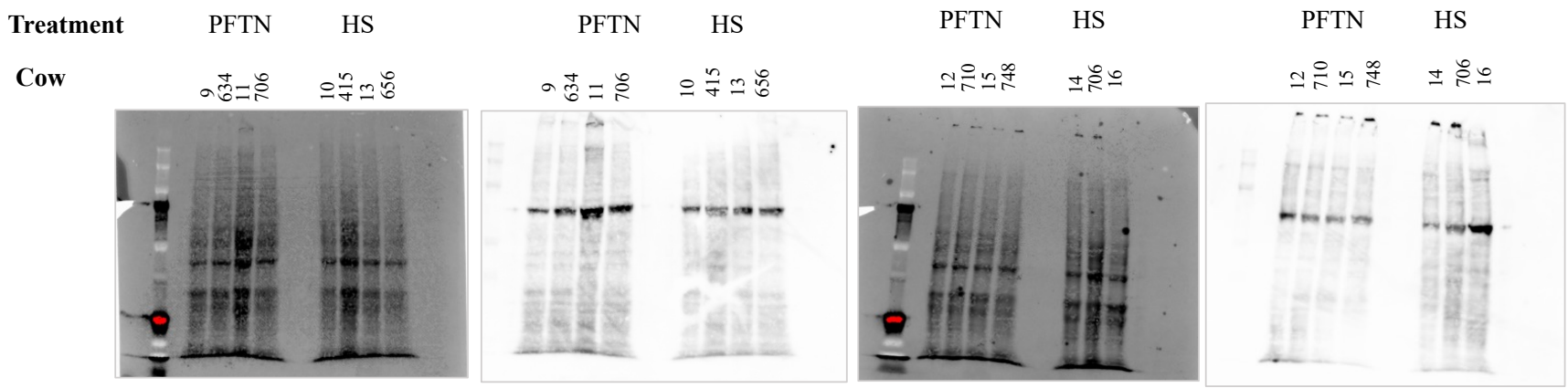


LC3 II
Stain free
Blot 1

LC3 II
Chemiluminescence
Blot 1

LC3 II
Stain free
Blot 2

LC3 II
Chemiluminescence
Blot 2



ATG 7
Stain free
Blot 1

ATG 7
Chemiluminescence
Blot 1

ATG 7
Stain free
Blot 2

ATG 7
Chemiluminescence
Blot 2

CHARACTERIZATION OF MILK-DERIVED CELL HETEROGENEITY IN MILK FROM HEALTHY BOVINE MAMMARY GLAND

INTRODUCTION

Bovine lactation is characterized by substantial milk production over a prolonged period and has resulted in extensive research, leading to a broad understanding of milk synthesis regulation in the bovine mammary gland. Milk production is an integrative response to the functional interaction of many cell types in the bovine mammary gland supporting milk synthesis including secretory mammary epithelial cells (**MEC**), myoepithelial cells, adipocytes, fibroblasts, endothelial cells, and immune cells. Milk also contains cells that originate from the mammary gland and are referred to as somatic cells. The somatic cells found in milk primarily consist of immune cells (Sharma et al., 2011) and secretory mammary epithelial cells (Boutinaud and Jammes, 2002a).

The characterization of specific molecular mechanisms within cell subpopulations has been challenging due to technology limitations. Moreover, the difficulty of cell identification is further exacerbated when dealing with uncommon cell subpopulations. However, in recent years, advancements in molecular technologies have facilitated the identification of numerous cell types within immune and epithelial cells present in human and bovine milk (Becker et al., 2021; Gleeson et al., 2022). The diverse range of cell subtypes present within these two primary subpopulations in milk makes milk a valuable and easily accessible sample source for gaining insight into fundamental aspects of lactation. This non-invasive approach allows for the examination of molecular and cellular features, providing a deeper understanding of the intricate processes occurring in the mammary gland (Martin Carli et al., 2020). As a result, the utilization of milk samples for investigating and elucidating the mechanisms occurring in the mammary gland has gained significant relevance. Still, somatic cells found in bovine milk exhibit complex

heterogeneity which hinders the precise delineation of individual cell type functionality and their roles within a heterogeneous group.

Technologies such as fluorescence-activated cell sorting (**FACS**) and RNA sequencing help address the challenges posed by cell heterogeneity (Chattopadhyay et al., 2014). However, these technologies have limitations including limits on the number of subpopulations that can be identified and the dilution of rare cell types in the bulk gene expression pattern reducing the complexity and diversity of cell heterogeneity. Recently, the development of new research technologies such as single-cell RNA sequencing (**scRNA-seq**) with the capacity to identify low abundance cell types and compare the transcriptome profile of individual cells within a heterogeneous sample has facilitated tracing molecular identities occurring at the individual cell level (Villani et al., 2017; Papalexi and Satija, 2018). This study aimed to identify and recognize the functional diversity within epithelial and hematopoietic cell subpopulations in milk from healthy Holstein cows using scRNA-seq technologies.

MATERIALS AND METHODS

Experimental design

The use of animals and all procedures for this investigation were approved by the Virginia Tech Institutional Animal Care and Use Committee (21-220). Twenty five healthy Holstein cows across different parities, lactation day, milk yield and pregnancy status were used in the experiment. Cows remained in the main dairy herd and were housed in a sand-bedded free-stall barn at the Virginia Tech Dairy Science Complex - Kentland Farm throughout the experiment. Cows were milked twice daily at 0100 and 1300 h and milk yield was recorded at the morning milking. Cows had ad libitum access to a total mixed ration balanced for milk production and composition. Cows were fed once daily (0800 to 1000h) and had free access to clean water. The trial was conducted from February to July 2022.

Flow cytometry and fluorescence activated cell sorting

On the day prior to milk sample collection, health and consistent milk production in the last week were assessed for each cow using the farm records (PCDART Software; program available from Dairy Records Management Systems (Raleigh, NC)). On collection day, individual representative samples (3.8 L) from 2 to 4 eligible cows were collected at the morning milking. A milk subsample from each cow (35 mL) was sent to the Lancaster Dairy Herd Improvement Association (Manheim, PA) and analyzed for somatic cell count, fat, protein, and solids (CombiFoss™ 7, Foss North America). Milk samples (n = 25) were processed in the laboratory for milk cell isolation (Lengi et al., 2021). Briefly, milk samples containing a final concentration of 0.5 mM ethylenediaminetetraacetic acid **(EDTA)** were centrifuged (850 g for 10 minutes) to pellet total cells present in milk. The pellet was washed once with Dulbecco's phosphate-buffered saline (**DPBS**) and EDTA (0.5 mM final concentration), centrifuged, re-suspended in red blood

cell lysis buffer for 15 min, and filtered through 100- and 40-µm sterile cell strainers (Genesee Scientific) to remove noncellular debris. Cell number was evaluated in each sample using a hemocytometer and samples were standardized to contain 2×10^6 and 2×10^7 cells for flow cytometry and fluorescence activated cell sorting (**FACS**) analysis, respectively. In order to label individual cells, primary antibodies for hematopoietic cell surface protein (**CD45** clone CACTB51A, Kingfisher Biotech, 3.1 ng/µL), macrophages (**CD14** clone CAM 36A, Kingfisher Biotech, 1.25 ng/µL), and mammary epithelial cells (Butyrophilin 1A1 (**BTN**), clone MAB8467 conjugated to APC, NOVUS Biologicals, 7 ng/µL) were used. Secondary antibodies used were rat anti-mouse IgG2a-phycoerythrin ((**PE**) clone SB84a, Southernn Biotech Associates, 1.0 ng/µL) and goat anti-mouse IgG1-AlexaFluor 488 ((**AF488**), polyclonal, Southern Biotech Associates, 1.25 ng/µL). Cell viability was determined using propidium iodide (PI; BD Biosciences, 5 µg/mL) dye, and Hoechst 33342 (Invitrogen, 10 µg/mL) was used as a nucleic acid stain.

For primary and secondary antibody labeling, cells were re-suspended in 100 and 1000 µL of cell staining buffer (BD Biosciences) for flow cytometry or FACS and incubated in the dark for 1 hour at room temperature. Cells were washed between antibody incubations, centrifuged at 850 g for 10 min, and re-suspended in Hoechst and PI for 60 min. After a final wash, cells were re-suspended in 100 or 1000 µL of Cell Staining Buffer and examined by flow cytometry or sorted by FACS. Flow cytometry and FACS analyses were performed on a BD FACSAria (BD Biosciences) using FACSDiva software (BD Biosciences). Side and forward scatter (SSC and FSC) thresholds were defined to eliminate cellular debris and aggregates. Gates used to discriminate positive and negative staining cells were set and these gates were applied consistently to all samples, allowing for minor adjustments for SSC variability. Live and nucleated cells were

selected by gating on PI and Hoechst, and different subpopulations were selected by gating on cells triple-labeled with CD45-PE, CD14-AF488, and BTN-APC.

All samples (n=25) were subjected to flow cytometry analysis, but only five of these samples were further sorted using FACS. Sorted samples were used for single-cell RNA sequencing analysis. Sample selection for scRNA-seq was based on cell composition consistency and levels of BTN^+ cells. Using preliminary data, we estimated that 40% of somatic cells are immune cells ($CD45^+$) and 1.5% are mammary epithelial cells (BTN^+). We chose to select cows with consistently higher proportions of BTN+ cells to reduce the dilution effect of $CD45^+$ cells on BTN^+ cells.

Single-cell RNA library construction

Sorted cells, obtained approximately 12 h after sampling time, were prepared for further scRNA-seq library preparation following the manufacturer's instructions (Chromium Next GEM Single Cell 3' Low Throughput, 10X Genomics, Pleasanton, CA, United States). Sorted cells ($BTN^+CD14^+CD45^-$ or $BTN^-CD14^-CD45^+$) from individual samples were diluted in resuspension buffer to achieve a concentration of 100-600 cells/µl for downstream analysis. Cell suspension was loaded into a master mix containing reverse transcription reagent B, template switch oligo, reducing agent B, and reverse transcription enzyme C plus nuclease-free water with a targeted cell recovery after sequencing for all samples from 500 to 1000 cells (1000 cells maximum cell number allowed per library). For library preparation, cell suspension, barcoded coated gel beads with oligonucleotides and partitioning oil were loaded into a 10X Chromium Next GEM Chip L (10X Genomics) and combined in the Chromium Controller (10X Genomics) using a microfluidics-based method to generate single-cell gel beads-in-emulsions (**GEM**). Within GEM and for each sample, polyadenylated mRNA was reverse transcribed into cDNA, and the resulting cDNA was

amplified for a total of 12 cycles (98°C for 3 min, 98°C for 3 secs, 63°C for 20 secs and 72°C for 1 min). Post cDNA amplification, cDNA concentration and sample quality were assessed using an Agilent TapeStation (High Sensitivity D5000). A fraction (10 µl) of the amplified and cleaned cDNA was fragmented using fragmentation buffer, fragmentation enzyme, buffer EB, and incubated at 32°C for 5 min, at 65°C for 30 min, and kept at 4°C until further analysis. Adaptor ligation used ligation buffer, DNA ligase, and adaptor oligos, followed by a 30°C incubation for 30 min. After post-ligation clean up (SPRIselect, Beckman Coulter, IN, USA), individual sample index sets (Dual Index Plate TT Set A, 10X Genomics) were added and incubated for 10 to 12 cycles depending on cDNA input (150 to 1000 ng) for 45 secs at 98°C, 20 secs at 98°C, 30 secs at 54°C, and 20 secs at 72°C. Resulting cDNA sequencing libraries were evaluated for DNA concentration using high sensitivity Agilent TapeStation (D5000) analysis, DNA peak was set to be between 240 and 460 bp.

Single-cell RNA sequencing and bioinformatics analysis

Individual sequencing libraries were sent to Novogene Sequencing Center for pair-end sequencing on an Ilumina HiSeq 6000 platform system (Novogene, Sacramento, CA, United States) using one lane per sample. The sequenced reads were subjected to processing and analysis using the Cell Ranger pipeline v7.0.0 by 10X Genomics, as described in Zheng et al. (2017). In summary, the FASTQ files were aligned to the Bos Taurus ARS-UCD1.2 genome using the default parameters and the Spliced Transcripts Alignment to a Reference (**STAR**; Du et al., 2020) aligner, as implemented in the cell ranger count pipeline. The subsequent step involved filtering the cell barcodes to ensure data quality and reliability. Mapped sequences from each library were used for unique molecular identifiers (**UMI**) counting. Reads generated by barcode-associated cells were quantified and used for establishing a UMI count matrix.

The aggregate pipeline from Cell Ranger by 10X Genomics was used to combine the data from the three MEC libraries. Further analysis for MEC data was completed by the Cell Ranger pipeline which included initial clustering, optimization, and hierarchical clustering in principal component analysis (**PCA**). For differential gene expression between clusters, the software method used was sSeq and edgeR. Mammary epithelial cells were clustered based on enriched expression ($P < 0.05$) of cell type specific markers. Data visualization for clustering and gene expression was carried out using Loupe Cell Browser Software (10X Genomics).

Data obtained from immune cell libraries (n = 2), were analyzed individually. To identify cell populations present in both immune cell datasets, we followed the scRNA-seq integration pipeline described by Stuart et al. (2019) using the Seurat package (v 4.3) from R (v. 4.2.2). The count matrix was converted to an object using the Seurat package for individual downstream dataset analysis following the recommended pipeline (Butler et al., 2018; Slovin et al., 2021). Briefly, data was normalized and scaled based on read quality control metrics, dimensionality reduction was carried out, clustering analysis and visualization.

RESULTS AND DISCUSSION

Heterogeneity of cells in bovine milk samples identified by flow cytometry

The standard technique to study the bovine mammary gland, a heterogeneous organ, at the cell level is through mammary gland biopsies. Nevertheless, bovine mammary biopsies can introduce a variety of risks and pitfalls (i.e., pain and discomfort in the animal, post-biopsy infection, and tissue fibrosis in biopsy site, non-representative sample of the gland). Recently, the use of non-invasive "milk liquid biopsies" that can be regularly obtained easily eliminate related surgical biopsy pitfalls and mimic the results obtained from mammary gland biopsies (Martin Carli et al., 2020). The collection of somatic cells present in milk is composed of secretory mammary epithelial cells and immune cells. It is known that secretory mammary epithelial cells result from the desquamation of the mammary epithelium of alveoli and ducts (Alhussien and Dang, 2018). In contrast, immune cells of a hematopoietic origin are white blood cells that enter the mammary gland in response to an injury or infection (Boutinaud and Jammes, 2002b; Sharma et al., 2011). Bovine MEC present in milk originate from the mammary gland. These cells are responsible for milk synthesis and are extensively studied due to their well-defined function. The definition of the extended cell profile and transcript expression of the bovine mammary gland during lactation is essential for a better understanding of the factors determining milk production.

Fresh milk samples from twenty-five multiparous Holstein cows were sampled across 6 months. Descriptive statistics for the cows used for milk collection and milk sample characteristics are presented in Table 5.1. To evaluate heterogeneity of the cells present in milk by flow cytometry, we used three cell surface markers for MEC (BTN), cells of hematopoietic origin (CD45), and macrophages (CD45 and CD14) that resulted in eight subpopulations (Table 5.2). According to flow cytometry analysis, the overall average including both live and dead MEC, total

immune cells, and macrophages observed in this study was 2.5%, 55.9%, and 8.7%, respectively. As expected, the main cell type present in milk was live hematopoietic cells ($CD45^+$) with 34.3% of the total cells, while the lowest concentration cell type present in milk was live MEC (BTN^+) with 0.8% of the total cells. The utilization of BTN, a protein involved in fat droplet secretion, ensured the isolation of only secretory MEC, excluding ductal epithelial cells, which are traditionally considered non-secretory epithelial cells and therefore not expressing BTN due to lack of milk fat secretion. The average cell yield was $152x10^6$ cells and the concentration was 8389 cells/mL independent of cell type.

The hematopoietic cell proportion in somatic cells observed here is smaller than previous reports with 87% of hematopoietic cells in milk samples from Holstein (< $100x10^3$ SCC/mL). From the total hematopoietic cells, 42%, 11%, and 34% were granulocytes, lymphocytes, and monocytes (Koess and Hamann, 2008). Similarly, macrophage percentages present in milk samples in this study were smaller than what was observed by De Matteis et al. (2020) where milk samples from multiparous Holstein cows at 2 weeks postpartum contained 14% macrophages identified by flow cytometry ranging from 2.3% to 36%. On the other hand, the average of MEC observed in this study aligns with previous reports where live MEC in milk from healthy Holstein cows averaged 1.2% and were the least abundant of the somatic cell types (Lengi et al., 2021).

Excluding the three main subpopulations measured, 32.4% of remaining cells had an unidentified identity that expressed a combination of the cell surface markers used in this study suggesting potential heterogeneity within each subpopulation. However, due to technical capabilities such as wavelength overlap between available fluorescent dyes for flow cytometry, there is limited use of markers for subpopulation identification. To further explore cell identity between the main subpopulations identified by flow cytometry, milk samples from five cows were

used for further scRNA-seq analysis. Milk samples from three and two different Holstein cows were used for the evaluation of the MEC (BTN^+) and hematopoietic cells ($CD45^+$) subpopulations, respectively.

Heterogeneity within mammary epithelial cells – BTN^+

To complete single cell analysis of MEC, fresh milk samples were obtained from three multiparous Holstein cows (2.7±0.6 lactations), averaging 361±46 DIM, milk yield of 14.2±4.7 kg/milking, and averaging a somatic cell count of 115±39x10^3 cells/mL (Table 5.3). Milk samples were processed and sorted by FACS using BTN as the cell surface marker for MEC analysis. Subsequently, sorted MEC were used to construct three individual sequencing libraries. Because of the type of chip used (Low Throughput) with a maximum target cell recovery of 1000 cells, the low amount of MEC obtained in two out of the three sampled cows, and the inherent small amount of MEC present in bovine milk, data obtained after sequencing analysis for each library were aggregated and analyzed as one dataset. After data quality evaluation and normalization, the aggregate dataset yielded a total of 839 cells for scRNA-seq analysis. As depicted in Figure 5.1A, from the 839 cells, the three sampled cows contributed 526, 195, and 118 cells and generated 7,092 total features analyzed.

Based on initial nearest-neighbor cluster analysis, sorted bovine MEC in milk showed an overall homogeneity displaying one principal cluster that contained four heterogeneous subclusters of cells expressing transcripts at distinctive levels (Figure 5.1B). The subcluster conformational proportion for MEC present in milk was 35.9%, 22.4%, 21.2%, and 20.5%, for A, B, C, and D subclusters, respectively. From the three cows sampled, one cow contributed to 63% of the total MEC present in the aggregate sample. We recognize that some of the differences detected between the four subclusters could be influenced by the intrinsic variation existing between cows. Although

cells from the three sampled cows are present in each cell subcluster, cells from cows 1 and 2 are present in a greater proportion in subclusters A and C, while cells from cow 1 make up the greatest proportion for subclusters B and D.

To show subcluster differentiation within the aggregate sample we explore the top seventeen up and downregulated genes visualized by heatmap analysis (Figure 5.1C). The mitochondrial *ND* genes which provide instructions for the NADH dehydrogenase synthesis were specifically upregulated in subcluster A, while in this subcluster the casein-related genes including *CSN1*, *CSN2*, and *CSN3* were downregulated, and a notable downregulation of *H2AC6* and *MT2A* was observed. Contrary to this, in cluster B, the *ND* genes were downregulated significantly and casein-related genes and *GLYCAM1*, *PAEP*, and *H2AC6* were upregulated. Subcluster C showed a similar pattern to cluster A, however, casein genes in this subcluster were undifferentiated, and the *ND* related genes upregulation expression was not extensive. Finally, subcluster D showed a mild downregulation of the *ND* and casein-related genes with an upregulation of the *MT2A* gene.

To further explore the differentiation between subclusters within MEC and identify cell heterogeneity, we analyzed cell and subcluster expression of the main transcripts differentiating MEC subclusters using violin and feature plots (Figure 5.2). These results are associated with the heat map analysis depicting an upregulation of *ND5* and *ND1* in most of the cells present in subcluster A. The milk protein related genes *CSN2*, *CSN3*, and *CSN1S1* had a similar expression in subclusters B, C, and D, and limited expression in cluster A. However, the expression of *CSN1S2* was only upregulated in most of the cells present in clusters B and C, while *LALBA* was expressed at low levels in subclusters B, C, and D. Other genes, including *COX2* and *ATP6* were present in all subclusters with cells depicting upregulation in clusters A and C. Furthermore, we evaluated the transcript expression of classically recognized genes expressed in secretory MEC

and one apoptosis related gene including *PRLR*, *BTN1A1*, *ESR1*, *ESR2*, *PGR*, and *STAT3* (Figure 5.3).

The differential expression of hormone receptors and MEC distinctive genes in milk derived bovine MEC in this study was minor. The minimal expression of these classical transcripts in MEC including *PRLR*, *BTN1A1*, *ESR1*, and *PGR* in our sample was unanticipated. Previous studies using scRNA-seq analyses reported an upregulation of gene expression of *ESR1*, *PRLR*, *PGR*, *CSN2*, and *LALBA*, in MEC sorted using the epithelial cell adhesion activating molecule (EpCAM) as cell surface marker from mammary gland tissue samples in pregnant, lactating, and gestating mice (Bach et al., 2017b). Nevertheless, agreeing with what was observed in this study on bovine MEC in milk, human MEC display no expression and very little expression of progesterone receptor (*PGR)* and estrogen receptor (*ESR1)*, and only around of 25% of the cells expressed *PRLR*. The differential expression of hormone receptors can be attributed to the type of sample used. It is plausible that MEC present in milk exhibit a distinct state that leads to cell shedding into milk, which may not occur in MEC found in functional and actively secreting mammary tissue. On the other hand, the expression of *LALBA*, *CSN2* and *CSN3* were expressed in all milk derived human MEC analyzed, and *BTN1A1* was only expressed in 75% of the cells (Martin Carli et al., 2020). Furthermore, in mononuclear mammary epithelial cells from bovine milk, only *CSN1S1-S2*, *CSN2*, *CSN3*, and *LALBA* were upregulated, however, the expression of hormone receptors was not reported (Becker et al., 2021).

The limited expression of classical mammary epithelial cell MEC transcripts, despite using a validated cell surface marker for sorting (Lengi et al., 2021), may be attributed to a distinct profile of MEC shed in milk compared to MEC obtained through biopsy. MEC obtained through biopsy, which are attached to the basement membrane, are considered functional cells. It is plausible that

MEC shed in milk are no longer functional and may be undergoing cell death and detachment or cell death as a result of detachment. The mammary epithelium parenchyma is characterized by a bilayer hollow cavity that is enclosed by a basal membrane. The inner monolayer is formed by luminal cuboidal cells facing the central apical cavity and surrounded by an external basal monolayer of myoepithelial cells. If a cell is damaged, loses functionality, or is dying, it can be a threat to the tight barrier that epithelia form. To preserve the integrity of the MEC barrier, live or dying cells are apically or basallly extruded in response to apoptotic stimuli or homeostasis regulation (Slattum and Rosenblatt, 2014; Mleynek et al., 2018). When cells are detached from the basement membrane and no longer have communication with the extracellular matrix and neighboring MEC, this produces an impairment of cell–matrix interaction and the loss of essential signals for survival leading the cells to a programed cell death known as anoikis (Frisch and Francis, 1994). Anoikis would result from detachment of viable epithelial cells from the basement membrane (Bretland et al., 2001) and has been implicated in luminal clearance during mammary gland development in mice (Humphreys et al., 1996). Nevertheless, the occurrence of this cell death type in the bovine mammary gland during lactation remains unexplored. Further investigation is needed to shed light on this aspect.

Although only live and viable MEC were used for library construction, the foundation of MEC sorting selection using FACS is based on dye exclusion using propidium iodide by membrane permeability. Propidium iodide penetrates the cell membrane with loss of integrity, entering the cell nucleus and binding double-stranded nucleic acid, while intact membranes from viable cells prevent PI dye penetration and staining. While it has been reported that certain dyes may not effectively label early apoptotic cells (De Schutter et al., 2021), there is no specific evidence of such limitations with propidium iodine. Additionally, it is important to note that not

all cell death types described in the literature display membrane rupture as a characteristic feature, as there are over 50 different mechanisms of cell death documented. In fact, during apoptosis – the major form of programmed cell death described and studied – cell membrane integrity is retained, while non-apoptotic cell death like pyroptosis is mostly characterized by membrane rupture (Zhang et al., 2018; Yan et al., 2020). The differentiation between subclusters might be influenced by the cell stage and the biological processes occurring in it. If the MEC captured and analyzed were undergoing cell death, these cells will exhibit different gene expression levels based on cell death stage. An example of this is the upregulation of the *MT2A* gene in subcluster D. In vitro studies suggest that the upregulation of *MT2A* induces cell apoptosis in promyeloblasts (HL-60 cells) by inhibition and upregulation of Bax and Bcl2 expression (Pan et al., 2021).

Furthermore, the presence of cell death-related transcripts in MEC observed in this study raises the possibility that sorting cells using FACS may have damaged cells or caused death. While limited literature exists on this topic, previous research has shown a significant increase in necrosis and apoptosis, attributed to loss of membrane integrity, in human skin fibroblasts after sorting (Seidl et al., 1999). Additionally, a computer simulation evaluation of energy dissipation and hydrodynamic forces during cell sorting has demonstrated that hydrodynamic forces contribute to substantial cell death in a small fraction of the sorted cells (Mollet et al., 2008). In this study we encountered challenges in obtaining a substantial number of MEC from milk samples. To overcome these challenges and enhance the study of this specific cell type, future investigations could consider utilizing biopsy samples. Biopsy samples could be a better approach as they help elucidate and minimize the variability observed between animals and cell states. Furthermore, the use of functional secretory and viable MEC obtained through biopsies will enable the collection of a greater number of MEC for analysis. This approach will contribute to a deeper understanding

of the heterogeneity of MEC in the bovine mammary gland and enhance our comprehension of milk synthesis capacity.

Heterogeneity within hematopoietic cells – CD45$^+$ positive cells

To address the molecular diversity within cells from hematopoietic origin, fresh milk samples were obtained from two Holstein multiparous cows averaging 2.5±0.7 lactations, 320±72 DIM, 14.5±5.8 kg/milking at collection, and 139±33x10^3 cells/mL (Table 5.3). Cells were sorted by FACS using the CD45$^+$ cell surface marker. After sorting, individual libraries were sequenced, and individual data were integrated and analyzed as one dataset to explore the general heterogeneity within CD45$^+$ cells following the integration method pipeline. The cluster analysis for the integrated data presented in Figure 5.4A exhibited 11 distinctive cell communities across both libraries. T cells were the most abundant cell type and depicted 7 distinctive types within the T cell subpopulation. The remaining 4 clusters were composed of granulocytes, including neutrophils, as well as macrophages and B cells. Integration analysis identified shared cell populations across sample sets, therefore both animals showed the same cell types, however, cell population proportion was different between them (Figure 5.4B). As presented in the cluster graph per sample in Figure 5.4C, cow one depicted a higher number of macrophages, neutrophils, *CD4* helper T cells, and *CD4* Cytotoxic T cells, while cow two had a higher amount of T cells with low expression of CD96 and high expression of *CD3E* (Figure 5.4C). To gain a deeper understanding of the heterogeneity observed between the two cows, we conducted a more detailed exploration of the hematopoietic cell heterogeneity within each milk sample.

The dataset for Cow 1 included 851 cells with 12,156 features where seven clusters were identified. Initially, we conducted verification of the expression of the CD45$^+$ marker, also known by its gene name *PTPRC*. This verification was completed using a violin plot and a feature plot

(Figure 5.5), which supported the accurate sorting of cells. After clustering analysis, seven distinct clusters were identified and are presented in Figure 5.6. According to identified differentially expressed genes after heatmap analysis (Figure 5.7) and the individual analysis of the main expression of canonical immune subpopulation markers (Figure 5.8) reported in the literature (Azizi et al., 2018; Becker et al., 2021), the putative cell subpopulations identified were granulocytes expressing *TLR4* and *CD68* (22.8%), macrophages expressing *CD14* (6.9%), B cells expressing *CD19* and *CD22* (2.1%), and T cells expressing *CD96*, *CD3E*, and *CD3D* with four different identities: regulatory T cells expressing *CD4* and *IL2RA* (26.9%), cytotoxic T cells expressing *CD8A* and *CD8B* (21.7%), helper T cells expressing *CD4* (10.2%), and a group of T cells expressing *CD8A*, *CD8B* and the *ZBTB16* (9.4%).

The dataset for Cow 2 included 923 cells with 17,771 features and eight differentiated clusters. Seven out of the eight clusters identified express the transcript *PTPRC* (encoding CD45) representative of hematopoietic cells as shown in Figure 5.9. The putative identity for each population depicted by cluster analysis is macrophages expressing *CD68*, *TLR4*, and *IL1B* (8.3%), dendritic cells expressing *ICAM1* and *RF4* (4.7%), B cells expressing *CD19*, *CD22*, and *CD79A* (4.3%), and five populations expressing T cells features classified as, helper T cells expressing *CD4* and *IL2RA* (10.9%), cytotoxic T cells expressing *TNF* and *CD8B* (16.6%), a subgroup of T cells expressing *TNF*, *CD28*, and *CD69* (23.9%), T cells expressing *KIT*, *CD69*, and *CD2* (7.2%), and T cells expressing *CAMK4* and *CD69* (24.1%) (Figure 5.10). Population and cell identity were based on the top 10 up- and down- regulated transcripts shown by heatmap analysis (Figure 5.11) and analysis of individual recognized markers of immune cells identities (Figure 5.12).

The presence of hematopoietic cells in milk has been widely reported in multiple species (Witkowska-Zimny and Kaminska-El-Hassan, 2017; Alhussien and Dang, 2018), but the

exploration of cellular heterogeneity within the cell subpopulations has been characterized mainly in cells present in human milk and only one study for cells present in bovine milk is present in the literature. Similar to what has been observed in this study, immune cells present in human milk are composed of the main immune cell types including a high proportion of T cells, monocytes, macrophages and B cells (Twigger et al., 2022). Similarly, immune cell identities including T cells, B cells, macrophages, and dendritic cells have been reported in milk samples from 15 different human donors from 3 to 630 days in lactation. Nevertheless, the immune cell subpopulations described in that study showed a wider range of cell types including *CSN1S1* macrophages, fibroblast, eosinophils, and Langerhans cells (Nyquist et al., 2022).

Expecting possible differences between species, immune cells present in bovine milk from scRNA-seq analysis reported in the literature showed a similar profile to that observed in this study and in immune cells from human milk depicting immune cell communities of macrophages, monocytes, T cells, B cells, dendritic cells, and natural killer cells which have not been reported before in milk (Becker et al., 2021). Clustering analysis from the literature showed five, two, and three different subpopulations of monocytes, macrophages, and T cells, respectively. Within this immune population, most cells in the dataset were monocytes and macrophage, contrasting with the results observed in our study where the major immune cell type identified was T cells, and only a main community of macrophages was identified, while no monocytes were observed. However, it is important to note that the study done by Becker et al. (2021) used a single residual milk sample (200 mL) from one udder quarter which might increase the variation between what was observed in this study in comparison with our investigation.

Neutrophils, the primary type of granulocyte found in milk (Alhussien et al., 2021), play a vital role in the innate immune response by primarily targeting and combatting bacterial infections

(Kobayashi and DeLeo, 2009). Given their known abundance in milk, it was anticipated that a greater number of these cells would be observed in the study. However, neutrophils are terminally differentiated cells with a remarkably short lifespan, serving as the frontline defenders against invading pathogens (Paape et al., 2003).

The limited lifespan of neutrophils, possibly due to their fragility relative to T cells, could produce a difference in the proportions of live cells after cell sorting leading to an enriched profile of T cells in this study. Furthermore, the increased infiltration of neutrophils to infection sites with these samples obtained from clinically healthy animals could have influenced the relative contribution of other immune cells, such as T cells, to the overall sample composition. Consequently, the viability and presence of neutrophils could have played a significant role in shaping the observed immune cell profile. Further investigation and analysis are warranted to explore this intriguing relationship fully.

The observed differences in the broader heterogeneity of immune cells in milk between humans and cattle may be attributed to inherent species-specific variances, as well as the number of cells analyzed. In this study we have the capacity to analyze only 1000 cells from a representative sample, while other studies in the literature include more than 8,000 cells which might have increased the diversity and the amount of rare immune cells. Also, a greater amount of cell types and cell number would add diversity to the dataset allowing for a more delineated identification of greater immune cell communities within a cell population and a greater ability to detect minor populations.

CONCLUSION

In conclusion, the emergence of advanced technologies like scRNA-seq has revolutionized our ability to examine the molecular intricacies of the bovine mammary gland at a single-cell level within a diverse population of cells. By employing scRNA-seq, we can now unravel the complex molecular mechanisms taking place in the mammary gland, providing invaluable insights into the interconnections and variations within specific cell subpopulations. This knowledge is particularly crucial for understanding the diverse roles played by mammary epithelial and immune cells in healthy Holstein cows. By comprehensively characterizing immune and secretory MEC cellular and transcriptional diversity, we gain a deeper understanding of the state and productive capacity of both the animal and the bovine mammary gland. Moving forward, this research clears the way for further investigations into the intricate cellular processes underlying mammary gland function and opens up new possibilities for improving animal health and milk production in the future.

ACKNOWLEDGMENTS

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TABLES AND FIGURES

Table 5.1. Descriptive statistics of parity, days in milk, milk yield and components of lactating Holstein cows (n = 25) sampled for milk analysis using flow cytometry throughout the experiment

Item	Mean ± SD 1	Minimum	Maximum
Parity	3.4 ± 1.1	2	6
DIM	278 ± 70	109	414
Milk yield per milking, kg	19.1 ± 4.9	9.1	30.2
SCCx10^3 cells/mL	134 ± 90	13	460
Fat, %	3.3 ± 0.7	2.3	5.6
Protein, %	3.7 ± 0.3	2.8	3.9

Table 5.2. Cell subpopulations expressed in percentage, yield and concentration of single nucleated live and dead cells present in milk from Holstein multiparous cows identified by flow cytometry analysis

	Cell labeling	Subpopulation, %	Yield, cells x10^6	Concentration, cells/mL
Live	**$BTN^+CD45^+CD14^-$**	0.3	6.5	378
	$BTN^+CD45^+CD14^+$	0.4	7.1	415
	$BTN^+CD45^-CD14^-$	0.8	17.5	946
	$BTN^+CD45^-CD14^+$	0.9	19.1	1,212
	$BTN^-CD45^+CD14^-$	34.3	876.2	49,238
	$BTN^-CD45^+CD14^+$	5.4	131.7	7,099
	$BTN^-CD45^-CD14^-$	5.6	122.6	7,054
	$BTN^-CD45^-CD14^+$	3.6	71.6	4,096
	TOTAL	51.3	1,252	70,438
Dead	**$BTN^+CD45^+CD14^-$**	0.5	10.0	591
	$BTN^+CD45^+CD14^+$	1.1	20.6	1,146
	$BTN^+CD45^-CD14^-$	1.7	32.6	1,827
	$BTN^+CD45^-CD14^+$	2.5	47.3	2,637
	$BTN^-CD45^+CD14^-$	21.6	611.5	31,958
	$BTN^-CD45^+CD14^+$	3.3	82.2	4,473
	$BTN^-CD45^-CD14^-$	11.2	257.4	14,222
	$BTN^-CD45^-CD14^+$	6.3	123.0	6,940
	TOTAL	48.2	1,184.5	63,794
		99.5	**2,436**	**134,232**

Table 5.3. Descriptive statistics of parity, days in milk, milk yield and somatic cell count of Holstein cows sampled for milk analysis of mammary epithelial cells (MEC; n = 3) and immune cells (n = 2) using scRNA-seq

Item	MEC libraries Mean±SD	Immune Cells libraries Mean±SD
Parity	2.7±0.6	2.5±0.7
DIM	361±46	320±72
MY per milking, kg	14.2±4.7	14.5±5.8
SCCx10^3 cells/mL	115±39	139±33

Figure 5.1. Clustering of aggregated mammary epithelial cells (MEC) in milk from three Holstein cows (A) carried out using the uniform manifold approximation and projection (UMAP) dimension reduction technique. The total 839 MEC composed one cluster divided into four subclusters (B). Cell proximity represents gene expression similarity and identification of cell types was done by analyzing significantly enriched expression of established cell markers. Heatmap of transcriptome similarities between cell clusters (C). Rows represent cell clusters and columns represent representative genes. Numbers and colors on the right represent log2 fold changes relative to the median gene expression level across all clusters. Color scheme is based on z-score distribution from –10 (blue) to 10 (red). Right margin color bars highlight gene sets specific to the respective cluster subset

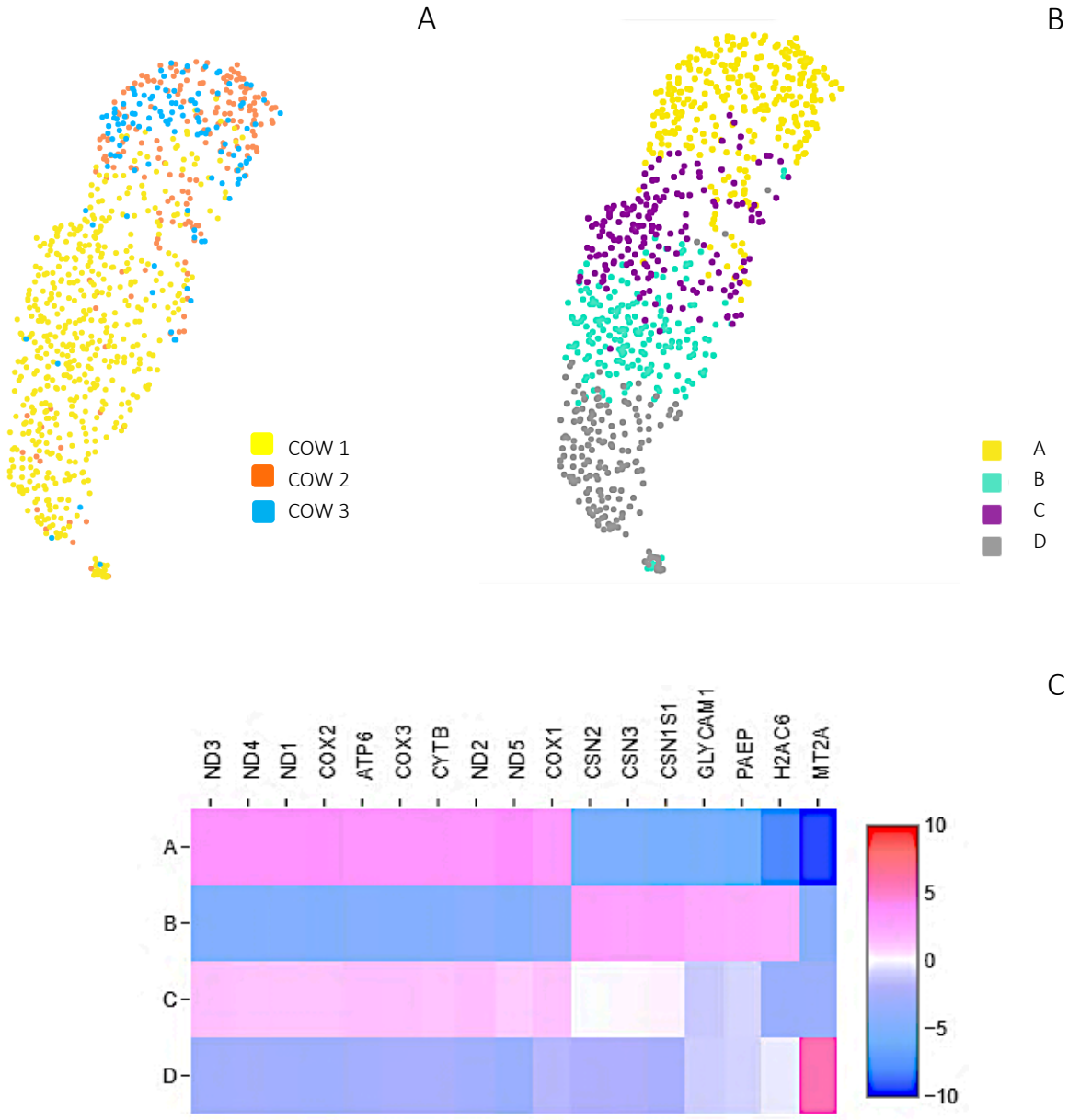

A
COW 1
COW 2
COW 3
B
A
B
C
D
C
ND3
ND4
ND1
COX2
ATP6
COX3
CYTB
ND2
ND5
COX1
CSN2
CSN3
CSN1S1
GLYCAM1
PAEP
H2AC6
MT2A
A
B
C
D
10
5
0
−5
−10

Figure 5.2. Violin plots per subcluster and feature plot for representative marker genes *ND5*, *ND1*, *GLYCAM1*, *CSN3*, *CSN2*, *CSN1S1*, *CSN1S2*, and *LALBA* of mammary epithelial cells (MEC) sorted by FACS in milk samples from two healthy Holstein cows

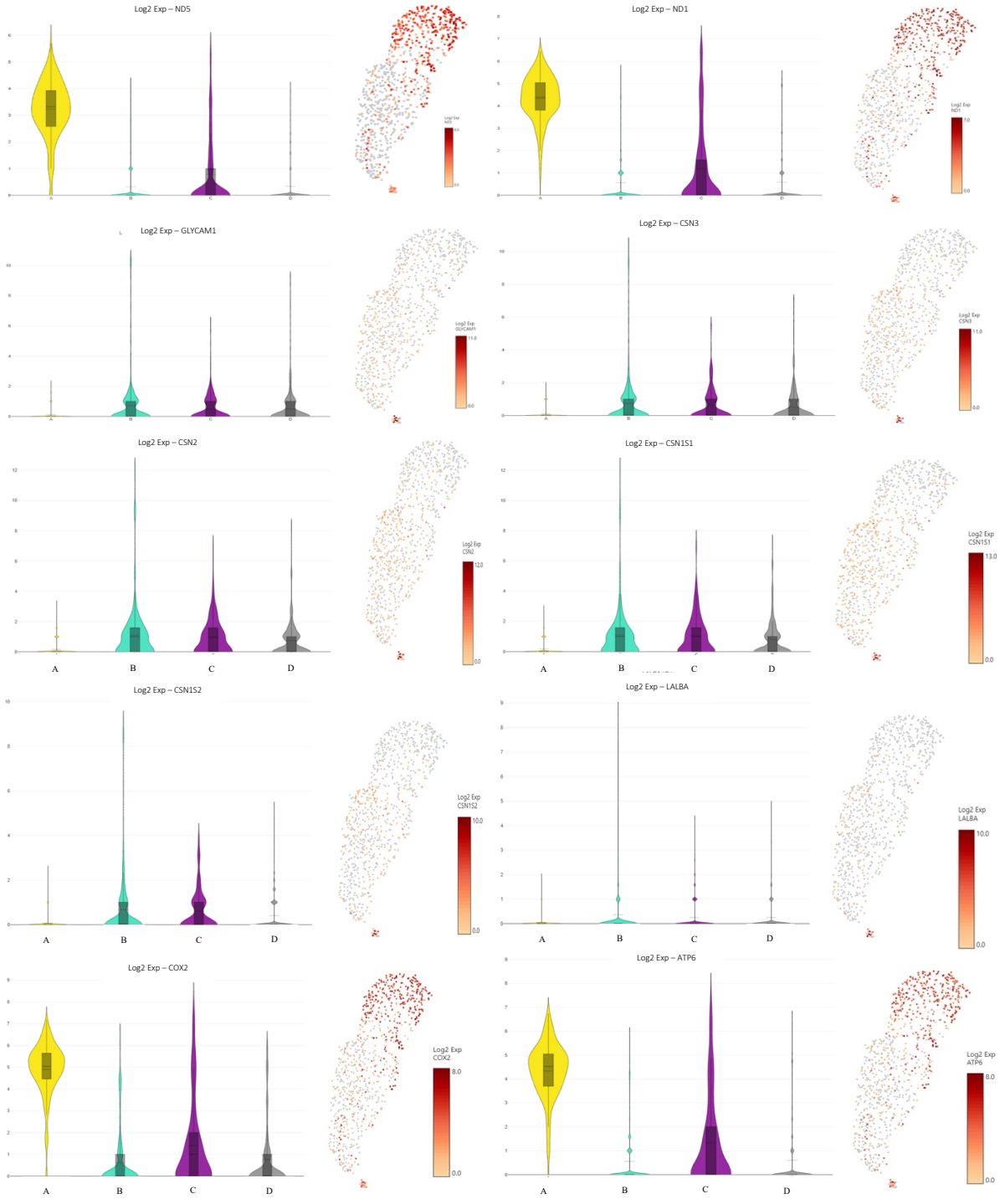

Log2 Exp – ND5
Log2 Exp – ND1
Log2 Exp – GLYCAM1
Log2 Exp – CSN3
Log2 Exp – CSN2
Log2 Exp – CSN1S1
Log2 Exp – CSN1S2
Log2 Exp – LALBA
Log2 Exp – COX2
Log2 Exp – ATP6
A
B
C
D

Figure 5.3. Violin plots per subcluster and feature plot for representative marker genes *PRLR*, *BTN1A1*, *ESR1*, *ESR2*, *PGR*, and *STAT3* of mammary epithelial cells (MEC) sorted by FACS in milk samples from two healthy Holstein cows

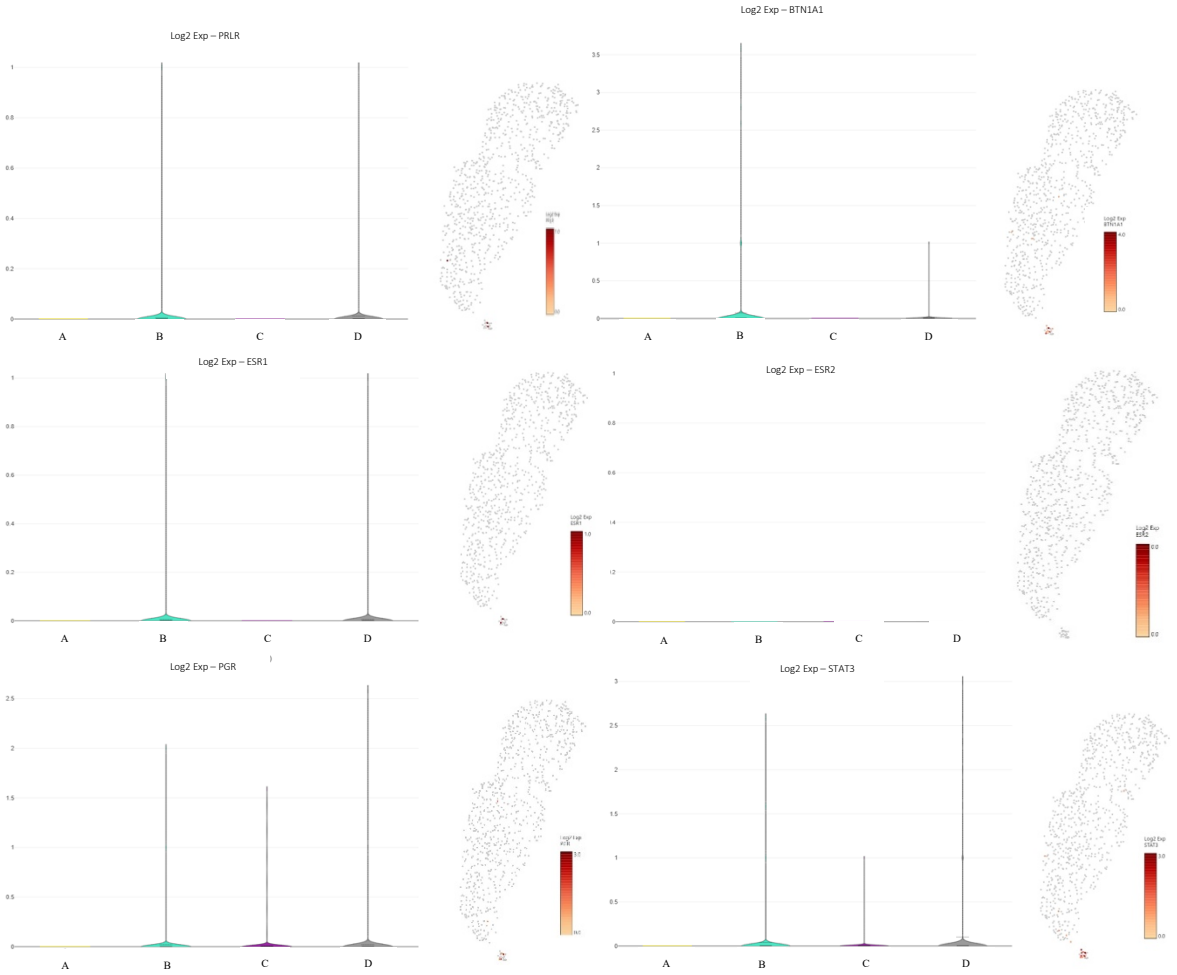


Figure 5.4. Clustering of integrated immune cells in milk (A) from two Holstein cows (B) carried out using the uniform manifold approximation and projection (UMAP) dimension reduction technique. The total 1,774 immune divided into 11 clusters and differential clustering expression per cow (C). Cell proximity represents gene expression similarity and identification of cell types was done by analyzing significantly enriched expression of established cell markers.

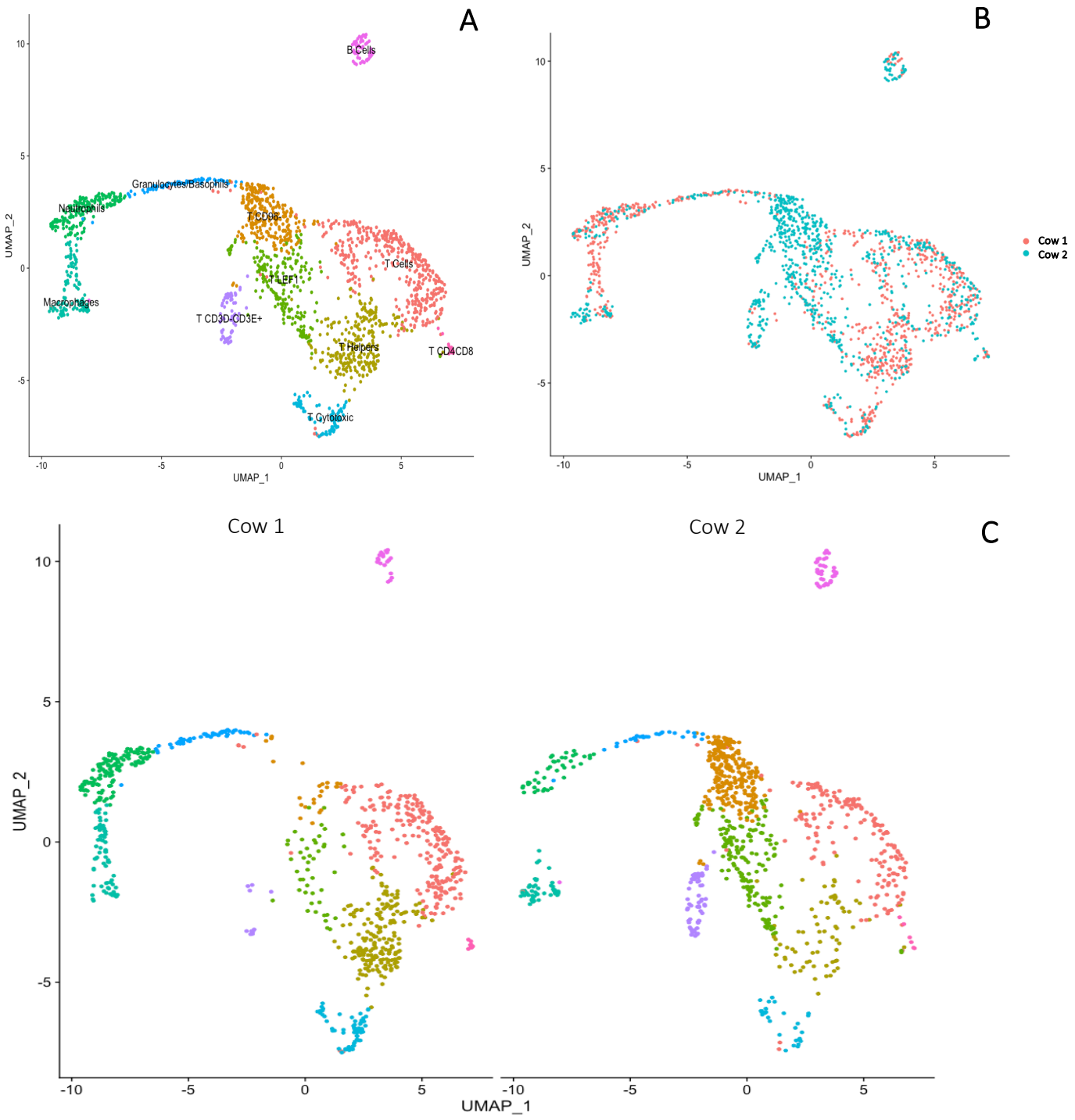

A
B
C
B Cells
Granulocytes/Basophils
Neutrophils
T CD8E
T Cells
T LEF1
Macrophages
T CD3D-CD3E+
T Helpers
T CD4CD8
T Cytotoxic
UMAP_1
UMAP_2
Cow 1
Cow 2

Figure 5.5. Expression of immune marker *PTPRC* (CD45) across all clusters, shown by violin plots (A) and feature plots (B) using the using the uniform manifold approximation and projection (UMAP).

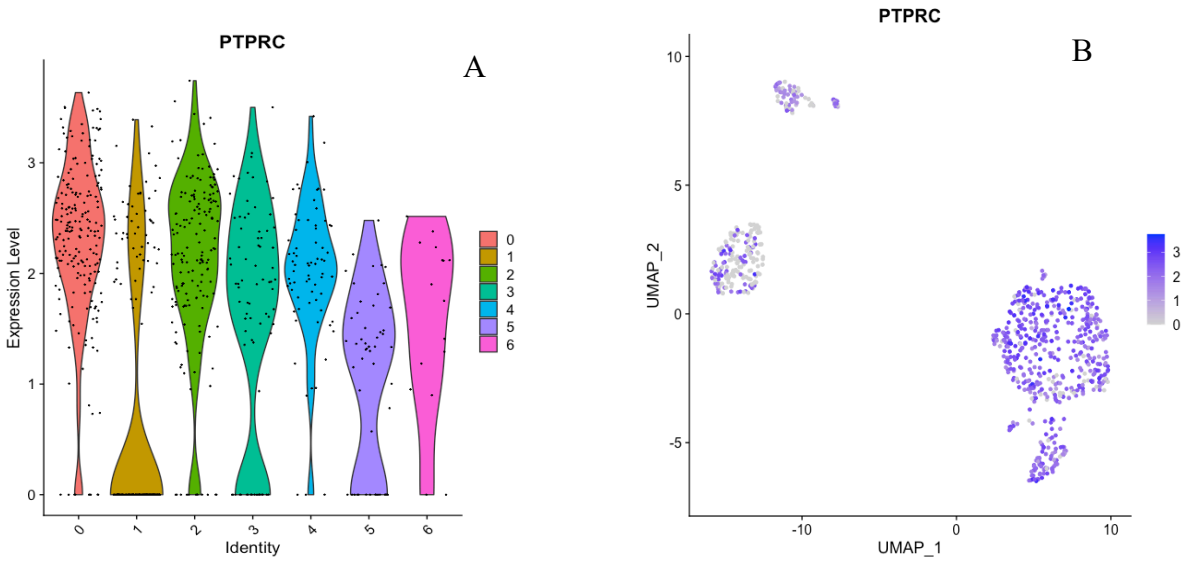


Figure 5.6. Clustering immune cells in milk from one Holstein cow carried out using the uniform manifold approximation and projection (UMAP) dimension reduction technique. The total 851 cells were divided into seven clusters. Cell proximity represents gene expression similarity and identification of cell types was done by analyzing significantly enriched expression of established cell markers.

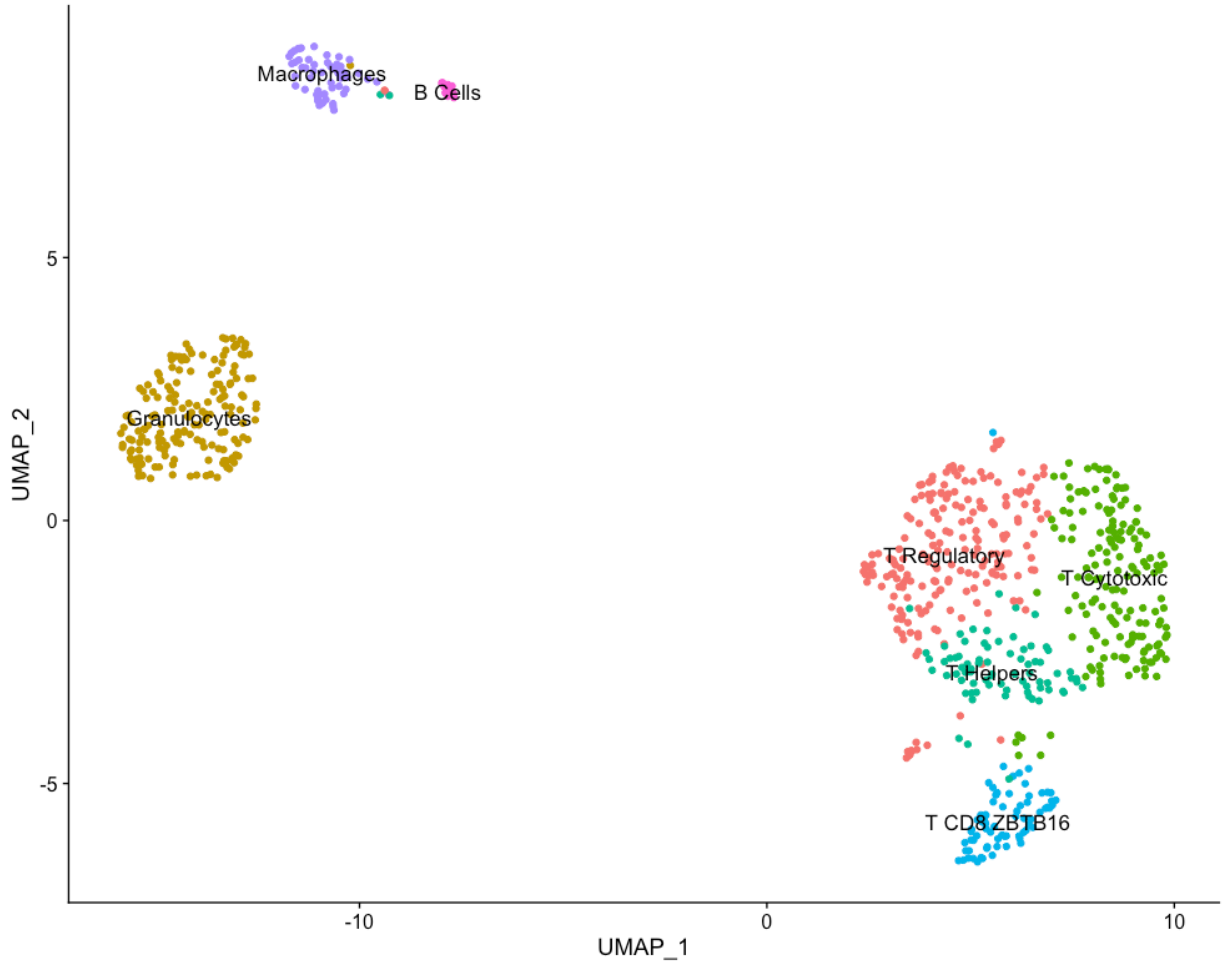


Figure 5.7. Heatmap of transcriptome similarities between cell clusters. Rows represent representative genes and columns represent cell clusters. Numbers and colors on the right represent log2 fold changes relative to the median gene expression level across all clusters. Color scheme is based on z-score distribution from –2 (purple) to 2 (yellow). Right margin color bars highlight gene sets specific to the respective cluster subset.

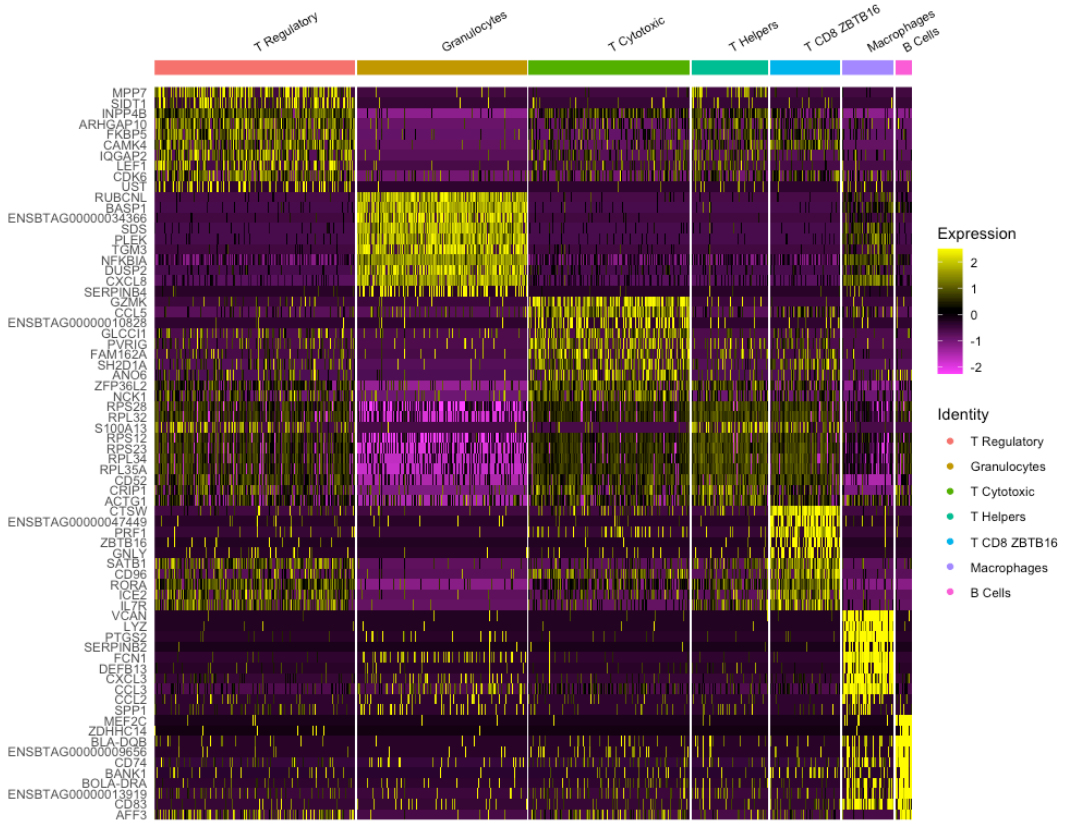


Figure 5.8. Violin plots for representative genes of clusters identified from immune cells ($CD45^{+}$) sorted using FACS present in milk from a healthy Holstein cow

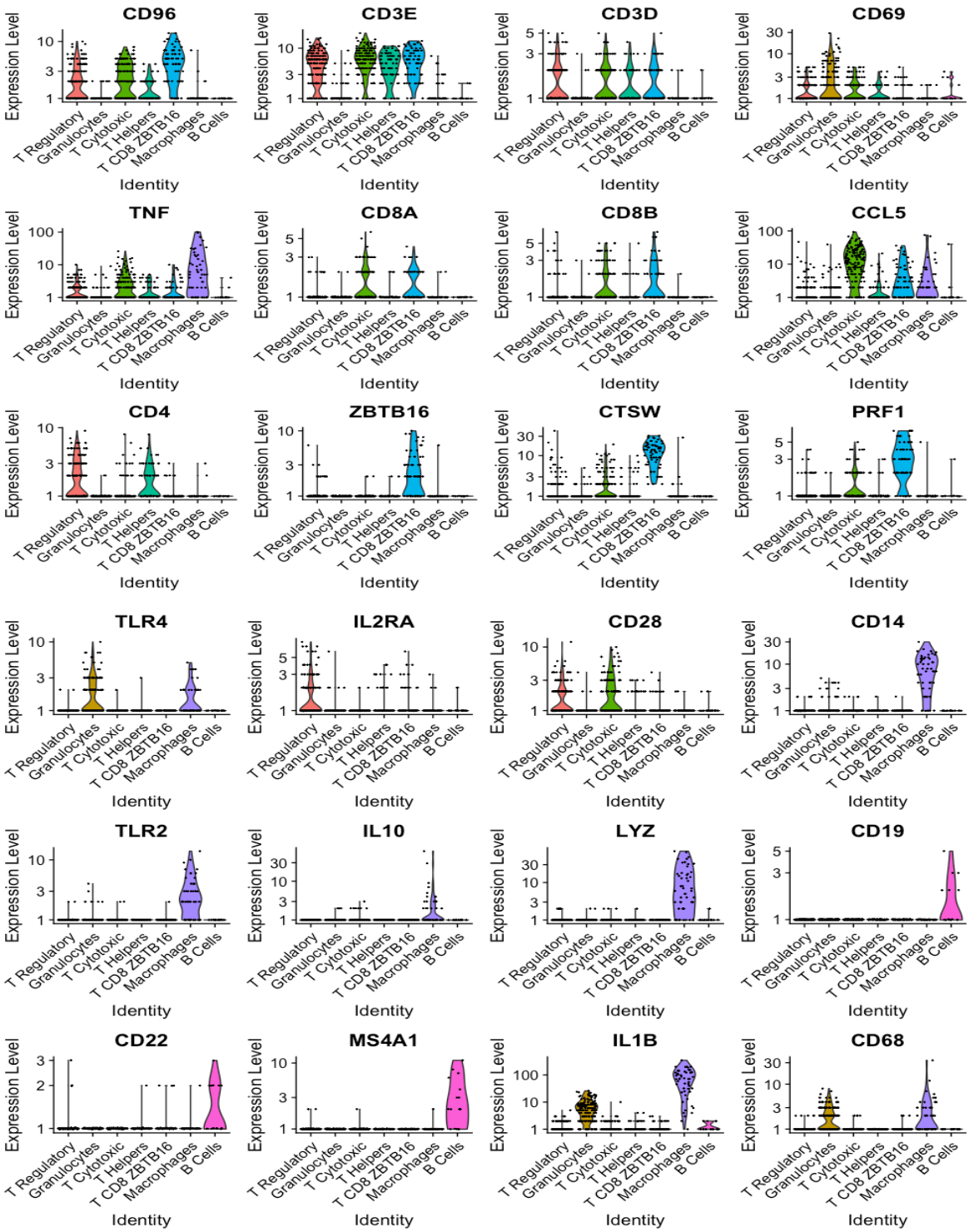


Figure 5.9. Expression of immune marker *PTPRC* (CD45) across all clusters, shown by violin plots (A) and feature plots (B) using the using the uniform manifold approximation and projection (UMAP).

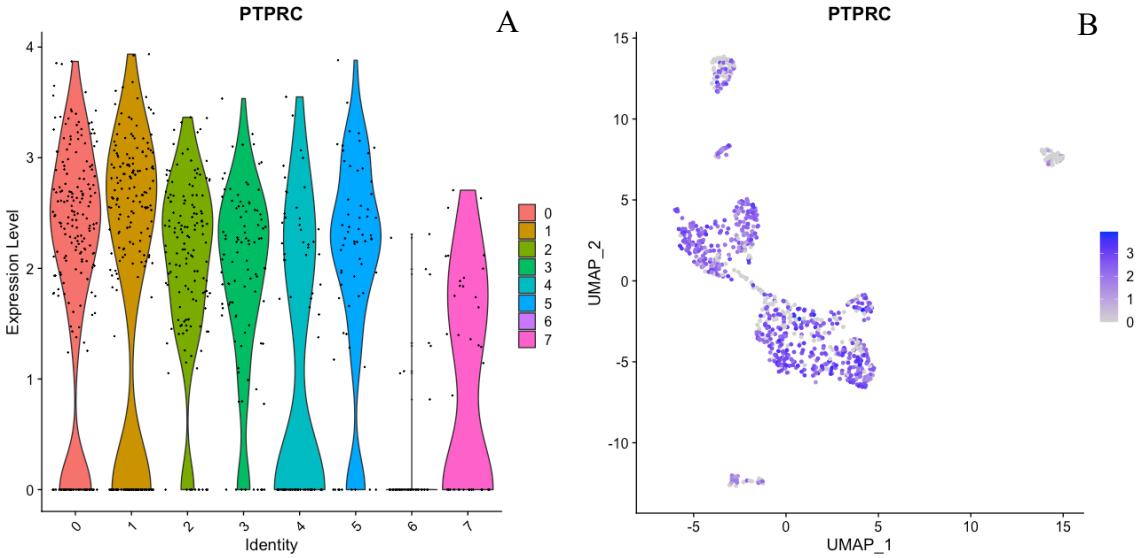


Figure 5.10. Clustering immune cells in milk from one Holstein cow carried out using the uniform manifold approximation and projection (UMAP) dimension reduction technique. The total 923 cells were divided into eight clusters. Cell proximity represents gene expression similarity and identification of cell types was done by analyzing significantly enriched expression of established cell markers.

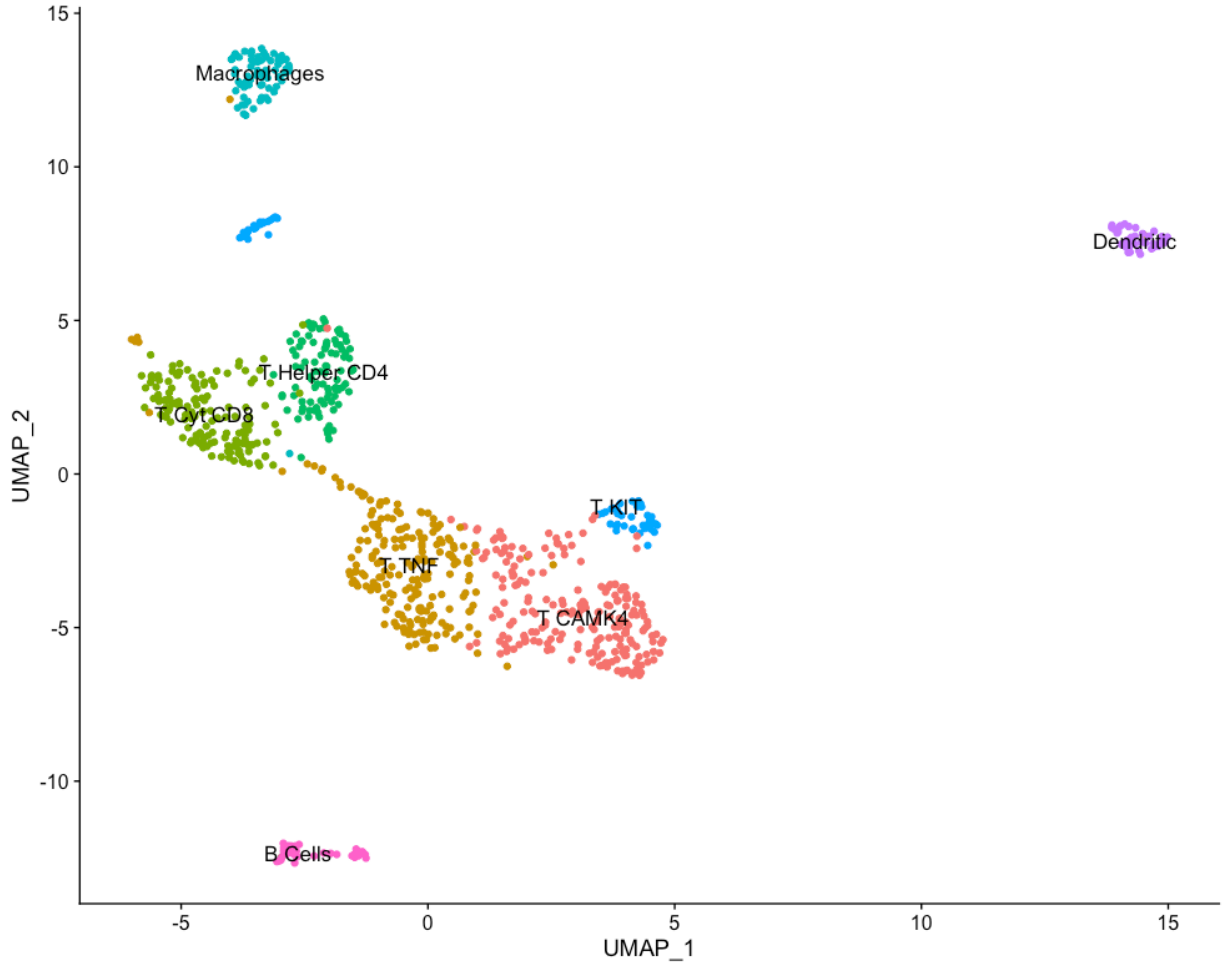


Figure 5.11. Heatmap of transcriptome similarities between cell clusters. Rows represent representative genes and columns represent cell clusters. Numbers and colors on the right represent log2 fold changes relative to the median gene expression level across all clusters. Color scheme is based on z-score distribution from –2 (purple) to 2 (yellow). Right margin color bars highlight gene sets specific to the respective cluster subset.

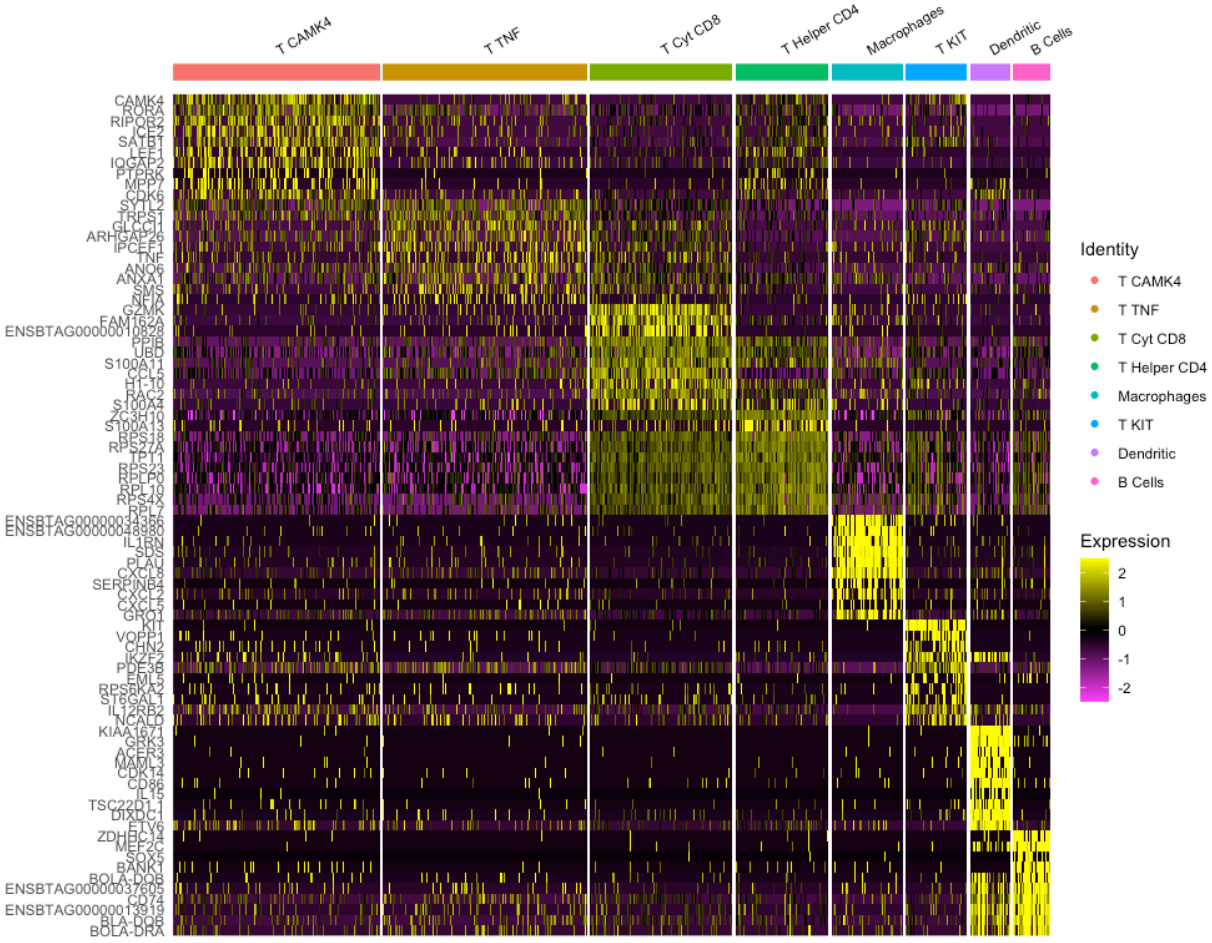
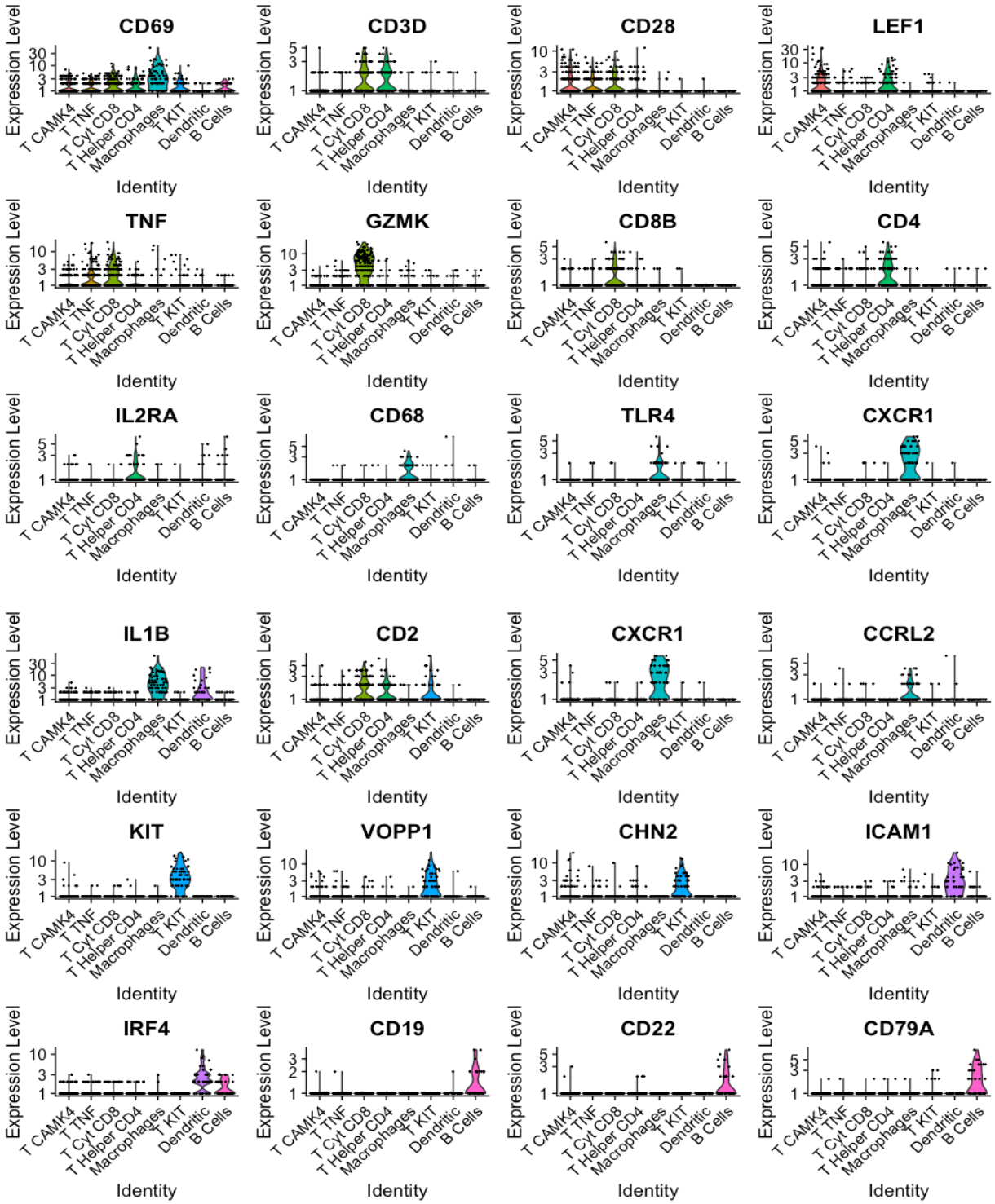


Figure 5.12. Violin plots for representative genes of clusters identified from immune cells ($CD45^{+}$) sorted using FACS present in milk from a healthy Holstein cow



INTEGRATIVE SUMMARY

The mammary gland is the primary organ responsible for milk production and consists of both non-secretory connective and glandular secretory tissue. These components contain multiple cell types, including epithelial cells and immune cells. The coordinated functioning of mammary epithelial cells plays a crucial role in the efficient production of various components of milk, such as proteins, lipids, carbohydrates, and bioactive elements. Milk production in dairy cows is a highly regulated and complex process that holds noteworthy importance due to its immense economic and agricultural significance. Several factors can influence the capacity of the mammary gland to produce milk including environmental stressors, management practices, and individual cow characteristics. Therefore, this work aimed to investigate the effects of increased milking frequency (**IMF**), heat stress (**HS**), and cell diversity on milk production at the mammary gland level in lactating Holstein cows, as these factors are considered key contributors to the regulation of milk production.

The first study aimed to examine the effects of IMF on milk, protein, and fat yields in udder halves milked 2× and 4× during early and mid-lactation. It also investigated the association between milking frequency and changes in mammary cisternal and alveolar capacities. The results showed that the difference in milk yield between udder halves in cows milked at IMF during early and mid-lactation was 2.27 kg higher compared to cows milked at IMF only during early lactation. Udder halves milked 4× produced more milk and protein than those milked 2× at 170, 200, 230, and 260 DIM during early and mid-lactation. However, there was no significant effect on cumulative and carryover udder half differences in milk yield. At 140 DIM, the alveolar volume was similar between udder halves milked 2× or 4×, while the cistern volume was larger for udder halves milked 4× during early lactation. There were no differences in alveolar or cistern volume

proportions in udder halves milked 2× or 4× before mid-lactation IMF. After 20 days of IMF, the alveolar volume remained similar between cows milked at IMF during early lactation and those milked at IMF during early and mid-lactation, regardless of udder half milking frequency. However, cows milked at IMF during early and mid-lactation had 4.9 kg more cistern milk than cows milked at IMF only during early lactation. Overall, IMF during early and mid-lactation impacted milk and protein yield, particularly during differential milking frequency regimens. Based on the findings, it can be concluded that udder halves subjected to early and mid-lactation IMF exhibited increased cistern volume capacity. Further studies focusing on udder compartment capacity, elasticity, and the hormonal and behavioral aspects (i. e., conditional responses) related to IMF implementation are necessary to deepen our understanding of these processes.

The second study evaluated the impact of HS on the structure of the secretory mammary epithelium tissue and the loss of cells in milk. This was achieved by comparing cows exposed to HS with cows under normal temperature conditions using a pair-feeding model. The results of the study demonstrated that exposure to HS for four days resulted in a reduction in MY of 4.3 kg per day per cow. Additionally, HS caused morphological changes in the tissue structure of the mammary gland and altered the profile of cell loss in milk. These changes included a 25% decrease in alveolar area and a threefold increase in the loss of putative progenitor cell in milk. Gene and protein expression analyses offered further understanding into the mechanisms underlying the decrease in milk yield during heat stress conditions. Alterations in pathways related to protein synthesis (pSTAT5), cell survival (pS6K1), and autophagy (LC3 II) were observed, which could help explain the decline in milk production under HS conditions. To enhance the understanding of milk production across lactation, more studies are needed to investigate the effects of HS on specific cell identities and the functionality of heterogeneous subpopulations within the bovine

mammary gland. Such studies would contribute to a better comprehension of the role played by these cell populations in milk production.

The last study aimed to characterize the functional diversity of epithelial and hematopoietic cell subpopulations in milk samples obtained from healthy Holstein cows by employing fluorescence-activated cell sorting (**FACS**) and single-cell RNA sequencing (**scRNA-seq**) techniques. The FACS analysis revealed that the predominant cell type present in milk was live hematopoietic cells, constituting 34% of the total cells. On the other hand, live MEC exhibited the lowest concentration with 0.8% of the total cells in milk. Based on the scRNA-seq analyses, MEC in milk showed overall homogeneity displaying one main cluster containing four heterogeneous subclusters with differential expression of classical MEC transcripts including *CSN1*, *CSN2*, *CSN3, and LALBA*. Additionally, the study revealed the presence of diverse populations of immune cells within the milk samples, including T cells (helper and cytotoxic), macrophages, neutrophils, and granulocytes/basophils. The comprehensive understanding of cell diversity provided valuable insights into the functionality of the mammary gland during lactation. These findings contribute to a deeper comprehension of the intricate interactions and roles played by different cell types within the mammary gland and the complex mechanisms underlying milk production in lactating cows.

Cell number and cell activity of secretory MEC are the primary determinants of the mammary gland's synthetic capacity. This study demonstrates that both cell activity and cell number can be influenced positively or negatively by systemic or local factors, including environmental stressors, management practices, and individual cow characteristics. An increase in milking frequency, for example, acts as a positive regulator of milk production by enhancing milk yield and enlarging the mammary gland's cistern capacity. This effect may be attributed to

increased MEC secretion and emptying, subsequently boosting the secretory activity of MEC. However, the potential impact of this regulator on MEC number and activity remains unexplored in this study. Conversely, negative regulators such as HS can disrupt the microstructure of the mammary gland, potentially diminishing the synthetic capacity of MEC regulated by HS. Moreover, HS adversely affects different cell subpopulations, including putative progenitors, which directly influences MEC proliferation rates and, consequently, cell number. Additionally, the diverse genetic, state, and functional variations among the cells present in milk offer valuable resources for investigating the effects of this heterogeneity on the milk synthetic capacity of the gland. Understanding the factors that influence mammary epithelial cell number and activity is crucial for optimizing milk synthesis, improving milk production efficiency, and ensuring the overall health and productivity of lactating dairy cows. Ongoing research in this field continues to deepen our understanding of the intricate interplay between these factors, providing valuable insights into strategies for enhancing milk production in dairy farming. By comprehensively considering and addressing these factors, dairy producers can work towards maximizing the milk-producing capacity of the mammary gland and achieving optimal and profitable production outcomes.

REFERENCES

Akers, M.R., D.E. Bauman, G.T. Goodman, A.V. Capuco, and H.A. Tucker. 1981. Prolactin Regulation of Cytological Differentiation of Mammary Epithelial Cells in Periparturient Cows*. Endocrinology 109:31–40. doi:10.1210/endo-109-1-31.

Akers, R.M. 2016. Lactation and the Mammary Gland: Akers/Lactation and the Mammary Gland. Blackwell Publishing Company, Ames, Iowa.

Alex, A.P., J.L. Collier, D.L. Hadsell, and R.J. Collier. 2015. Milk yield differences between 1× and 4× milking are associated with changes in mammary mitochondrial number and milk protein gene expression, but not mammary cell apoptosis or SOCS gene expression. J. Dairy Sci. 98:4439–4448.

Alhussien, M.N., and A.K. Dang. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. Vet. World 11:562–577. doi:10.14202/vetworld.2018.562-577.

Alhussien, M.N., B.S.K. Panda, and A.K. Dang. 2021. A Comparative Study on Changes in Total and Differential Milk Cell Counts, Activity, and Expression of Milk Phagocytes of Healthy and Mastitic Indigenous Sahiwal Cows. Front. Vet. Sci. 8:670811. doi:10.3389/fvets.2021.670811.

Allen, D.B., E.J. DePeters, and R.C. Laben. 1986. Three times a day milking: effects on milk production, reproductive efficiency, and udder health.. J. Dairy Sci. 69:1441–1446. doi:10.3168/jds.S0022-0302(86)80553-7.

Andón, F.T., and B. Fadeel. 2013. Programmed Cell Death: Molecular Mechanisms and Implications for Safety Assessment of Nanomaterials. Acc. Chem. Res. 46:733–742. doi:10.1021/ar300020b.

Ayadi, M., G. Caja, X. Such, and C.H. Knight. 2003a. Effect of omitting one milking weekly on lactational performances and morphological udder changes in dairy cows. J. Dairy Sci. 86:2352–2358.

Ayadi, M., G. Caja, X. Such, and C.H. Knight. 2003b. Use of ultrasonography to estimate cistern size and milk storage at different milking intervals in the udder of dairy cows. J. Dairy Res. 70:1–7. doi:10.1017/S0022029902005873.

Azizi, E., A.J. Carr, G. Plitas, A.E. Cornish, C. Konopacki, S. Prabhakaran, J. Nainys, K. Wu, V. Kiseliovas, and M. Setty. 2018. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. Cell 174:1293-1308. e36.

Bach, K., S. Pensa, M. Grzelak, J. Hadfield, D.J. Adams, J.C. Marioni, and W.T. Khaled. 2017. Differentiation dynamics of mammary epithelial cells revealed by single-cell RNA sequencing. Nat. Commun. 8:2128. doi:10.1038/s41467-017-02001-5.

Baumgard, L.H., and R.P. Rhoads Jr. 2013. Effects of heat stress on postabsorptive metabolism and energetics. Annu Rev Anim Biosci 1:311–337.

Baumgard, L.H., and R.P. Rhoads. 2012. Ruminant nutrition symposium: ruminant production and metabolic responses to heat stress. J. Anim. Sci. 90:1855–1865.

Becker, D., R. Weikard, F. Hadlich, and C. Kühn. 2021. Single-cell RNA sequencing of freshly isolated bovine milk cells and cultured primary mammary epithelial cells. Sci. Data 8:177. doi:10.1038/s41597-021-00972-1.

Ben-Jonathan, N., C.R. LaPensee, and E.W. LaPensee. 2008. What Can We Learn from Rodents about Prolactin in Humans?. Endocr. Rev. 29:1–41. doi:10.1210/er.2007-0017.

Berchtold, D., and T.C. Walther. 2009. TORC2 Plasma Membrane Localization Is Essential for Cell Viability and Restricted to a Distinct Domain. Mol. Biol. Cell 20:1565–1575. doi:10.1091/mbc.e08-10-1001.

Bernabucci, U., S. Biffani, L. Buggiotti, A. Vitali, N. Lacetera, and A. Nardone. 2014. The effects of heat stress in Italian Holstein dairy cattle. J. Dairy Sci. 97:471–486. doi:10.3168/jds.2013-6611.

Bernier-Dodier, P., L. Delbecchi, G.F. Wagner, B.G. Talbot, and P. Lacasse. 2010. Effect of milking frequency on lactation persistency and mammary gland remodeling in mid-lactation cows1. J. Dairy Sci. 93:555–564. doi:10.3168/jds.2009-2320.

Betterman, K.L., S. Paquet-Fifield, M.-L. Asselin-Labat, J.E. Visvader, L.M. Butler, S.A. Stacker, M.G. Achen, and N.L. Harvey. 2012. Remodeling of the Lymphatic Vasculature during Mouse Mammary Gland Morphogenesis Is Mediated via Epithelial-Derived Lymphangiogenic Stimuli. Am. J. Pathol. 181:2225–2238. doi:10.1016/j.ajpath.2012.08.035.

Biswas, S.K., S. Banerjee, G.W. Baker, C.-Y. Kuo, and I. Chowdhury. 2022. The Mammary Gland: Basic Structure and Molecular Signaling during Development. Int. J. Mol. Sci. 23:3883. doi:10.3390/ijms23073883.

Blatteis, C., J. Boulant, M. Cabanac, B. Cannon, R. Freedman, C.J. Gordon, J.R.S. Hales, M. Horowitz, M. Iriki, and L. Janský. 2001. Glossary of terms for thermal physiology. Jpn. J. Physiol. 51:245–280.

Bourges, D., F. Meurens, M. Berri, C. Chevaleyre, G. Zanello, B. Levast, S. Melo, V. Gerdts, and H. Salmon. 2008. New insights into the dual recruitment of IgA+ B cells in the developing mammary gland. Mol. Immunol. 45:3354–3362. doi:10.1016/j.molimm.2008.04.017.

Boutinaud, M., L. Galio, V. Lollivier, L. Finot, S. Wiart, D. Esquerré, and E. Devinoy. 2013a. Unilateral once daily milking locally induces differential gene expression in both mammary tissue and milk epithelial cells revealing mammary remodeling. Physiol. Genomics 45:973–985. doi:10.1152/physiolgenomics.00059.2013.

Boutinaud, M., J. Guinard-Flament, and HélèneJammes. 2004a. The number and activity of mammary epithelial cells, determining factors for milk production. Reprod. Nutr. Dev. 44:499–508. doi:10.1051/rnd:2004054.

Boutinaud, M., L. Herve, and V. Lollivier. 2015. Mammary epithelial cells isolated from milk are a valuable, non-invasive source of mammary transcripts. Front. Genet. 6:323.

Boutinaud, M., L. Herve, H. Quesnel, V. Lollivier, L. Finot, F. Dessauge, E. Chanat, P. Lacasse, C. Charton, and J. Guinard-Flament. 2019. Review: the cellular mechanisms underlying

mammary tissue plasticity during lactation in ruminants. Animal 13:s52–s64. doi:10.1017/S1751731119000624.

Boutinaud, M., and H. Jammes. 2002a. Potential uses of milk epithelial cells: a review. Reprod. Nutr. Dev. 42:133–147. doi:10.1051/rnd:2002013.

Boutinaud, M., and H. Jammes. 2002b. Potential uses of milk epithelial cells: a review. Reprod. Nutr. Dev. 42:133–147.

Boutinaud, M., J. Shand, M. Park, K. Phillips, J. Beattie, D. Flint, and G. Allan. 2004b. A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. J. Mol. Endocrinol. 33:195–207. doi:10.1677/jme.0.0330195.

Boutinaud, M., L. Yart, P. Debournoux-Poton, S. Wiart, L. Finot, E. Le Guennec, P.-G. Marnet, F. Dessauge, and V. Lollivier. 2013b. Exfoliation of mammary epithelial cells in milk is linked with lactation persistency in dairy cows. Page np in 64. Annual Meeting of the European Federation of Animal Science (EAAP). Wageningen Academic Publishers.

Bretland, A.J., J. Lawry, and R.M. Sharrard. 2001. A study of death by anoikis in cultured epithelial cells: *Anoikis in epithelial cells*. Cell Prolif. 34:199–210. doi:10.1046/j.1365-2184.2001.00198.x.

Bruckmaier, R.M., and J.W. Blum. 1992. B-mode ultrasonography of mammary glands of cows, goats and sheep during α- and β-adrenergic agonist and oxytocin administration. J. Dairy Res. 59:151–159. doi:10.1017/S0022029900030399.

Bruckmaier, R.M., and J.W. Blum. 1996. Simultaneous recording of oxytocin release, milk ejection and milk flow during milking of dairy cows with and without prestimulation. J. Dairy Res. 63:201–208. doi:10.1017/S0022029900031708.

Bruckmaier, R.M., and J.W. Blum. 1998. Oxytocin release and milk removal in ruminants. J. Dairy Sci. 81:939–949.

Bruckmaier, R.M., E. Rothenanger, and J.W. Blum. 1994. Measurement of mammary gland cistern size and determination of the cisternal milk fraction in dairy cows. Milchwissenschaft 49:543–546.

Burgos, S.A., M. Dai, and J.P. Cant. 2010. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. J. Dairy Sci. 93:153–161. doi:10.3168/jds.2009-2444.

Butler, A., P. Hoffman, P. Smibert, E. Papalexi, and R. Satija. 2018. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. 36:411–420.

Cai, M., Y. Hu, T. Zheng, H. He, W. Xiao, B. Liu, Y. Shi, X. Jia, S. Chen, J. Wang, and S. Lai. 2018. MicroRNA-216b inhibits heat stress-induced cell apoptosis by targeting Fas in bovine mammary epithelial cells. Cell Stress Chaperones 23:921–931. doi:10.1007/s12192-018-0899-9.

Caja, G., M. Ayadi, and C.H. Knight. 2004. Changes in cisternal compartment based on stage of lactation and time since milk ejection in the udder of dairy cows. J. Dairy Sci. 87:2409–2415.

Capper, J.L., R.A. Cady, and D.E. Bauman. 2009. The environmental impact of dairy production: 1944 compared with 20071. J. Anim. Sci. 87:2160–2167. doi:10.2527/jas.2009-1781.

Capuco, A.V., and R.M. Akers. 1999. [No title found]. J. Mammary Gland Biol. Neoplasia 4:137–144. doi:10.1023/A:1018769022990.

Capuco, A.V., and R.M. Akers. 2009. The origin and evolution of lactation. J. Biol. 8:37. doi:10.1186/jbiol139.

Capuco, A.V., R.M. Akers, and J.J. Smith. 1997. Mammary Growth in Holstein Cows During the Dry Period: Quantification of Nucleic Acids and Histology. J. Dairy Sci. 80:477–487. doi:10.3168/jds.S0022-0302(97)75960-5.

Capuco, A.V., and R.K. Choudhary. 2020. Symposium review: Determinants of milk production: Understanding population dynamics in the bovine mammary epithelium. J. Dairy Sci. 103:2928–2940. doi:10.3168/jds.2019-17241.

Capuco, A.V., S.E. Ellis, S.A. Hale, E. Long, R.A. Erdman, X. Zhao, and M.J. Paape. 2003. Lactation persistency: insights from mammary cell proliferation studies. J. Anim. Sci. 81:18–31.

Capuco, A.V., D.L. Wood, R. Baldwin, K. Mcleod, and M.J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. J. Dairy Sci. 84:2177–2187.

Chattopadhyay, P.K., T.M. Gierahn, M. Roederer, and J.C. Love. 2014. Single-cell technologies for monitoring immune systems. Nat. Immunol. 15:128–135. doi:10.1038/ni.2796.

Chedly, H.B., P. Lacasse, P.G. Marnet, and M. Boutinaud. 2013. The decrease in milk yield during once daily milking is due to regulation of synthetic activity rather than apoptosis of mammary epithelial cells in goats. Animal 7:124–133.

Chen, C.-C., D.B. Stairs, R.B. Boxer, G.K. Belka, N.D. Horseman, J.V. Alvarez, and L.A. Chodosh. 2012. Autocrine prolactin induced by the Pten–Akt pathway is required for lactation initiation and provides a direct link between the Akt and Stat5 pathways. Genes Dev. 26:2154–2168. doi:10.1101/gad.197343.112.

Chen, K.-L., H.-L. Wang, L.-Z. Jiang, Y. Qian, C.-X. Yang, W.-W. Chang, J.-F. Zhong, and G.-D. Xing. 2020. Heat stress induces apoptosis through disruption of dynamic mitochondrial networks in dairy cow mammary epithelial cells. Vitro Cell. Dev. Biol. - Anim. 56:322–331. doi:10.1007/s11626-020-00446-5.

Cleverley, J.D., and S.J. Folley. 1970. THE BLOOD LEVELS OF OXYTOCIN DURING MACHINE MILKING IN COWS WITH SOME OBSERVATIONS ON ITS HALF-LIFE IN THE CIRCULATION. J. Endocrinol. 46:347–361. doi:10.1677/joe.0.0460347.

Colbert, D.C., M.P. McGarry, K. O'neill, N.A. Lee, and J.J. Lee. 2005. Decreased size and survival of weanling mice in litters of IL-5–/–mice are a consequence of the IL-5 deficiency in nursing dams. J. Am. Assoc. Lab. Anim. Sci. 44:53–55.

Collier, R.J., J.L. Collier, R.P. Rhoads, and L.H. Baumgard. 2008. Invited review: genes involved in the bovine heat stress response. J. Dairy Sci. 91:445–454.

Collier, R.J., and K.G. Gebremedhin. 2015. Thermal Biology of Domestic Animals. Annu. Rev. Anim. Biosci. 3:513–532. doi:10.1146/annurev-animal-022114-110659.

Collier, R.J., L.W. Hall, S. Rungruang, and R.B. Zimbleman. 2012. Quantifying heat stress and its impact on metabolism and performance. Dep. Anim. Sci. Univ. Ariz. 68:1–11.

Collier, R.J., B.J. Renquist, and Y. Xiao. 2017. A 100-Year Review: Stress physiology including heat stress. J. Dairy Sci. 100:10367–10380. doi:10.3168/jds.2017-13676.

Connor, E.E., S. Siferd, T.H. Elsasser, C.M. Evock-Clover, C.P. Van Tassell, T.S. Sonstegard, V.M. Fernandes, and A.V. Capuco. 2008. Effects of increased milking frequency on gene expression in the bovine mammary gland. BMC Genomics 9:362. doi:10.1186/1471-2164-9-362.

Council, N.R. 2000. Nutrient requirements of dairy cattle: 2001.

Cowley, F.C., D.G. Barber, A.V. Houlihan, and D.P. Poppi. 2015. Immediate and residual effects of heat stress and restricted intake on milk protein and casein composition and energy metabolism. J. Dairy Sci. 98:2356–2368.

Cristea, S., and K. Polyak. 2018. Dissecting the mammary gland one cell at a time. Nat. Commun. 9:2473. doi:10.1038/s41467-018-04905-2.

Crowley, W.R., and W.E. Armstrong. 1992. Neurochemical regulation of oxytocin secretion in lactation. Endocr. Rev. 13:33–65.

Dado-Senn, B., A.L. Skibiel, G.E. Dahl, S.I. Arriola Apelo, and J. Laporta. 2021. Dry Period Heat Stress Impacts Mammary Protein Metabolism in the Subsequent Lactation. Animals 11. doi:10.3390/ani11092676.

Dado-Senn, B., A.L. Skibiel, T.F. Fabris, G.E. Dahl, and J. Laporta. 2019. Dry period heat stress induces microstructural changes in the lactating mammary gland. PLoS One 14:e0222120.

Dado-Senn, B., A.L. Skibiel, T.F. Fabris, Y. Zhang, G.E. Dahl, F. Peñagaricano, and J. Laporta. 2018. RNA-Seq reveals novel genes and pathways involved in bovine mammary involution during the dry period and under environmental heat stress. Sci. Rep. 8:1–11.

Dahl, G.E., R.L. Wallace, R.D. Shanks, and D. Lueking. 2004. Hot topic: effects of frequent milking in early lactation on milk yield and udder health.. J. Dairy Sci. 87:882–885. doi:10.3168/jds.S0022-0302(04)73232-4.

Davis, S.R., V.C. Farr, P.J. Copeman, V.R. Carruthers, C.H. Knight, and K. Stelwagen. 1998. Partitioning of milk accumulation between cisternal and alveolar compartments of the bovine udder: relationship to production loss during once daily milking.. J. Dairy Res. 65:1–8. doi:10.1017/s0022029997002562.

De Matteis, G., F. Grandoni, M.C. Scatà, G. Catillo, B. Moioli, and L. Buttazzoni. 2020. Flow Cytometry-Detected Immunological Markers and on Farm Recorded Parameters in Composite Cow Milk as Related to Udder Health Status. Vet. Sci. 7:114. doi:10.3390/vetsci7030114.

De Schutter, E., B. Cappe, B. Wiernicki, P. Vandenabeele, and F.B. Riquet. 2021. Plasma membrane permeabilization following cell death: many ways to dye!. Cell Death Discov. 7:183. doi:10.1038/s41420-021-00545-6.

Dessauge, F., V. Lollivier, B. Ponchon, R. Bruckmaier, L. Finot, S. Wiart, E. Cutullic, C. Disenhaus, S. Barbey, and M. Boutinaud. 2011. Effects of nutrient restriction on mammary cell turnover and mammary gland remodeling in lactating dairy cows. J. Dairy Sci. 94:4623–4635. doi:10.3168/jds.2010-4012.

Do Amaral, B.C., E.E. Connor, S. Tao, M.J. Hayen, J.W. Bubolz, and G.E. Dahl. 2011. Heat stress abatement during the dry period influences metabolic gene expression and improves immune status in the transition period of dairy cows. J. Dairy Sci. 94:86–96.

Du, Y., Q. Huang, C. Arisdakessian, and L.X. Garmire. 2020. Evaluation of STAR and Kallisto on single cell RNA-Seq data alignment. G3 Genes Genomes Genet. 10:1775–1783.

Elmore, S. 2007. Apoptosis: A Review of Programmed Cell Death. Toxicol. Pathol. 35:495–516. doi:10.1080/01926230701320337.

Fabris, T.F., J. Laporta, F.N. Corra, Y.M. Torres, D.J. Kirk, D.J. McLean, J.D. Chapman, and G.E. Dahl. 2017. Effect of nutritional immunomodulation and heat stress during the dry period on subsequent performance of cows. J. Dairy Sci. 100:6733–6742. doi:10.3168/jds.2016-12313.

Fabris, T.F., J. Laporta, A.L. Skibiel, F.N. Corra, B.D. Senn, S.E. Wohlgemuth, and G.E. Dahl. 2019. Effect of heat stress during early, late, and entire dry period on dairy cattle. J. Dairy Sci. 102:5647–5656.

Fabris, T.F., J. Laporta, A.L. Skibiel, B. Dado-Senn, S.E. Wohlgemuth, and G.E. Dahl. 2020. Effect of heat stress during the early and late dry period on mammary gland development of Holstein dairy cattle. J. Dairy Sci. 103:8576–8586.

Fan, C., R. Hu, H. Fan, Y. Yang, B. Gong, S. Zhang, J. Ding, Y. Su, Z. Zhuo, and J. Cheng. 2021. Effects of seasonal ambient heat stress on expression of microRNAs in the mammary gland of Holstein cows. Int. J. Biometeorol. 65:235–246. doi:10.1007/s00484-020-02025-5.

Feugeas, J.-P., J. Tourret, A. Launay, O. Bouvet, C. Hoede, E. Denamur, and O. Tenaillon. 2016. Links between Transcription, Environmental Adaptation and Gene Variability in Escherichia coli: Correlations between Gene Expression and Gene Variability Reflect Growth Efficiencies. Mol. Biol. Evol. 33:2515–2529. doi:10.1093/molbev/msw105.

Fink, S.L., and B.T. Cookson. 2005. Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. Infect. Immun. 73:1907–1916. doi:10.1128/IAI.73.4.1907-1916.2005.

Frisch, S., and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124:619–626. doi:10.1083/jcb.124.4.619.

Ganger, M.T., G.D. Dietz, and S.J. Ewing. 2017. A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. BMC Bioinformatics 18:1–11.

Gao, S.T., J. Guo, S.Y. Quan, X.M. Nan, M.S. Fernandez, L.H. Baumgard, and D.P. Bu. 2017. The effects of heat stress on protein metabolism in lactating Holstein cows. J. Dairy Sci. 100:5040–5049.

Gao, S.T., L. Ma, Z. Zhou, Z.K. Zhou, L.H. Baumgard, D. Jiang, M. Bionaz, and D.P. Bu. 2019. Heat stress negatively affects the transcriptome related to overall metabolism and milk protein synthesis in mammary tissue of midlactating dairy cows. Physiol. Genomics 51:400–409.

García Solá, M.E., M. Stedile, I. Beckerman, and E.C. Kordon. 2021. An integrative single-cell transcriptomic atlas of the post-natal mouse mammary gland allows discovery of new developmental trajectories in the luminal compartment. J. Mammary Gland Biol. Neoplasia 26:29–42.

Gleeson, J.P., N. Chaudhary, K.C. Fein, R. Doerfler, P. Hredzak-Showalter, and K.A. Whitehead. 2022. Profiling of mature-stage human breast milk cells identifies six unique lactocyte subpopulations. Sci. Adv. 8:eabm6865. doi:10.1126/sciadv.abm6865.

Gouon-Evans, V., M.E. Rothenberg, and J.W. Pollard. 2000. Postnatal mammary gland development requires macrophages and eosinophils. Development 127:2269–2282. doi:10.1242/dev.127.11.2269.

Green, K.A., and C.H. Streuli. 2004. Apoptosis regulation in the mammary gland. Cell. Mol. Life Sci. 61:1867–1883. doi:10.1007/s00018-004-3366-y.

Gregor, M.F., E.S. Misch, L. Yang, S. Hummasti, K.E. Inouye, A.-H. Lee, B. Bierie, and G.S. Hotamisligil. 2013. The Role of Adipocyte XBP1 in Metabolic Regulation during Lactation. Cell Rep. 3:1430–1439. doi:10.1016/j.celrep.2013.03.042.

Guo, Z., S. Gao, J. Ouyang, L. Ma, and D. Bu. 2021. Impacts of Heat Stress-Induced Oxidative Stress on the Milk Protein Biosynthesis of Dairy Cows. Animals 11:726. doi:10.3390/ani11030726.

Hadsell, D., J. George, and D. Torres. 2007. The declining phase of lactation: peripheral or central, programmed or pathological?. J. Mammary Gland Biol. Neoplasia 12:59–70.

Hale, S.A., A.V. Capuco, and R.A. Erdman. 2003. Milk yield and mammary growth effects due to increased milking frequency during early lactation. J. Dairy Sci. 86:2061–2071.

Hammami, H., J. Vandenplas, M.-L. Vanrobays, B. Rekik, C. Bastin, and N. Gengler. 2015. Genetic analysis of heat stress effects on yield traits, udder health, and fatty acids of Walloon Holstein cows. J. Dairy Sci. 98:4956–4968. doi:10.3168/jds.2014-9148.

Hanling, H.H., M.L. McGilliard, and B.A. Corl. 2021. Uneven milking intervals are adequate to achieve the benefits of increased milking frequency in early lactation. J. Dairy Sci. 104:9355–9361. doi:10.3168/jds.2020-20100.

Hassiotou, F., A.R. Hepworth, P. Metzger, C. Tat Lai, N. Trengove, P.E. Hartmann, and L. Filgueira. 2013. Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. Clin. Transl. Immunol. 2:e3. doi:10.1038/cti.2013.1.

Herve, L., H. Quesnel, V. Lollivier, and M. Boutinaud. 2016. Regulation of cell number in the mammary gland by controlling the exfoliation process in milk in ruminants. J. Dairy Sci. 99:854–863.

Herve, L., H. Quesnel, M. Veron, J. Portanguen, J.J. Gross, R.M. Bruckmaier, and M. Boutinaud. 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows. J. Dairy Sci. 102:2670–2685. doi:10.3168/jds.2018-15398.

Hogan, C., S. Dupré-Crochet, M. Norman, M. Kajita, C. Zimmermann, A.E. Pelling, E. Piddini, L.A. Baena-López, J.-P. Vincent, Y. Itoh, H. Hosoya, F. Pichaud, and Y. Fujita. 2009. Characterization of the interface between normal and transformed epithelial cells. Nat. Cell Biol. 11:460–467. doi:10.1038/ncb1853.

Hovey, R.C., and L. Aimo. 2010. Diverse and Active Roles for Adipocytes During Mammary Gland Growth and Function. J. Mammary Gland Biol. Neoplasia 15:279–290. doi:10.1007/s10911-010-9187-8.

Hu, H., Y. Zhang, N. Zheng, J. Cheng, and J. Wang. 2016. The effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells: Heat Stress on Mammary Epithelial Cells. Anim. Sci. J. 87:84–91. doi:10.1111/asj.12375.

Humphreys, R.C., M. Krajewska, S. Krnacik, R. Jæger, H. Weiher, S. Krajewski, J.C. Reed, and J.M. Rosen. 1996. Apoptosis in the terminal endbud of the murine mammary gland: a

mechanism of ductal morphogenesis. Development 122:4013–4022. doi:10.1242/dev.122.12.4013.

Hurley, W.L. 1989. Mammary Gland Function During Involution. J. Dairy Sci. 72:1637–1646. doi:10.3168/jds.S0022-0302(89)79276-6.

Inman, J.L., C. Robertson, J.D. Mott, and M.J. Bissell. 2015. Mammary gland development: cell fate specification, stem cells and the microenvironment. Development 142:1028–1042. doi:10.1242/dev.087643.

Kakumoto, K., J. Ikeda, M. Okada, E. Morii, and C. Oneyama. 2015. mLST8 Promotes mTOR-Mediated Tumor Progression. PLOS ONE 10:e0119015. doi:10.1371/journal.pone.0119015.

Keown, J.F., R.W. Everett, N.B. Empet, and L.H. Wadell. 1986. Lactation Curves. J. Dairy Sci. 69:769–781. doi:10.3168/jds.S0022-0302(86)80466-0.

Kéri, S., and I. Kiss. 2011. Oxytocin response in a trust game and habituation of arousal. Physiol. Behav. 102:221–224.

Key, N., S. Sneeringer, and D. Marquardt. 2014. Climate change, heat stress, and US dairy production. USDA-ERS Econ. Res. Rep.

Keys, J.E., A.V. Capuco, R.M. Akers, and J. Djiane. 1989. Comparative study of mammary gland development and differentiation between beef and dairy heifers. Domest. Anim. Endocrinol. 6:311–319. doi:10.1016/0739-7240(89)90025-8.

Kim, J.Y., T.-M. Cho, J.M. Park, S. Park, M. Park, K.D. Nam, D. Ko, J. Seo, S. Kim, E. Jung, L. Farrand, C.-T. Nguyen, V.-H. Hoang, M. Thanh La, J. Ann, G. Nam, H.-J. Park, J. Lee, Y.-J. Kim, and J.H. Seo. 2022. A novel HSP90 inhibitor SL-145 suppresses metastatic triple-negative breast cancer without triggering the heat shock response.. Oncogene 41:3289–3297. doi:10.1038/s41388-022-02269-y.

Knight, C.H., D. Hirst, and R.J. Dewhurst. 1994. Milk accumulation and distribution in the bovine udder during the interval between milkings. J. Dairy Res. 61:167–177.

Knight, C.H., and M. Peaker. 1984. MAMMARY DEVELOPMENT AND REGRESSION DURING LACTATION IN GOATS IN RELATION TO MILK SECRETION. Q. J. Exp. Physiol. 69:331–338. doi:10.1113/expphysiol.1984.sp002809.

Knight, C.H., M. Peaker, and C.J. Wilde. 1998. Local control of mammary development and function. Rev. Reprod. 3:104–112.

Kobayashi, K., Y. Tsugami, K. Matsunaga, T. Suzuki, and T. Nishimura. 2018. Moderate High Temperature Condition Induces the Lactation Capacity of Mammary Epithelial Cells Through Control of STAT3 and STAT5 Signaling. J. Mammary Gland Biol. Neoplasia 23:75–88. doi:10.1007/s10911-018-9393-3.

Kobayashi, S.D., and F.R. DeLeo. 2009. Role of neutrophils in innate immunity: a systems biology-level approach. WIREs Syst. Biol. Med. 1:309–333. doi:10.1002/wsbm.32.

Koess, C., and J. Hamann. 2008. Detection of mastitis in the bovine mammary gland by flow cytometry at early stages. J. Dairy Res. 75:225–232. doi:10.1017/S0022029908003245.

Krishna, M., and H. Narang. 2008. The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell. Mol. Life Sci. 65:3525–3544. doi:10.1007/s00018-008-8170-7.

Kuipers, D., A. Mehonic, M. Kajita, L. Peter, Y. Fujita, T. Duke, G. Charras, and J.E. Gale. 2014. Epithelial repair is a two-stage process driven first by dying cells and then by their neighbours. J. Cell Sci. jcs.138289. doi:10.1242/jcs.138289.

Lacetera, N., U. Bernabucci, D. Scalia, L. Basiricò, P. Morera, and A. Nardone. 2006. Heat stress elicits different responses in peripheral blood mononuclear cells from Brown Swiss and Holstein cows. J. Dairy Sci. 89:4606–4612.

Lecchi, C., N. Rota, A. Vitali, F. Ceciliani, and N. Lacetera. 2016. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Vet. Immunol. Immunopathol. 182:89–94.

Leitner, G., R. Eligulashvily, O. Krifucks, S. Perl, and A. Saran. 2003. Immune Cell Differentiation in Mammary Gland Tissues and Milk of Cows Chronically Infected with *Staphylococcus aureus*: Immunohistochemistry of bovine supra-mammary lymph node. J. Vet. Med. Ser. B 50:45–52. doi:10.1046/j.1439-0450.2003.00602.x.

Lengi, A.J., M. Makris, and B.A. Corl. 2021. A flow cytometric method for measuring and isolating mammary epithelial cells from bovine milk. JDS Commun. 2:426–430.

Lengi, A.J., J.W. Stewart, M. Makris, M.L. Rhoads, and B.A. Corl. 2022a. Heat Stress Increases Mammary Epithelial Cells and Reduces Viable Immune Cells in Milk of Dairy Cows. Animals 12:2810. doi:10.3390/ani12202810.

Lengi, A.J., J.W. Stewart, M. Makris, M.L. Rhoads, and B.A. Corl. 2022b. Heat Stress Increases Mammary Epithelial Cells and Reduces Viable Immune Cells in Milk of Dairy Cows. Animals 12:2810.

Li, L., Y. Wang, C. Li, and G. Wang. 2017. Proteomic analysis to unravel the effect of heat stress on gene expression and milk synthesis in bovine mammary epithelial cells. Anim. Sci. J. 88:2090–2099.

Li, X., G. Li, X. Du, X. Sun, Z. Peng, C. Zhao, Q. Xu, A.M. Abdelatty, F.F. Mohamed, Z. Wang, and G. Liu. 2020. Increased autophagy mediates the adaptive mechanism of the mammary gland in dairy cows with hyperketonemia. J. Dairy Sci. 103:2545–2555. doi:10.3168/jds.2019-16910.

Lilla, J.N., and Z. Werb. 2010. Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. Dev. Biol. 337:124–133. doi:10.1016/j.ydbio.2009.10.021.

Linzell, J.L. 1966. Measurement of Udder Volume in Live Goats as an Index of Mammary Growth and Function. J. Dairy Sci. 49:307–311. doi:10.3168/jds.S0022-0302(66)87853-0.

Lollivier, V., P. Lacasse, J.A. Arizala, P. Lamberton, S. Wiart, J. Portanguen, R. Bruckmaier, and M. Boutinaud. 2015. In vivo inhibition followed by exogenous supplementation demonstrates galactopoietic effects of prolactin on mammary tissue and milk production in dairy cows. J. Dairy Sci. 98:8775–8787.

Lollivier, V., P. Marnet, S. Delpal, D. Rainteau, C. Achard, A. Rabot, and M. Ollivier-Bousquet. 2006a. Oxytocin stimulates secretory processes in lactating rabbit mammary epithelial cells. J. Physiol. 570:125–140. doi:10.1113/jphysiol.2005.097816.

Lollivier, V., P.-G. Marnet, S. Delpal, D. Rainteau, C. Achard, A. Rabot, and M. Ollivier-Bousquet. 2006b. Oxytocin stimulates secretory processes in lactating rabbit mammary epithelial cells. J. Physiol. 570:125–140.

Lush, J.L., and R.R. Shrode. 1950. Changes in milk production with age and milking frequency.. J. Dairy Sci. 33:338–357.

Macias, H., and L. Hinck. 2012. Mammary gland development: Mammary gland development. Wiley Interdiscip. Rev. Dev. Biol. 1:533–557. doi:10.1002/wdev.35.

Makarem, M., N. Kannan, L.V. Nguyen, D.J.H.F. Knapp, S. Balani, M.D. Prater, J. Stingl, A. Raouf, O. Nemirovsky, P. Eirew, and C.J. Eaves. 2013. Developmental Changes in the in Vitro Activated Regenerative Activity of Primitive Mammary Epithelial Cells. PLoS Biol. 11:e1001630. doi:10.1371/journal.pbio.1001630.

Mane, S., S. Taneja, J.S. Madala, S. Agarkhedkar, and M. Khetan. 2022. Study of Stem Cells in Human Milk. Cureus 14.

Martin Carli, J.F., G.D. Trahan, K.L. Jones, N. Hirsch, K.P. Rolloff, E.Z. Dunn, J.E. Friedman, L.A. Barbour, T.L. Hernandez, P.S. MacLean, J. Monks, J.L. McManaman, and M.C. Rudolph. 2020. Single Cell RNA Sequencing of Human Milk-Derived Cells Reveals Sub-Populations of Mammary Epithelial Cells with Molecular Signatures of Progenitor and Mature States: a Novel, Non-invasive Framework for Investigating Human Lactation

Physiology. J. Mammary Gland Biol. Neoplasia 25:367–387. doi:10.1007/s10911-020-09466-z.

Martin Carli, J.F., G.D. Trahan, and M.C. Rudolph. 2021. Resolving human lactation heterogeneity using single milk-derived cells, a resource at the ready. J. Mammary Gland Biol. Neoplasia 26:3–8.

McFadden, T.B., R.M. Akers, and G.W. Kazmer. 1987. Alpha-Lactalbumin in Bovine Serum: Relationships with Udder Development and Function. J. Dairy Sci. 70:259–264. doi:10.3168/jds.S0022-0302(87)80005-X.

Mleynek, T.M., M. Redd, A. Chan, Y. Gu, D.Y. Li, and J. Rosenblatt. 2018. Endothelia extrude apoptotic cells to maintain a constant barrier. Cell Biology.

Mollet, M., R. Godoy-Silva, C. Berdugo, and J.J. Chalmers. 2008. Computer simulations of the energy dissipation rate in a fluorescence-activated cell sorter: Implications to cells. Biotechnol. Bioeng. 100:260–272. doi:10.1002/bit.21762.

Montazer-Torbati, F., M. Boutinaud, N. Brun, C. Richard, A. Neveu, F. Jaffrézic, D. Laloë, D. LeBourhis, M. Nguyen, S. Chadi, H. Jammes, J.-P. Renard, S. Chat, A. Boukadiri, and E. Devinoy. 2016. Differences during the first lactation between cows cloned by somatic cell nuclear transfer and noncloned cows. J. Dairy Sci. 99:4778–4794. doi:10.3168/jds.2015-10532.

Motyl, T., M. Gajewska, J. Zarzynska, A. Sobolewska, and B. Gajkowska. 2007. Regulation of Autophagy in Bovine Mammary Epithelial Cells. Autophagy 3:484–486. doi:10.4161/auto.4491.

Murney, R., K. Stelwagen, T.T. Wheeler, J.K. Margerison, and K. Singh. 2015. The effects of milking frequency in early lactation on milk yield, mammary cell turnover, and secretory activity in grazing dairy cows. J. Dairy Sci. 98:305–311. doi:10.3168/jds.2014-8745.

Nakamura, K., and S.F. Morrison. 2008. A thermosensory pathway that controls body temperature. Nat. Neurosci. 11:62–71. doi:10.1038/nn2027.

Nanavati, B.N., A.S. Yap, and J.L. Teo. 2020. Symmetry Breaking and Epithelial Cell Extrusion. Cells 9:1416. doi:10.3390/cells9061416.

Nickerson, S.C., and R.M. Akers. 2011. MAMMARY GLAND | Anatomy. J.W. Fuquay, ed. Academic Press, San Diego.

Noble, M.S., and W.L. Hurley. 1999. Effects of Secretion Removal on Bovine Mammary Gland Function Following an Extended Milk Stasis. J. Dairy Sci. 82:1723–1730. doi:10.3168/jds.S0022-0302(99)75402-0.

Nørgaard, J., A. Sørensen, M.T. Sørensen, J.B. Andersen, and K. Sejrsen. 2005a. Mammary cell turnover and enzyme activity in dairy cows: Effects of milking frequency and diet energy density. J. Dairy Sci. 88:975–982.

Nørgaard, J., A. Sørensen, M.T. Sørensen, J.B. Andersen, and K. Sejrsen. 2005b. Mammary cell turnover and enzyme activity in dairy cows: Effects of milking frequency and diet energy density. J. Dairy Sci. 88:975–982.

Nyquist, S.K., P. Gao, T.K.J. Haining, M.R. Retchin, Y. Golan, R.S. Drake, K. Kolb, B.E. Mead, N. Ahituv, M.E. Martinez, A.K. Shalek, B. Berger, and B.A. Goods. 2022. Cellular and

transcriptional diversity over the course of human lactation. Proc. Natl. Acad. Sci. 119:e2121720119. doi:10.1073/pnas.2121720119.

O'Brien, J., H. Martinson, C. Durand-Rougely, and P. Schedin. 2012. Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. Development 139:269–275. doi:10.1242/dev.071696.

Oftedal, O.T. 2002. The mammary gland and its origin during synapsid evolution. J. Mammary Gland Biol. Neoplasia 7:225–252.

Oltenacu, P., and D. Broom. 2010. The impact of genetic selection for increased milk yield on the welfare of dairy cows. Anim. Welf. 19:39–49. doi:10.1017/S0962728600002220.

Ouellet, V., I.M. Toledo, B. Dado-Senn, G.E. Dahl, and J. Laporta. 2021. Critical Temperature-Humidity Index Thresholds for Dry Cows in a Subtropical Climate. Front. Anim. Sci. 2. doi:10.3389/fanim.2021.706636.

Oviedo-Boyso, J., J.J. Valdez-Alarcón, M. Cajero-Juárez, A. Ochoa-Zarzosa, J.E. López-Meza, A. Bravo-Patiño, and V.M. Baizabal-Aguirre. 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. J. Infect. 54:399–409. doi:10.1016/j.jinf.2006.06.010.

Paape, M.J., Bannerman, D. D., X. Zhao, X., and Lee, J. 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res. 34:597–627. doi:10.1051/vetres:2003024.

Pan, Y.-Q., M. Niu, S. Liu, Y.-X. Bao, K. Yang, X.-B. Ma, L. He, Y.-X. Li, J.-X. Cao, X. Zhang, and Y. Du. 2021. Effect of MT2A on apoptosis and proliferation in HL60 cells. Int. J. Med. Sci. 18:2910–2919. doi:10.7150/ijms.57821.

Papalexi, E., and R. Satija. 2018. Single-cell RNA sequencing to explore immune cell heterogeneity. Nat. Rev. Immunol. 18:35–45. doi:10.1038/nri.2017.76.

Pearson, G., F. Robinson, T. Beers Gibson, B.E. Xu, M. Karandikar, K. Berman, and M.H. Cobb. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.. Endocr. Rev. 22:153–183. doi:10.1210/edrv.22.2.0428.

Pearson, R.E., L.A. Fulton, P.D. Thompson, and J.W. Smith. 1979. Three Times a Day Milking during the First Half of Lactation1. J. Dairy Sci. 62:1941–1950. doi:10.3168/jds.S0022-0302(79)83526-2.

Penry, J.F., E.L. Endres, B. de Bruijn, A. Kleinhans, P.M. Crump, D.J. Reinemann, and L.L. Hernandez. 2017. Effect of incomplete milking on milk production rate and composition with 2 daily milkings.. J. Dairy Sci. 100:1535–1540. doi:10.3168/jds.2016-11935.

Pilla, R., D. Schwarz, S. König, and R. Piccinini. 2012. Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples. J. Dairy Sci. 95:4410–4420. doi:10.3168/jds.2012-5331.

Pollard, J.W., and L. Hennighausen. 1994. Colony stimulating factor 1 is required for mammary gland development during pregnancy.. Proc. Natl. Acad. Sci. 91:9312–9316. doi:10.1073/pnas.91.20.9312.

Ravagnolo, O., I. Misztal, and G. Hoogenboom. 2000. Genetic component of heat stress in dairy cattle, development of heat index function. J. Dairy Sci. 83:2120–2125.

Reed, J.R., and K.L. Schwertfeger. 2010. Immune Cell Location and Function During Post-Natal Mammary Gland Development. J. Mammary Gland Biol. Neoplasia 15:329–339. doi:10.1007/s10911-010-9188-7.

Rhoads, M.L., R.P. Rhoads, M.J. VanBaale, R.J. Collier, S.R. Sanders, W.J. Weber, B.A. Crooker, and L.H. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin1. J. Dairy Sci. 92:1986–1997. doi:10.3168/jds.2008-1641.

Rivas, A.L., F.W. Quimby, J. Blue, and O. Coksaygan. 2001. Longitudinal Evaluation of Bovine Mammary Gland Health Status by Somatic Cell Counting, Flow Cytometry, and Cytology. J. Vet. Diagn. Invest. 13:399–407. doi:10.1177/104063870101300506.

Rovai, M., X. Such, J. Piedrafita, G. Caja, and M.R. Pujol. 1999. Evolution of mammary morphology traits during lactation and its relationship with milk yield of Manchega and Lacaune dairy sheep. Publ.-Eur. Assoc. Anim. Prod. 95:107–112.

Rowson, A.R., K.M. Daniels, S.E. Ellis, and R.C. Hovey. 2012. Growth and development of the mammary glands of livestock: A veritable barnyard of opportunities. Semin. Cell Dev. Biol. 23:557–566. doi:10.1016/j.semcdb.2012.03.018.

Schmidt, G.H. (Glen H. 1971. Biology of Lactation / [by] G. H. Schmidt. A Series of books in animal science. W. H. Freeman, San Francisco.

Schwanhäusser, B., D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, and M. Selbach. 2011. Global quantification of mammalian gene expression control. Nature 473:337–342. doi:10.1038/nature10098.

Seebacher, F. 2009. Responses to temperature variation: integration of thermoregulation and metabolism in vertebrates. J. Exp. Biol. 212:2885–2891. doi:10.1242/jeb.024430.

Seidl, J., R. Knuechel, and L.A. Kunz-Schughart. 1999. Evaluation of membrane physiology following fluorescence activated or magnetic cell separation. Cytometry 36:102–111. doi:10.1002/(SICI)1097-0320(19990601)36:2<102::AID-CYTO3>3.0.CO;2-D.

Seldin, L., A. Le Guelte, and I.G. Macara. 2017. Epithelial plasticity in the mammary gland. Curr. Opin. Cell Biol. 49:59–63. doi:10.1016/j.ceb.2017.11.012.

Sharma, N., N.K. Singh, and M.S. Bhadwal. 2011. Relationship of Somatic Cell Count and Mastitis: An Overview. Asian-Australas. J. Anim. Sci. 24:429–438. doi:10.5713/ajas.2011.10233.

Silanikove, N., F. Shapiro, and D. Shinder. 2009. Acute heat stress brings down milk secretion in dairy cows by up-regulating the activity of the milk-borne negative feedback regulatory system. BMC Physiol. 9:1–9.

Singh, K., J. Dobson, C.V.C. Phyn, S.R. Davis, V.C. Farr, A.J. Molenaar, and K. Stelwagen. 2005. Milk accumulation decreases expression of genes involved in cell–extracellular matrix communication and is associated with induction of apoptosis in the bovine mammary gland. Livest. Prod. Sci. 98:67–78. doi:10.1016/j.livprodsci.2005.10.016.

Singh, K., I. Vetharaniam, J.M. Dobson, M. Prewitz, K. Oden, R. Murney, K.M. Swanson, R. McDonald, H.V. Henderson, and K. Stelwagen. 2016. Cell survival signaling in the bovine mammary gland during the transition from lactation to involution. J. Dairy Sci. 99:7523–7543. doi:10.3168/jds.2015-10515.

Sirka, O.K., E.R. Shamir, and A.J. Ewald. 2018. Myoepithelial cells are a dynamic barrier to epithelial dissemination. J. Cell Biol. 217:3368–3381.

Skibiel, A.L., F. Peñagaricano, R. Amorín, B.M. Ahmed, G.E. Dahl, and J. Laporta. 2018. In utero heat stress alters the offspring epigenome. Sci. Rep. 8:14609.

Slattum, G.M., and J. Rosenblatt. 2014. Tumour cell invasion: an emerging role for basal epithelial cell extrusion. Nat. Rev. Cancer 14:495–501. doi:10.1038/nrc3767.

Slepicka, P.F., A.V.H. Somasundara, and C.O. Dos Santos. 2021. The molecular basis of mammary gland development and epithelial differentiation. Semin. Cell Dev. Biol. 114:93–112. doi:10.1016/j.semcdb.2020.09.014.

Slovin, S., A. Carissimo, F. Panariello, A. Grimaldi, V. Bouché, G. Gambardella, and D. Cacchiarelli. 2021. Single-cell RNA sequencing analysis: a step-by-step overview. RNA Bioinforma. 343–365.

Smith, D.L., T. Smith, B.J. Rude, and S.H. Ward. 2013. Short communication: Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. J. Dairy Sci. 96:3028–3033. doi:10.3168/jds.2012-5737.

Smith, S. 1994. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. FASEB J. 8:1248–1259.

Sobhani, A., N. Khanlarkhani, M. Baazm, F. Mohammadzadeh, A. Najafi, S. Mehdinejadiani, and F.S. Aval. 2017. Multipotent stem cell and current application. Acta Med. Iran. 6–23.

Soloff, M.S. 1982. Oxytocin receptors and mammary myoepithelial cells. J. Dairy Sci. 65:326–337.

Sorensen, A., D.D. Muir, and C.H. Knight. 2008. Extended lactation in dairy cows: effects of milking frequency, calving season and nutrition on lactation persistency and milk quality.. J. Dairy Res. 75:90–97. doi:10.1017/S0022029907002944.

Spiers, D.E., J.N. Spain, J.D. Sampson, and R.P. Rhoads. 2004. Use of physiological parameters to predict milk yield and feed intake in heat-stressed dairy cows. Int. Therm. Physiol. Symp. Physiol. Pharmacol. Temp. Regul. 29:759–764. doi:10.1016/j.jtherbio.2004.08.051.

Stefanon, B., M. Colitti, G. Gabai, C.H. Knight, and C.J. Wilde. 2002. Mammary apoptosis and lactation persistency in dairy animals. J. Dairy Res. 69:37–52.

Stein, T., J.S. Morris, C.R. Davies, S.J. Weber-Hall, M.-A. Duffy, V.J. Heath, A.K. Bell, R.K. Ferrier, G.P. Sandilands, and B.A. Gusterson. 2004. [No title found]. Breast Cancer Res. 6:R75. doi:10.1186/bcr753.

Stelwagen, K., C.V.C. Phyn, S.R. Davis, J. Guinard-Flament, D. Pomiès, J.R. Roche, and J.K. Kay. 2013. Invited review: Reduced milking frequency: Milk production and management implications. J. Dairy Sci. 96:3401–3413. doi:10.3168/jds.2012-6074.

Sternlicht, M.D. 2005. Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis. Breast Cancer Res. 8:201. doi:10.1186/bcr1368.

St-Pierre, N.R., B. Cobanov, and G. Schnitkey. 2003. Economic losses from heat stress by US livestock industries. J. Dairy Sci. 86:E52–E77.

Stuart, T., A. Butler, P. Hoffman, C. Hafemeister, E. Papalexi, W.M. Mauck, Y. Hao, M. Stoeckius, P. Smibert, and R. Satija. 2019. Comprehensive Integration of Single-Cell Data. Cell 177:1888-1902.e21. doi:10.1016/j.cell.2019.05.031.

Sutherland, M.A., and M. Tops. 2014. Possible involvement of oxytocin in modulating the stress response in lactating dairy cows. Front. Psychol. 5:951.

Tao, S., J.W. Bubolz, B.C. Do Amaral, I.M. Thompson, M.J. Hayen, S.E. Johnson, and G.E. Dahl. 2011. Effect of heat stress during the dry period on mammary gland development. J. Dairy Sci. 94:5976–5986.

Tao, S., A.P.A. Monteiro, I.M. Thompson, M.J. Hayen, and G.E. Dahl. 2012. Effect of late-gestation maternal heat stress on growth and immune function of dairy calves. J. Dairy Sci. 95:7128–7136. doi:10.3168/jds.2012-5697.

Tao, S., R.M. Orellana, X. Weng, T.N. Marins, G.E. Dahl, and J.K. Bernard. 2018. Symposium review: The influences of heat stress on bovine mammary gland function. J. Dairy Sci. 101:5642–5654.

Toledo, I.M., X. Zhao, and P. Lacasse. 2020. Effects of milking frequency and domperidone injections on milk production and prolactin signaling in the mammary gland of dairy cows. J. Dairy Sci. 103:1969–1981. doi:10.3168/jds.2019-17330.

Trend, S., E. De Jong, M.L. Lloyd, C.H. Kok, P. Richmond, D.A. Doherty, K. Simmer, F. Kakulas, T. Strunk, and A. Currie. 2015. Leukocyte Populations in Human Preterm and Term Breast Milk Identified by Multicolour Flow Cytometry. PLOS ONE 10:e0135580. doi:10.1371/journal.pone.0135580.

Twigger, A.-J., L.K. Engelbrecht, K. Bach, I. Schultz-Pernice, S. Pensa, J. Stenning, S. Petricca, C.H. Scheel, and W.T. Khaled. 2022. Transcriptional changes in the mammary gland during lactation revealed by single cell sequencing of cells from human milk. Nat. Commun. 13:562. doi:10.1038/s41467-021-27895-0.

Villani, A.-C., R. Satija, G. Reynolds, S. Sarkizova, K. Shekhar, J. Fletcher, M. Griesbeck, A. Butler, S. Zheng, S. Lazo, L. Jardine, D. Dixon, E. Stephenson, E. Nilsson, I. Grundberg, D. McDonald, A. Filby, W. Li, P.L. De Jager, O. Rozenblatt-Rosen, A.A. Lane, M. Haniffa, A. Regev, and N. Hacohen. 2017. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science 356:eaah4573. doi:10.1126/science.aah4573.

Visvader, J.E. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev. 23:2563–2577. doi:10.1101/gad.1849509.

Visvader, J.E., and J. Stingl. 2014. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev. 28:1143–1158. doi:10.1101/gad.242511.114.

Wagner, K.-U., A. Krempler, A.A. Triplett, Y. Qi, N.M. George, J. Zhu, and H. Rui. 2004. Impaired Alveologenesis and Maintenance of Secretory Mammary Epithelial Cells in Jak2 Conditional Knockout Mice. Mol. Cell. Biol. 24:5510–5520. doi:10.1128/MCB.24.12.5510-5520.2004.

Wakasa, H., Y. Tsugami, T. Koyama, L. Han, T. Nishimura, N. Isobe, and K. Kobayashi. 2022. Adverse Effects of High Temperature On Mammary Alveolar Development In Vitro. J. Mammary Gland Biol. Neoplasia 27:155–170.

Wall, E.H., J.P. Bond, and T.B. McFadden. 2013. Milk yield responses to changes in milking frequency during early lactation are associated with coordinated and persistent changes in mammary gene expression. BMC Genomics 14:296. doi:10.1186/1471-2164-14-296.

Wall, E.H., H.M. Crawford, S.E. Ellis, G.E. Dahl, and T.B. McFadden. 2006. Mammary response to exogenous prolactin or frequent milking during early lactation in dairy cows. J. Dairy Sci. 89:4640–4648.

Wall, E.H., and T.B. McFadden. 2007a. The Milk Yield Response to Frequent Milking in Early Lactation of Dairy Cows Is Locally Regulated. J. Dairy Sci. 90:716–720. doi:10.3168/jds.S0022-0302(07)71555-2.

Wall, E.H., and T.B. McFadden. 2007b. Optimal Timing and Duration of Unilateral Frequent Milking During Early Lactation of Dairy Cows. J. Dairy Sci. 90:5042–5048. doi:10.3168/jds.2007-0356.

Wall, E.H., and T.B. McFadden. 2008. Use it or lose it: Enhancing milk production efficiency by frequent milking of dairy cows. J. Anim. Sci. 86:27–36.

Wall, E.H., and T.B. McFadden. 2010. The effects of milk removal or four-times-daily milking on mammary expression of genes involved in the insulin-like growth factor-I axis.. J. Dairy Sci. 93:4062–4070. doi:10.3168/jds.2010-3162.

Wall, E.H., and T.B. McFadden. 2012. TRIENNIAL LACTATION SYMPOSIUM: A local affair: How the mammary gland adapts to changes in milking frequency1,2. J. Anim. Sci. 90:1695–1707. doi:10.2527/jas.2011-4790.

Watson, C.J., and T.G. Burdon. 1996. Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway.. Rev. Reprod. 1:1–5. doi:10.1530/ror.0.0010001.

Watson, C.J., and W.T. Khaled. 2008. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. Development 135:995–1003. doi:10.1242/dev.005439.

Weaver, S.R., and L.L. Hernandez. 2016. Autocrine-paracrine regulation of the mammary gland1. J. Dairy Sci. 99:842–853. doi:10.3168/jds.2015-9828.

Wellnitz, O., and R.M. Bruckmaier. 2021. Invited review: The role of the blood–milk barrier and its manipulation for the efficacy of the mammary immune response and milk production. J. Dairy Sci. 104:6376–6388. doi:10.3168/jds.2020-20029.

WELLNITZ, O., R.M. BRUCKMAIER, C. ALBRECHT, and J.W. BLUM. 1999. Atosiban, an oxytocin receptor blocking agent: pharmacokinetics and inhibition of milk ejection in dairy cows. J. Dairy Res. 66:1–8.

Weng, X., A.P.A. Monteiro, J. Guo, C. Li, R.M. Orellana, T.N. Marins, J.K. Bernard, D.J. Tomlinson, J.M. DeFrain, S.E. Wohlgemuth, and S. Tao. 2018. Effects of heat stress and dietary zinc source on performance and mammary epithelial integrity of lactating dairy cows. J. Dairy Sci. 101:2617–2630. doi:10.3168/jds.2017-13484.

West, J.W., B.G. Mullinix, and J.K. Bernard. 2003. Effects of Hot, Humid Weather on Milk Temperature, Dry Matter Intake, and Milk Yield of Lactating Dairy Cows. J. Dairy Sci. 86:232–242. doi:10.3168/jds.S0022-0302(03)73602-9.

Wheelock, J.B., R.P. Rhoads, M.J. VanBaale, S.R. Sanders, and L.H. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. J. Dairy Sci. 93:644–655.

Witkowska-Zimny, M., and E. Kaminska-El-Hassan. 2017. Cells of human breast milk. Cell. Mol. Biol. Lett. 22:11. doi:10.1186/s11658-017-0042-4.

Wohlgemuth, S.E., Y. Ramirez-Lee, S. Tao, A.P.A. Monteiro, B.M. Ahmed, and G.E. Dahl. 2016. Short communication: Effect of heat stress on markers of autophagy in the

mammary gland during the dry period. J. Dairy Sci. 99:4875–4880. doi:10.3168/jds.2015-10649.

Wright, J.B., E.H. Wall, and T.B. McFadden. 2013. Effects of increased milking frequency during early lactation on milk yield and udder health of primiparous Holstein heifers.. J. Anim. Sci. 91:195–202. doi:10.2527/jas.2012-5692.

Yan, G., M. Elbadawi, and T. Efferth. 2020. Multiple cell death modalities and their key features (Review). World Acad. Sci. J.. doi:10.3892/wasj.2020.40.

Yue, S., Z. Wang, L. Wang, Q. Peng, and B. Xue. 2020. Transcriptome functional analysis of mammary gland of cows in heat stress and thermoneutral condition. Animals 10:1015.

Zhang, Y., X. Chen, C. Gueydan, and J. Han. 2018. Plasma membrane changes during programmed cell deaths. Cell Res. 28:9–21. doi:10.1038/cr.2017.133.

Zhao, F.-Q. 2014. Biology of glucose transport in the mammary gland. J. Mammary Gland Biol. Neoplasia 19:3–17.

Zhao, F.-Q., P.J. Miller, E.H. Wall, Y.-C. Zheng, B. Dong, M.C. Neville, and T.B. McFadden. 2004. Bovine glucose transporter GLUT8: cloning, expression, and developmental regulation in mammary gland. Biochim. Biophys. Acta BBA-Gene Struct. Expr. 1680:103–113.

Zheng, G.X., J.M. Terry, P. Belgrader, P. Ryvkin, Z.W. Bent, R. Wilson, S.B. Ziraldo, T.D. Wheeler, G.P. McDermott, and J. Zhu. 2017. Massively parallel digital transcriptional profiling of single cells. Nat. Commun. 8:14049.

Zhou, Y., A.V. Capuco, and H. Jiang. 2008. Involvement of connective tissue growth factor (CTGF) in insulin-like growth factor-I (IGF1) stimulation of proliferation of a bovine mammary epithelial cell line. Domest. Anim. Endocrinol. 35:180–189. doi:10.1016/j.domaniend.2008.05.003.

Zimbelman, R.B., R.J. Collier, and M.L. Eastridge. 2011. Feeding strategies for high-producing dairy cows during periods of elevated heat and humidity. Pages 111–126 in Tri-State Dairy Nutrition Conference, Grand Wayne Center.

Zimbelman, R.B., R.P. Rhoads, M.L. Rhoads, G.C. Duff, L.H. Baumgard, and R.J. Collier. 2009. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. Pages 158–169 in Proceedings of the Southwest Nutrition Conference (ed. RJ Collier).