Blood and Urinary Metabotyping Reveals Potential Predictive Biomarkers for Identifying Dairy Cows at Risk of Subclinical Mastitis During the Dry-Off Period

by

Klevis Haxhiaj

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Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Subclinical mastitis (SCM) remains one of the most important infectious diseases of dairy cows as it is associated with considerable losses in milk production and financial revenue. Currently, most SCM research and practices focus on diagnosing this intramammary infection (IMI) by counting somatic cells (SCC) in milk throughout lactation. Therefore, this study aimed to identify metabolic alterations in the serum and urine of pre-SCM cows during the dry period, along with developing panels of screening biomarkers for lab-based and pen-side tests. Early identification of susceptible cows will enable better preventative and management strategies for SCM.

A combination of flow injection and liquid chromatography coupled with tandem mass spectrometry (FIA/LC-MS/MS) analysis were used to characterize 580 blood and urine samples collected from 145 Holstein cows at –8 and –4 wks before the expected date of calving. Cows enrolled in this nested-case control study were then monitored for the development of postpartum diseases. Fifteen cows were free of any condition (CON), and just 10 cows presented with only SCM (characterized by high SCC) after calving and were free of other diseases. Metabolomics identified 126 serum metabolites from which 59 at –8 wks and 47 at –4 wks were found altered ($P \leq 0.05$) in pre-SCM cows compared to CON cows. Using FDR adjusted P values, 32 metabolites at –8 wks and 17 at –4 wks were in the range of $q < 0.005$. The main metabolite classes that were altered were related to lipid metabolism, such as acylcarnitines (ACs), lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs) and sphingomyelins (SMs). Others were amino acids (AAs), methyl donor compounds, organic acids (OAs), and several carbohydrate species. Univariate, multivariate, and machine learning analysis indicated that a panel of 4 serum metabolites including alanine, leucine, betaine, and ornithine (AUC = 0.92; $P < 0.001$) at

–8 wks and alanine, pyruvate, methylmalonate, and lactate (AUC = 0.92, $P < 0.01$) at –4 wks before parturition might serve as the best predictive serum biomarkers for SCM for a pen-side test. On the other hand, a total of 82 metabolites were found in the urine samples, and only 27 compounds ($P \leq 0.05$) were different at each sampling period. At $q < 0.005$ only 4 metabolites were altered from each week. The most discriminating metabolites were ACs, several AAs and their derivatives, glucose, and OAs. Further regression analysis showed that four metabolites: ADMA, proline, leucine, and homovanillate (AUC=0.88; $P = 0.02$) at –8 wks and another four metabolites: ADMA, spermidine, methylmalonic acid and citrate (AUC = 0.88, $P = 0.03$) at –4 wks as specific urinary biomarkers for SCM.

Overall, these data indicated systemic metabolic alterations occur in pre-SCM cows. They also showed that differentiation of pre-SCM cows against CON cows is possible and the data provided more information on the pathobiology of SCM. These predictive biomarkers also offer the potential to develop lab-based and pen-side tests to identify cows at risk of SCM during the dry period. The health status dataset for all the cows enrolled in this study demonstrated that several other cows were positive for SCM and at least one or more other diseases, including ketosis, leukosis, retained placenta, lameness, and milk fever. This complicates the development of lab and pen-side tests and warrants more research to explore the possibility of identifying specific metabolites for SCM alone that can separate SCM cows from the other diseases.

Preface

The idea, project proposal, and experimental design were developed by my supervisor Dr. Burim N. Ametaj and Dr. David Wishart. All experiments and research activities of the study were conducted at Dr. Ametaj's lab, Dr. Wishart's lab – The Metabolomics Innovation Centre (TMIC) and Sunalta Farms Inc. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee for Livestock (Animal Use Protocol No. 00006283).

Dr. Ametaj supervised the training and the experimental activities as well as the statistical analysis, reviewing, writing, and editing of the thesis. Dr. Wishart and his team at TMIC contributed to sample analyses, statistical processing and document revision. The interdepartmental and laboratory work was facilitated by laboratory managers Dr. Rupasri Mandal and Suzanna M. Dunn. Preparation of field materials and the collection of samples was fully or partially conducted with the help of Dr. Ametaj, Suzanna M. Dunn, Dr. Zhili Li, and undergraduate students Jacqueline Zhang and Sandy Chow. I was responsible for collecting the samples, preparing and maintaining the databases of samples, collecting the metabolomic data, and cow cards, performing the statistical analysis, and writing the thesis and related documents.

To my mom, dad, and sister,

thank you for the trust,

and allowing me to walk my own path.

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List of Abbreviations

-	Minus
+	Plus
$	Dollar
%	Percentage
<	Less than
>	More than
≤	Less than or equal to
°C	degree Celsius
1H-NMR	Proton nuclear magnetic resonance
2D-NMR	Two-dimensional nuclear magnetic resonance
3-NPH	3-Nitrophenylhydrazine
AAs	Amino acids
ACAA2	Acetyl-CoA acyltransferase-2
ACN	Acetonitrile
ACs	Acylcarnitines
ADMA	Asymmetric dimethylarginine
AGP	Antimicrobial growth promoters
Ala	Alanine
AMR	Antimicrobial resistance
AMS	Automated milking systems
AMS	Animal antimicrobial stewardship
APC	Antigen presenting cells
APCI	Atmospheric-pressure chemical ionization
APPs	Acute phase proteins
APR	Acute phase response
Arg	Arginine

Asp	Aspartic acid
AUC	The area under the ROC curve
BCAAs	Branched-chain amino acids
BHBA	Beta-hydroxybutyric acid
BMDB	Bovine metabolome database
BSC	Body condition score
BTSCC	Bulk tank milk somatic cell count
C0	DL-Carnitine
C2	Acetyl-L-carnitine
C3	Propionyl-L-carnitine
C3:1	Propenyl-L-carnitine
C3-DC (C4-OH)	Malonyl-L-carnitine / Hydroxy butyryl-L-carnitine
C3-OH	Hydroxy propionyl-L-carnitine
C4	Butyryl-L-carnitine
C4:1	Butenyl-L-carnitine
C5	Valeryl-L-carnitine
C5:1	Tiglyl-L-carnitine
C5:1-DC	Glutaconyl-L-carnitine
C5-DC (C6-OH)	Glutaryl-L-carnitine / Hydroxyhexanoyl-L-carnitine
C5-M-DC	Methylglutaryl-L-carnitine
C5-OH (C3-DC-M)	Methylmalonyl-L-carnitine/Hydroxyvaleryl-L-carnitine
C6 (C4:1-DC)	Fumaryl-L-carnitine / Hexanoyl-L-carnitine
C6:1	Hexenoyl-L-carnitine
C7-DC	Pimelyl-L-carnitine
C8	Octanoyl-L-carnitine
C9	Nonayl-L-carnitine
C10	Decanoyl-L-carnitine
C10:1	Decenoyl-L-carnitine

C10:2	Decadienyl-L-carnitine
C12	Dodecanoyl-L-carnitine
C12:1	Dodecenoyl-L-carnitine
C12-DC	Dodecanedioyl-L-carnitine
C14	Tetradecanoyl-L-carnitine
C14:1	Tetradecenoyl-L-carnitine
C14:1-OH	Hydroxy tetradecenoyl-L-carnitine
C14:2	Tetradecadienyl-L-carnitine
C14:2-OH	Hydroxy tetradecadienyl-L-carnitine
C16	Hexadecanoyl-L-carnitine
C16:1	Hexadecenoyl-L-carnitine
C16:1-OH	Hydroxy hexadecenoyl-L-carnitine
C16:2	Hexadecadienyl-L-carnitine
C16:2-OH	Hydroxy hexadecadienyl-L-carnitine
C16-OH	Hydroxy hexadecanoyl-L-carnitine
C18	Octadecenoyl-L-carnitine
C18:1	Octadecenoyl-L-carnitine
C18:1-OH	Hydroxy octadecenoyl-L-carnitine
C18:2	Octadecadienyl-L-carnitine
C1P	Ceramide-1-phosphate
Cals	Calibration curve standards
CanWest DHI	Canada west dairy herd improvement
CD14	Cluster of differentiation 14
CD40L	Cluster of differentiation 40 ligand
CE-MS	Capillary electrophoresis mass spectrometry
CFU	Colony-forming units
CGRP	Calcitonin gene-related peptide
CI	Confidence interval

CIC	Citrate carrier
CLP	Cecal ligation and puncture
CM	Clinical mastitis
CMT	California mastitis test
CON	Healthy control
CRP	C-reactive protein
CV	Cross-validation
d	Day
DA	Displaced abomasum
DAMPs	Damage-associated molecular patterns
DCT	Dry cow therapy
DIM	Days in milk
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Electrical conductivity
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAO	Fatty acid oxidation
FIA/LC-MS/MS	Flow injection analysis/liquid chromatography - tandem mass spectrometry
FT-ICR	Fourier transform ion cyclotron resonance
GC-MS	Gas chromatography - mass spectrometry
GH	Growth hormone
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GSH	Glutathione

H	Hour(s)
Hcy	Homocysteine
HDL	High density lipoproteins
HILIC	Hydrophilic interaction liquid type of chromatography
His	Histidine
HMB	Hydroxy-beta-methyl butyrate
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
Hp	Haptoglobin
HPLC	High performance liquid chromatography
HSCC	Herd average of individual cow somatic cell count
HVA	Homovanillic acid
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IFN-γ	Interferon-gamma
IIR	Innate Immune Response
IL	Interleukin
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-17	Interleukin-17
Ile	Isoleucine
IMI	Intramammary infection
ISTD	Internal standards
kg	Kilogram
LAMP	Loop-mediated isothermal amplification
LBP	Lipopolysaccharide binding protein
LC	Liquid chromatography/chromatographer

LDA	Left displaced abomasum
Leu	Leucine
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Lys	Lysine
LPC	Lysophosphatidylcholine
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MCCV	Monte-Carlo cross-validation
MCDB	Milk composition database
MD2	Myeloid differential protein 2
MEC	Mammary epithelial cells
MeOH	Methanol
Met	Methionine
MG	Mammary gland
MHC I	Major histocompatibility complex 1
MHC II	Major histocompatibility complex 2
min	Minutes
mL	Milliliter
mM	Millimolar
MMA	Methylmalonic acid
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry/spectrometer
MSEA	Metabolite set enrichment analysis
mTOR	Mammalian target of rapamycin
MUT	Methylmalonyl-coA mutase
NAS	Non-aureus staphylococci
NEB	Negative energy balance

NEFA	Non-esterified fatty acids
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NK	Natural killer cells
NLR	Nucleotide-binding and oligomerization domain-like receptors
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NOS	Nitric oxide synthase
OAs	Organic acids
Orn	Ornithine
P. multocida	*Pasteurella multocida*
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffer saline
PC	Principal component
PC	Phosphatidylcholine
PC aa	Phosphatidylcholine diacyl
PC ae	Phosphatidylcholine acyl-alkyl
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEMT	Phosphatidylethanolamine N-methyltransferase
PGE2	Prostaglandin E2
Phe	Phenylalanine
PITC	Phenylisothiocyanate
PLS-DA	Partial least squares - discriminant analysis
PMN	Polymorphonuclear neutrophil
Pro	Proline

PRRs	Pattern recognition receptor
Q2	Quality of prediction
QC	Quality control
QIT	Quadrupole-iron trap
qPCR	Quantitative polymerase chain reaction
QqQ	Triple quadrupole
Q-TOF	Quadrupole-time of flight
R2	Correlation index
ROC	Receiver-operating characteristic
ROS	Reactive oxygen species
RP	Retained placenta
RPLC	Reversed-phase liquid chromatography
rpm	Rotations per minute
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
S. chromogens	*Staphylococci chromogens*
S. dysgalactiae	*Streptococcus dysgalactiae*
S. uberis	*Streptococcus uberis*
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SCC	Somatic cell count
SCM	Subclinical mastitis
SCM+	Subclinical mastitis and other diseases
SDMA/TDMA	Symmetric dimethylarginine/ Total dimethylarginine
SEM	Standard error of the mean
Ser	Serine
SM	Sphingomyelin
SM (OH)	Hydroxy sphingomyelin

SPC	Standard plate count
sPLA2	Secretory phospholipase A2
SVM	Support vector machine
TAG	Triacylglycerides/triglycerides
TCA cycle	Tricarboxylic acid / citric acid cycle
TG	Triglycerides
Thr	Threonine
TLRs	Toll-like receptors
TMAO	Trimethylamine-N-oxide
TMIC	The Metabolomics Innovation Centre
TMS	Trimethylsilane
TNF	Tumor necrosis factor
TNF-α	Tumour necrosis factor-alpha
Trp	Tryptophan
Tyr	Tyrosine
UPLC	Ultra-high performance liquid chromatography
Val	Valine
VIP	Variable importance in projection
VLDL	Very-low-density lipoproteins
Wks	Weeks
α	Alpha
β	Beta
γ	Gamma
μ	Micro
μL	microliter
μM	micromolar

Chapter 1. Literature Review

1.1 Introduction

1.1.1 The importance of mastitis in dairy cows

Mastitis is a multifactorial inflammatory disease of the mammary gland. This intramammary infections (IMI) is most commonly caused by bacteria such as *Escherichia coli, Staphylococcus aureus,* and *Streptococcus spp*. Mastitis is of particular concern to dairy cattle and dairy farmers. It is typically seen in dairy herds between the dry-off and early lactation period (Thompson-Crispi et al., 2013). Other rare causative agents for mastitis can be trauma or toxic chemicals (Reyher and Dohoo, 2011). Mastitis presents chemical and physical alterations to the milk and abnormal mammary gland appearance. Depending on the signs, symptoms and severity, mastitis can be classified as clinical mastitis (CM) or subclinical mastitis (SCM). Mastitis is monitored by measuring the somatic cell count (SCC) in milk, which, if the number of somatic cells exceed 200,000 cells/mL, the mammary gland is inflamed and this highly suggests infection (Dohoo and Leslie, 1991), as presented in Figure 1.1.

This disease can significantly impact the health of dairy cattle and dairy farm profitability (Ruegg and Petersson-Wolfe, 2018). Indeed, mastitis' financial impact is considerable, as it is estimated to cost a Canadian dairy farmer $662/year per cow (Aghamohammadi et al., 2018). In fact, reproductive disorders and mastitis are the most common reasons why dairy cows are prematurely culled from the herd (CDIC, 2020). The median incidence of CM was estimated to be 19 cases per 100 cows/year (Aghamohammadi et al., 2018), whereas the incidence of SCM is estimated to be up to 50 cases per 100 cows/year (Busanello et al., 2017). Farm management is a significant factor

in the occurrence of IMI; thus, different farms will have different levels of CM or SCM (Verbeke et al., 2014). Given the excessive costs and impact on dairy production, profitability and herd health, there is a strong global effort to lower mastitis incidence, reduce the negative impact and improve milk quality, production and farmer finances.

1.1.2 Etiology

In 1988, Watts listed 137 microorganisms as being causative for mastitis (Watts, 1988). Later Zadoks and colleagues reported more than 200 pathogens are associated with this pathology (Zadoks et al., 2011). The vast majority of mastitis cases in dairy cattle are thought to derive from contagious, environmental pathogens or opportunistic microbes such as non-aureus Staphylococci (NAS) (Moroni et al., 2018). Following is a more detailed list of some of the most important agents causing IMI, presented on Table 1.1

Staphylococcus aureus

Staphylococcus aureus is currently one of the most problematic mastitis pathogens. It is more predominant in chronic or subclinical forms of the disease, causing mild, moderate and severe infections that can cause sudden death (Schukken et al., 2011). It invades the mammary tissues and causes necrosis through the release of lipoteichoic acid (LTA) into the interstitial tissue of the mammary gland (MG) (Dego et al., 2002). *Staphylococcus aureus* can also produce biofilms as a barrier against the host immune response. These infections are more common during early lactation and are associated with continuous losses in milk production (Persson Waller et al., 2009; Heikkilä et al., 2018). LTA of Gram-positive bacteria such as *S. aureus* tend to elicit a weaker immune response.

This is because *S. aureus* can dampen the activation of the NF-kB signalling pathway, not eliciting a strong proinflammatory cytokine expression (Lara-Zárate et al., 2011; Giovannini et al., 2017). The antimicrobial treatment has generally not shown much efficacy against *S. aureus* infections, making this pathogen persistent in many dairy herds (Barkema et al., 2006).

Escherichia coli

Escherichia coli is the primary pathogen that causes clinical signs of mastitis (described in more detail in section 1.1.4). This Gram-negative bacterium is found in the environment and causes acute to peracute infections resulting in a rapid, sometimes fatal immune response (Pyörälä et al., 2011). Due to the systemic inflammation that follows *E. coli* infection, much more damage to the mammary gland and a substantial reduction in milk yield can occur compared to infections by other pathogens (Heikkilä et al., 2018). The mammary gland operates under low oxygen pressures, making it a suitable environment for this coliform bacterium to thrive and flourish (Hogan and Smith, 2003). *E. coli* can be found in a non-pathogenic form in the udder, gastrointestinal and reproductive tract. Under non-favourable conditions, lipopolysaccharide (LPS) produced by Gram-negative bacteria such as *E. coli* can translocate into the systemic circulation and cause endotoxemia (Eckel and Ametaj, 2016).

Streptococcus uberis

Streptococcus uberis, a common environmental pathogen that causes moderate clinical signs, which manifests as abnormal and visible changes in the mammary glands

with CM (Leigh, 1999). It is a Gram-positive microorganism present in pasture and free-stall systems, and because of its ubiquity in the environment, it can become a persistent causative agent of mastitis (Leigh, 1999; Bradley et al., 2007; Rato et al., 2011).

Klebsiella spp

Klebsiella spp. are Gram-negative, environmental pathogens that cause 2-9% of clinical mastitis cases (Oliveira et al., 2013; Levison et al., 2016). The importance of *Klebsiella spp.* is related to economic losses as these infections lower milk yield and increase veterinary bills. Furthermore, most cows that are positive for *Klebsiella spp.* are predisposed to life-threating mastitis and not a positive prognosis (Gröhn et al., 2004). The pathogenicity of *Klebsiella spp.* infection is proposed to be mediated by many virulence genes and biofilm formation. Furthermore, 42% of all mastitis samples with *Klebsiella spp.* demonstrate antimicrobial resistance (AMR) (Schönborn et al., 2017; Massé et al., 2020).

Opportunistic pathogens

Most opportunistic pathogens causing mastitis fall into the category of non-aureus staphylococci (NAS). These microbes are common residents of the teat skin. There are 48 known NAS, but few of them cause IMI. The best known of the causative NAS is *Staphylococci chromogens*. This microbe causes SCM but with only slight change to milk quality. An increase in NAS can occur because of poor hygiene, teat skin injuries and inadequate milking procedures. A NAS infection does not often demonstrate any clinical signs. As a result, it can only be discovered from a positive bacteriological culture.

Measures such as post milking teat dipping with iodine along with improved milking hygiene, minimizing teat damages, and dry cow therapy can keep NAS infections under control (Moroni et al., 2018). NAS pathogens are becoming the predominant mastitis bacteria in many countries, and these microbes are often associated with recurrent infections due to their ability to form biofilms (Tremblay et al., 2013).

Mastitis-causing pathogens can be categorized into two groups: major or minor, based on their pathogenicity. The major pathogens comprise *S. aureus, S. dysgalactiae, S. agalactiae, S. uberis* and the Enterobacteriaceae. Cows infected with these pathogens require intensive care, and these bacteria are not easily eliminated. The minor pathogens include *Corynebacterium spp*. and the NAS spp. Minor pathogens do not cause visible changes to the udder and can be kept under control, however, they trigger an elevated SCC in the milk, leading to IMI and sudden death (Reyher et al., 2012).

In Canada, most CM cases are caused by *Escherichia coli* and *Staphylococcus aureus* (Sargeant et al., 1998; Thompson-Crispi et al., 2013). In the Netherlands and the US, *E. coli* was found to be predominant over all other microbes (Barkema et al., 1998; Roberson et al., 2004). *S. aureus, S. dysgalactiae*, and *E. coli* are most frequently isolated in Norway and Sweden (Reksen et al., 2006). In New Zealand, *S. uberis* is the leading cause of CM and SCM (McDougall, 1999), whereas, in Albania, *S. aureus* and *S. agalactiae* were found to be the main cause of SCM (McDougall, 1999; Kopali et al., 2011). In China, Enterobacteriaceae were the most common pathogens leading to CM and SCM (He et al., 2020).

1.1.3 Pathogenesis

No matter the pathogen, whether it is environmental or contagious, the main route of mammary gland infection in mastitis is through the teat canal. The first anatomical structure that bacteria deal with is the teat end and teat canal. A sphincter muscle surrounds it and helps to maintain a tight closure not to let milk escape. Keratin, a waxy material derived from the epithelial lining, also lines the teat canal. This serves as a physical obstruction usually present during the non-lactating period when the teat end is completely closed with this substance. Keratin contains antibacterial fatty acids with bacteriostatic and bactericidal activities. Their activity is more intense towards some bacteria than others. (Breen et al., 2006; Sordillo, 2018). When mastitis-causing pathogens pass this barrier, an innate immune response (IIR) is initiated.

This immune response is mediated at the beginning by innate immunity. It includes both cellular (e.g., polymorphonuclear neutrophils (PMN), macrophages, natural killer (NK) cells, dendritic cells and mammary epithelial cells (MEC)) and humoral defences (complement system, cytokines, lactoferrin, transferrin, lysozyme, acute phase proteins (APPs) as well as reactive oxygen species (ROS) and antimicrobial peptides (Rainard and Riollet, 2006). Local mammary cell populations such as macrophages, dendritic cells, and epithelial cells have pathogen recognition receptors (PRRs) that interact with pathogen-associated molecular patterns (PAMPs). The latter are motifs or distinctive protein sequences on the surface of microbes (e.g., LPS or LTA) released by microorganisms when they replicate or degrade. Toll-like receptors (TLR) and nucleotide-binding and oligomerization domain-like receptors (NLR) are the two prominent families of PRRs (Wiersinga et al., 2014). LPS is recognized by TLR-4, whereas TLR-2 recognizes LTA. In

case of a Gram-negative infection, LPS will stimulate innate immunity, being recognized by TLR-4. Other proteins, including cluster of differentiation 14 (CD14), myeloid differential protein 2 (MD2), and lipopolysaccharide-binding protein (LBP), help activate the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signalling pathway (Miyake, 2007; Mani et al., 2012). This activation triggers the production of pro-inflammatory cytokines and the acute phase response (APR), which initiates a ubiquitous and rapid innate immune response (Brenaut et al., 2014).

Peripheral neutrophils contacted by IL-8 rapidly migrate to the infected area to phagocytose and destroy the intruding bacteria. The high concentration of SCCs found in cows' milk with CM or SCM is mostly made of neutrophils, indicating "a cellular battle" inside the mammary gland (Zhao and Lacasse, 2008). If microbes are eliminated, the host returns to a healthy, homeostatic state. If not, the adaptive immune system is activated. This response is mediated by T and B lymphocytes. T-cells are activated by encountering an antigen-presenting cell (APC), MHC II, plus various cytokines. Once activated, T-cells can exert their cytotoxic activity or activate B-cells, which produce antibodies to eliminate the bacterial intruder and create a memory of its specific antigen (Sordillo and Streicher, 2002; Ezzat Alnakip et al., 2014). Depending on the type and scale of pathogen invasion, this infection is presented either in a clinical or subclinical form. This process is presented schematically in Figure 1.2.

1.1.4 Clinical mastitis (CM)

Clinical mastitis is one of the most important diseases affecting dairy cows. Clinical mastitis manifests with clear visible external signs such as swelling, redness and/or pain in

the udder, and systemic fever. There are also detectable changes to the milk and its components (Leslie and Petersson-Wolfe, 2012). Variations in colour, consistency, the presence of clots, blood, and chemical changes can occur in milk (Zhao and Lacasse, 2008; Roberson, 2012). Other milk modifications may include increased conductivity, increased pH and changed water content.

Clinical mastitis is categorized based on its duration (subacute, acute, peracute) and severity (mild, moderate, severe) (Adkins and Middleton, 2018). With mild/subacute CM, most changes are found in the milk's colour and consistency. Moderate/acute CM is characterized by changes in the milk and mammary gland, including redness, swelling, heat and pain. Severe/peracute CM is characterized by abnormal milk and mammary gland along with systemic signs such as fever, loss of appetite, and an inability or unwillingness to move (Ruegg, 2011; Suojala et al., 2013).

1.1.5 Subclinical mastitis

Subclinical mastitis is another form of mastitis but doesn't present with any noticeable symptoms or signs such as external changes in the udder or milk (Forsbäck et al., 2009). As the milk does not show any SCM changes, farmers unintentionally decrease the bulk-tank milk quality while mixing this milk with milk from healthy cows (Leitner et al., 2008). Adding SCM milk contributes to an increase in somatic cells, decreases milk quality, and introduces pathogens into the normal milk. Since there are no visible signs of the disease with SCM, it can continue to persist. The only way to discover it is by doing bacterial cultures and weekly tests to measure the presence of SCC in the milk throughout the lactation period (Schukken et al., 2011).

Subclinical mastitis can be highly significant to a dairy farm as it can affect up to 50% of the herd (Pitkälä et al., 2004; Busanello et al., 2017). This has made SCM detection and management an enormous challenge for the past, present, and future dairy industry. Currently, SCM and IMI are monitored through continuous measurements of SCC in the milk. If the SCC is lower than the threshold of 200,000 cells/mL, a quarter of the udder can be considered non-infected. Therefore, the optimal value of SCC for a herd should be no more than 100,000 SCC/mL of milk as this would guarantee that most of the herd is not infected (Schukken et al., 2003; Rhoda and Pantoja, 2012).

1.2 Role of the dry period in the life of cows

1.2.1 Anatomical and physiological changes of the mammary gland

The dry or the nonlactating period is when metabolic and nutritional changes happen to the cow and the mammary gland (Dingwell et al., 2003a). This period prepares cows for the next calving and lactation cycle (Jones, 2009). The dry period helps the udder tissue involute and regenerate to provide better milk yields after calving (Capuco et al., 1997; Kuhn et al., 2005). This period should be at least 40 – 60 days long; most farmers practice a 6 – 8 week (42 – 56 days) dry period. It has been shown that cows dried less than 40 days typically produce less milk than the previous lactation (Jones, 2009).

Involution, steady-state, and colostrogenesis are the three phases that the mammary gland goes through during the dry-off period. Involution that starts with milk cessation is proceeded by dry cow therapy (DCT), where the farmers routinely administer antibiotics, teat sealants, and vaccines as measures to eliminate current infections and prevent new ones from developing during late gestation. Colostrogenesis prepares the udder and the

host for milk secretion while the mammary gland grows and repairs for the upcoming lactation. Intramammary infections that occur during the dry period damage the mammary tissue turning it into a non-functional gland that produces less milk. Another physiological system that is affected during the dry-off period is the gastrointestinal tract and its microflora. These systems must adapt to dietary changes introduced during the dry-off period (Dingwell et al., 2004; Akers et al., 2014).

The critical period for a cow's health is three weeks before and three weeks after calving. This is called the transition period. During this period, cows go through significant nutritional, metabolic, immune, and hormonal changes, making them more susceptible to periparturient diseases (Ingvartsen and Moyes, 2015). The periparturient period is when the fetus grows the most (Dingwell et al., 2004). It requires several major metabolic adaptions (e.g., mobilization of energy and proteins from maternal body reserves) for the cow to support this high growth and milk production level. The transition period from gestation to lactation is linked with increased nutritional demands but at the same time with appetite depression, which puts the cow in a negative energy balance (NEB) and under metabolic stress (Butler and Smith, 1989; Bell, 1995). To support their postpartum physiological requirements, cows typically experience lipolysis and ketogenesis, making them more prone to other metabolic diseases (Ametaj, 2005; von Keyserlingk et al., 2009).

During the periparturient period, alterations in hormone levels can cause changes in energy balance metabolites, specifically with non-esterified fatty acids (NEFAs). Hormonal profiles related to parturition and lactation are also altered. For instance, estrogen begins to increase while progesterone declines just 1-2 days before calving. Furthermore, as lactation is initiated, serum levels of growth hormone (GH) and

glucocorticoids increase while at the same time, they inhibit insulin and IGF-1 production, triggering mobilization of NEFAs (Lucy et al., 2001). Overproduction of the NEFAs leads to incomplete oxidation and accumulation in the liver due to the lack of re-esterification of the NEFAs back to triacylglycerides (TAGs). This, predisposes cows to ketosis or fatty liver disease (Ingvartsen, 2006).

On the other hand, a high concentration of NEFAs initiates a pro-inflammatory response, which can benefit placental detachment during calving. This helps fight infections or it can lead to self-harming if inflammation persists for a long time. Negative energy balance has been widely accepted as a failure to supply the energy requirements and sufficient dry matter intake (DMI) (Ingvartsen, 2006). In contrast, a growing body of evidence conducted on dairy cows during the periparturient period affirms that the energy deficiency comes from the host's response to systemic inflammation. Defining the origin of this systemic inflammation during the transition period is a work in progress.

1.2.2 Susceptibility to intramammary infections during the dry period

Many factors participate in the etiology of mastitis. Disease severity and extent depend on the balance between the host, the farm, and the pathogens (De Vliegher et al., 2018). A high incidence of IMI is strongly correlated with the immunosuppression that cows go through around parturition (Sordillo, 2005). Pathogens then use this opportunity to attack and establish an infection (Contreras and Rodríguez, 2011). The origin of these pathogens can be from the environment, the udder or an existing infection (Breen et al., 2009). Cows having dirty udders and farms with improper hygiene are more likely to develop clinical mastitis (Hogan et al., 1989). Sand bedding is preferred over organic

material as sand doesn't predispose cattle to environmental mastitis (Hogan et al., 1999). Thompson-Crispi et al. (2013) estimated that the incidence of CM in conventional dairy farms to be 26.3 cases per 100 cows.

In contrast, organic dairy farms tend to have fewer cases of mastitis (Levison et al., 2016). It is interesting to note that different studies report different SCC values between these two systems, with some stating that organic farms have higher SCC values (Levison et al., 2016). This may be due to the fact that organic farms have different standards and feeding ratios than conventional farms (Ruegg, 2009). Also, SCC levels are affected by breed, stage of lactation, parity and season (Verbeke et al., 2014). Holstein cows are more likely to be culled when they present with high SCCs than Jersey cows (Bannerman et al., 2008). Also, pure Holstein cows are more predisposed to become infected and to have a shorter lifespan than crossbreeds (Dezetter et al., 2017). A dairy farmer's financial interest is to make a profit by selecting cows that produce considerable amounts of milk. For many years, the dairy industry has focused on selecting cows for their production traits, but unintentionally, this has led to a negative impact on cow health and welfare. In particular, this has increased the average somatic cell count in milk and the number of mastitis cases while generating less milk yield and increased culling rates (Heringstad et al., 2005; Negussie et al., 2008).

As far back as in the 1950s, it was first noticed that cows could be genetically selected for mastitis resistance and general health optimization (Lush, 1950; Shook, 1989). Several studies have shown that susceptibility to IMI can be inherited. Subclinical mastitis has a low heritability from 0.03 to 0.17 (De Haas et al., 2008; Urioste et al., 2012; Narayana et al., 2018), as does CM, with a heritability of 0.07 to 0.1 (Heringstad et al., 2005; Hinrichs

et al., 2011). On the other hand, Svendsen and Heringstad reported that SCM cows with an SCC threshold between 50,000 - 200,000 SCC/mL shared a high genetic correlation (range = 0.89 - 0.92) (Svendsen and Heringstad, 2006).

Cows are susceptible to mastitis throughout lactation. However, they are most at risk two weeks postpartum, and as they grow older (Barkema et al., 1998; Riekerink et al., 2008). There is supporting evidence that many metabolic and immune alterations occur long before presenting CM or SCM after parturition. Indeed, many researchers have reported that the infection is typically acquired during the dry-off period. It has been demonstrated that mastitis-positive cows identified within 30-100 days in lactation likely had the infection since the dry period (Green et al., 2002; Bradley et al., 2008). This was later endorsed by Dervishi et al. (2015), who found activation of innate immunity and other metabolic changes in cows that went on to develop SCM after calving. Furthermore, these cases increase IMI persistence and recurrence in the herd (Jamali et al., 2018).

1.3 Impact of mammary gland infections on dairy herds

1.3.1 Mastitis – a threat to the dairy industry profitability

Mastitis costs the dairy industry dearly. In 1972, Foley and colleagues reported that the average cost to treat a mastitic cow was $30-$50 per year (Foley et al., 1972). They estimated that the total cost to the United States economy was a loss of between $300 million and $600 million per year. Since then, inflation has added to the estimated cost and the economic impact of mastitis. Aghamohammadi et al. (2018) reported that a Canadian dairy farmer would spend CAD $662 per cow in a year to treat or prevent mastitis. These

costs included the losses due to reduced milk production from SCM, treatment of CM, and prevention programs.

It is well known that one of the first consequences of an IMI infection is milk reduction (Hertl et al., 2014). On the other hand, even subclinical mastitis cases are accompanied by yield losses. While not as high as CM, SCM is longer in duration (Halasa et al., 2009). The reduction in yield comes from mammary tissue destruction, so the MEC can no longer synthesize or secrete milk. Most SCM cases caused by *S. aureus* turn into a significant concern as the secretory tissue transforms into useless fibrotic tissue (Botaro et al., 2014). Knowing that almost 50% of a dairy herd can be affected by SCM and that 20-50% of the cows will experience more than one disorder around parturition, the scale of these losses is disturbing (Leblanc, 2010). Older cows produce less milk than those in the first lactation. At the limit of 200,000 SCC/mL, primiparous cows can lose 0.31 kg/d, while multiparous cows lose 0.58 kg/d (Halasa et al., 2009). Due to yield reductions, milk composition changes, especially the loss of nutrient value, the milk from SCM or CM cows become unconsumable, so it must be discarded. Another reason milk (quarter level or bulk tank) from SCM or CM-affected herds is often discarded is due to the presence of high levels of antibiotics, making it a concern for public safety (Ruegg, 2003). A better solution for farmers is to remove or cull the infected cows and replace them with new ones. This further adds to the farm's profitability (Hogeveen et al., 2011). Other costs due to CM or SCM are related to veterinary assistance, extra labour, and preventive measurements (Halasa et al., 2007; Aghamohammadi et al., 2018).

A growing concern for the dairy industry, veterinary medicine, and the public is the antibiotic presence, usage of antimicrobial growth promoters (AGP) and antibiotic

resistance in livestock (Oliver and Murinda, 2012; Brown et al., 2017). Antibiotic resistance and antibiotic presence are a burden not only for the farmer's pocket and for the cow's welfare but also for global health and economic issues. Most farmers use systemic or intramammary antibiotics after confirming the presence of elevated SCC. Non-specific and extensive antibiotic use has led to the global problem of antimicrobial resistance (AMR) (Aga et al., 2016). Treatment of mastitis accounts for almost 80% of antibiotics used in dairy cows (Pol and Ruegg, 2007). Antimicrobial resistance presents a significant concern because some bacterial populations develop resistance genes and can pass this resistance to other bacterial communities.

Furthermore, the presence of antibiotic residues in milk means that this milk cannot enter the human food chain or be fed to calves (Maynou et al., 2017). Resistant bacteria can pass onto humans. For example, through the consumption of unpasteurized milk, there have been a number of disease outbreaks (Piddock, 1996; Oliver et al., 2005). The concept of One Health is becoming a central part of the agenda of health and veterinary discussions in the US, the European Union, and Canada (Martins et al., 2019). For example, the establishment of Animal Antimicrobial Stewardship (AMS) Canada (Otto et al., 2018) and other national or international cohorts will help develop policies to prevent AMR while at the same time finding more effective treatments for farm animals and the environment.

1.3.2 Mastitis in relation to other diseases

Cows are at a higher risk of developing one or more periparturient diseases after calving. As discussed in section 1.2, the most susceptible time to develop these diseases is during the dry period. Many of these diseases are then presented or manifested after

calving. Eckel and Ametaj (2016) suggested that bacterial endotoxin (LPS or LTA) might be implicated in the periparturient disease pathogenesis, proposing that the endotoxin produced by bacteria can translocate from the rumen, uterus, or mammary gland. Indeed, several papers have shown a good correlation between increased SCC and other metabolic diseases diagnosed simultaneously (Zhang et al., 2015, 2016; Dervishi et al., 2016b; a). Transition diseases are interlinked between one another (Mulligan and Doherty, 2008). For example, hypocalcaemia makes a cow more predisposed to most periparturient diseases, while acidotic cows are more susceptible to mastitis, laminitis, milk fever, and left displaced abomasum (LDA). Retained placenta (RP) leads to metritis, milk fever, and LDA (Gröhn et al., 1989). Ketotic cows have an increased concentration of NEFA. Beta-hydroxy butyrate (BHBA), in addition to being a diagnostic tool for ketosis, helps identify uterine infections and LDA.

Elevated levels of NEFA before parturition depresses feed intake, which impacts immune function and can lead to subclinical ketosis and metritis development. Metabolic changes and adaptations occurring during the transition period can be metaphorically compared to the domino effect – one falls over the other. Thus, incomplete oxidation of NEFA results in fatty liver and ketosis, which in ketotic cows with a high body condition score (BCS) increases the incidence of displaced abomasum (DA) (Ingvartsen, 2006). The increased lipolysis and BHBA impair immune functions, consequently making cows prone to infectious diseases like mastitis and metritis. This immunosuppression, combined with hypocalcaemia, leads to retention of fetal membranes or retained placenta (Mulligan and Doherty, 2008).

Another initiator of this cascade can be the ruminal environment. As the diet changes, so do the bacterial population in the gut, favouring the release and translocation of the Gram-negative bacteria endotoxin, LPS, to enter the bloodstream, initiating an immune response (Emmanuel et al., 2007; Zebeli et al., 2011; Saleem et al., 2012). To combat this, the host requires more energy, leading to a postpartum NEB. Recent studies have established that a decrease in feed intake in SCM cows begins to appear at least four weeks before parturition and decreases after calving. This corresponds to a drop in milk production. These fluctuations are linked to the increased presence of TNF, which as a pro-inflammatory mediator, decreases appetite and inhibits prolactin, therefore, reducing milk yield (Dervishi et al., 2015). Ketosis and fatty liver disease are the two most frequent disorders found simultaneously in dairy cows (Gröhn et al., 1989).

Dervishi and colleagues (2015) reported that subclinical mastitis was preceded by the systemic presence of an inflammatory insult during the dry-off period, which may make cows more susceptible to other diseases. The origin of the inflammation may be attributed to the translocation of LPS or pro-inflammatory cytokines into the systemic circulation (Eckel and Ametaj, 2016; Zwierzchowski et al., 2020a). In general, any metabolic disease, once it has occurred, presents a higher likelihood to develop again (Roche, 2006). Moreover, cows that suffered once from CM are more predisposed to present it again throughout lactation (Lam et al., 1997; Zadoks et al., 2001). These health disorders, including mastitis, poor reproductive performance and metabolic perturbations, increase dairy farmers' culling decisions (De Vries, 2017).

Mastitis also impacts the reproductive health of dairy cows (Ahmadzadeh et al., 2009). No matter when it happens during lactation, mastitis lowers a cow’s future

reproductive performance and conception rates. In particular, cows experience more days open and more days to first service, more abortions, and higher culling rates (Barker et al., 1998; Schrick et al., 2001). Several meta-analyses have studied the relation between udder and uterus health. Fourichon et al. (2000) initially concluded that reproductive performance was not affected by IMI. However, the latest study, which used far more data, proved that mastitis incidence was related to the incidence of uterine tract disorders (Dolecheck et al., 2019).

Prepartum augmentation of NEFA increases the odds that a cow can develop mastitis, RP, ketosis, and DA after calving (Cameron et al., 1998). Clinically mastitic cows continue to have high levels of NEFA and BHBA and lowered glucose at the beginning of lactation (Moyes et al., 2009). These authors connected the susceptibility of mastitis to immune suppression through hyperketonemia on neutrophil recruitment (Zarrin et al., 2014). Administration of LPS to the udder or naturally-occurring IMI causes local and generalized immune alterations (Wellnitz and Bruckmaier, 2012). Most artificial-mastitis cases are induced at the beginning of lactation, while not many are assessed during the dry-off period. Zebeli et al. (2011) applied LPS intravenously several times during the transition period in cows. They noticed increased levels of β-hydroxybutyric acid almost two weeks before parturition. Later, the elevation of BHBA was found together with higher numbers of SCC at dry-off. This implies that endotoxin could initiate the elevation in BHBA (Zhang et al., 2016). After an LPS challenge post-calving, a few authors noted plasma changes characterized by increased insulin levels (Waldron et al., 2006; Vernay et al., 2012). Another consistent finding is elevated plasma cortisol. This impacts insulin resistance, induced by pro-inflammatory cytokines such as IL-1, IL-6, and TNF (Waldron

et al., 2003; Huszenicza et al., 2004). These immune mediators are released upon exposure of host cells (leukocytes; MEC) to the pathogen and alert peripheral WBCs and the liver to initiate an acute phase response (APR) (Ezzat Alnakip et al., 2014). The acute phase proteins (APP) released from the liver include SAA, Hp, CGRP, serum albumin and LBP. As reported by Dervishi et al. (2015), these proteins were found to be elevated in cows two months before being diagnosed as mastitis positive. High-density lipoprotein-SAA complexes make endotoxin neutralization possible and safe removal of endotoxin from the circulation. This rapid removal of endotoxin shifted out from the liver makes it possible for a higher-than-average amount of lipids to pass through the liver, contributing to the deposition of fat to the liver or fatty liver disease (Ametaj, 2005; Ametaj et al., 2010b; Eckel and Ametaj, 2016).

1.4 Current diagnostic approaches

The inflammatory response seen in a cow's udder can indicate the presence of mastitis, whereas the identification of the bacterial pathogen causing the disease confirms the intramammary infection (Adkins and Middleton, 2018). As described in section 1.1.4, CM can be diagnosed via an abnormal appearance of the udder. Redness, swelling, and warm when touched due to inflammation are examples of common symptoms. Also, the milk changes its appearance from white to yellow and its consistency.

Subclinical diseases present a significant problem for monitoring the health of dairy herds. SCM cases are quite common in many conventional dairy farms. Two of the most routinely used methods to identify SCM cows are the California Mastitis Test (CMT) and Somatic Cell Count (SCC) test (Viguier et al., 2009). Estimation of SCCs is a traditional

approach to diagnose mastitis. In addition to SCC measurements, several other methods are being used. The count of somatic cells or CM biomarkers, such as *N*-acetyl-beta-D glycosaminidase and lactate dehydrogenase enzymes have shown positive outcomes. But scientists are always working to find better, faster, and cheaper methods. Immunoassays, hand-held biosensors, nucleic acid tests, and enzymatic assays as well as advances in genomics, proteomics, and metabolomics sciences has made it easier to detect mastitis at a much earlier time (Viguier et al., 2009).

1.4.1 Laboratory techniques

Somatic cell count

The somatic cell count or SCC indicates an IMI and gives an overview of a cow's udder health (Dohoo and Leslie, 1991). Data suggests that clean udders or quarters have approximately 70,000 cells/mL, and SCC measurements equal to or greater than 200,000 cells/mL indicate an SCM cow (Koeck et al., 2012). According to the Dairy Farmers of Canada website, last updated August 2017, it is required that a sample of raw milk must contain less than 400,000 cells/mL. In the United States, as of August 2018, the allowed amount of SCC is 750,000 cells/mL. In Albania and the European Union, the limit is 400,000 cells/mL (Berry and Hillerton, 2002; Beli, 2016). Normally, milk shouldn't have more than 150,000 cells/mL, and if the SCC is greater than 200,000 cells/mL, it shows some level of abnormality and that the immune system is activated (Vissio et al., 2014). There is a chance that high SCCs sometimes can be caused by factors other than an infection. High levels of animal stress, for example, can produce high SCCs, thus giving false-positive results (Schukken et al., 2003; Ruegg and Pantoja, 2013).

Under normal health conditions, the somatic cells found in the mammary gland are the macrophages, which make up 66-68% of detected cells. Other somatic cells may include neutrophils, mononuclear, and epithelial cells. The local concentration of neutrophils increases as the IMI progresses. The only cells that demonstrate the presence of high SCCs are leukocytes, specifically high numbers of neutrophils, seen in almost 90% of SCM cases (Pilla et al., 2012).

A random sampling procedure is typically followed by farmers when collecting milk samples for laboratory SCC evaluation. Somatic cells in milk can be analyzed in several ways, but most labs use flow cytometry or combine flow cytometry and fluorescence (Gunasekera et al., 2003). In Alberta, milk samples are processed by CanWest DHI, which has a standardized protocol. Samples can be taken from herd average of individual cows (HSCC) or bulk tank (BTSCC) (Dufour et al., 2011). Analyzing bulk tank milk is a convenient and inexpensive method to control milk quality and test for pathogen presence (Bauman et al., 2018). Measurement of BTSCC can help the farm evaluate its management policies, but the most accurate SCM detection in a cow is through quarter samples (Hernández-Castellano et al., 2017).

Bacterial culturing

After confirmation of contamination, the causative agent should be identified for treatment purposes and good management practices (Hope, 2000). The National Mastitis Council (2017) has described various methods for working with bacterial cultures. The most common method is still the standard plate count (SPC) with a healthy target of less

than 5,000 colony-forming units (CFUs)/mL. If the number of CFUs is over 20,000 per mL, then financial fees are levied on the farms (Murphy et al., 2016).

Pathogen identification by plate culture focuses on discovering *Staphylococcus aureus, Streptococcus agalactia, Escherichia coli*, *Mycoplasma spp*., *Corynebacterium spp*. and NAS (Klaas and Zadoks, 2018). When taking milk samples for culture, attention must be paid to avoiding contamination. Factors such as a dirty stall or a contaminated environment, poor udder preparation, or incorrectly performing the procedure may lead to milk contamination and the presence of a remarkably high number of bacteria on the plate, leading to false-positive results (Constable et al., 2017; Ashraf and Imran, 2018).

PCR – based methods

Although culture plate identification of bacteria is considered the gold standard for CM and SCM, it is not necessarily the best method. Compared to the polymerase chain reaction (PCR), culture plate methods were able to identify mastitis pathogens on only 47% of the no-growth milk samples (Bexiga et al., 2011). Nucleic acid-based detection or PCR has facilitated the detection of those pathogens that cannot be identified using standard bacterial culture plates. The superior sensitivity of PCR enables better farm management, too (Lui et al., 2009).

Electrospray ionization mass spectrometry ESI-MS is another common diagnostic method that can detect other microorganisms such as parasites, yeasts, and viruses, but PCR is still the preferred sequencing technique for bacterial identification (Perreten et al., 2013; Lange et al., 2015). Polymerase chain reaction methods are more rapid, but several times more expensive than conventional bacterial plate culture methods. However, it

should be kept in mind that PCR methods can detect only the species included in the PCR kit (Cantekin et al., 2015).

Other molecular techniques include real-time quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), and next-generation sequencing (NGS) methods (Graber et al., 2007; Li et al., 2017; Anis et al., 2018). As a secondary confirmatory test, matrix-assisted laser desorption/ionization time-of-flight (MALDI – TOF) mass spectrometry can be applied as a diagnostic technique for bacterial species identification in mastitis studies (Gonçalves et al., 2014; Cameron et al., 2017). But the cost of analysis, the required sample pre-treatment and the frequency of false-negative results mean that this approach is not readily adaptable for commercial use (Barreiro et al., 2017).

1.4.2 Cow-side tests

California Mastitis Test

The California Mastitis Test (CMT) is one of the oldest and easiest tests to use as a cow-side test. It evaluates the milk's alkalinity using a detergent (alkyl aryl sulfonate) combined with a pH indicator, bromocresol purple (Schalm and Noorlander, 1957). If mastitis is present, this results in the formation of a purple viscous mass due to the nucleic acids and other constituents released from the lysis of somatic cells. The interpretation of the CMT might be subjective as it gives variable results to identify IMI. The CMT kit is composed of a plastic paddle and four cups to put the milk from each quarter of the udder. The reagent is added, then the milk in the cup is stirred until a mixture is formed. The test

is most useful when a very high number of somatic cell counts, with an average of 500,000 cells/mL (Sargeant et al., 2001). The result of the CMT is read as negative (N) or trace (T) and then depending on the viscosity of the gel, with a grade from 1 to 3 to indicate the number of somatic cells, with three being the highest (>5 million SCC/mL) (Gordon et al., 1980). The CMT cannot identify whether the infection is from major or minor pathogens (Viguier et al., 2009).

Electrical Conductivity test (EC)

During inflammation, concentrations of many ions change. Increases in the concentration of ions such as potassium, magnesium, sodium, and calcium can increase the electrical conductivity (EC) in milk (Viguier et al., 2009). A high EC is based on high sodium and chloride concentrations in the milk and a loss of lactose and potassium. But the concentration of ions in milk can be influenced by factors other than mastitis. Conductivity is also affected by the cow's age and lactation stage (Pyörälä, 2003).

Many dairy farmers use automatic milking systems (AMS), which helps them increase milk production and minimize labour costs (Hovinen and Pyörälä, 2011; John et al., 2017). Automatic milking systems are equipped with sensors that measure EC to detect mastitis (Khatun et al., 2017). One drawback of this process is that just a part of the milk is measured. Sensors that identify mastitis in AMS farms do not consider the measurement of foremilk (Bruckmaier and Blum, 1998; Lehmann et al., 2015). Foremilk is the milk obtained in the first part of milking. This initial milk is discarded, whereas alveolar ejection is preceded by teat cleaning and stimulation. The time difference between cisternal and alveolar milk is between 50-100 seconds, and this can correlate negatively with EC and

SCC (Bruckmaier et al., 2004; Bansal et al., 2005; Lehmann et al., 2015). It has been shown that milk sampled before ejection improves mastitis detection (Khatun et al., 2019).

On-farm culture

Biplates and triplates are the main cow-side testing methods to confirm the presence of bacteria. The biplate contains two types of agars, one for Gram-negative and another one for Gram-positive bacteria. In contrast, the triplate type comprises three types of agars, which can differentiate Gram-negatives from Gram-positives and Gram-positive staphylococci from Gram-positive streptococci. The results of biplate or triplate tests are not as promising as one would hope. However, they can be used to discover whether the pathogen can grow in culture (Royster et al., 2014).

A milk sample is considered contaminated when three or more colonies are present. One quarter may be regarded as cured when the bacteria found present at the beginning of the plate test are no longer isolated from the milk sample. On the other hand, there are cases where cows with clinical mastitis have a bacteriologically negative sample. These results are present even when all the necessary protocols for collecting and performing the sample analysis were followed. The reasons might be due to a low concentration of the pathogen in the milk, presence of intracellular bacteria, or of growth-inhibitory substances in the milk. In cases where no pathogen can be detected, enzyme-linked immunosorbent assays (ELISA) methods can help identify *S. aureus, E. coli, S. dysgalactiae,* and *S. agalactiae* (Royster et al., 2014; Constable et al., 2017). However, it is essential to realize that in some cases, microorganisms in quarters of milk are due to microbiological

contamination during sampling rather than IMI, particularly in milk samples with low colony counts, <100 CFU/mL (Dohoo et al., 2011).

Different farms apply different on-farm techniques to monitor udder health. This includes measuring enzymes (N-acetyl-D glucosaminidase; lactate dehydrogenase), pH indicators, strip plates or portable SCC measurements (PortaCheck; BacSomatic; DeLaval Cell Counter) (Viguier et al., 2009; Moroni et al., 2018; Kandeel et al., 2019). These conventional methods are focused on discovering mastitis at the time of occurrence.

The need to detect and prevent mastitis or IMI as soon as possible is important for disease mitigation and spread control. Emerging innovations using a combination of biotechnology and nanotechnology are making this possible. Thanks to the invention of nanotechnology-based biosensors and lab-on-a-chip technologies, high-throughput analysis using proteomics and metabolomics is now possible (Boyd-Moss et al., 2016). These systems will offer farmers an all-in-one method – from processing the sample to analyzing the sample and giving an accurate result right on the farm (Sang et al., 2013). Most of the studies done with biosensor and biomarkers in the field of mastitis have been performed in milk. Martins et al. (2019) noted that milk samples negatively influenced biosensor detection performance and suggested that other biological fluids should be used. My focus in this thesis is on identifying and trying to validate new kinds of mastitis biomarkers using more user-friendly sample types such as blood and urine. This opens the door to detecting mastitis at its earliest stages, before milking even begins.

1.5 Prevention of mastitis

Eradicating mastitis infections is almost impossible. Much effort is still focused on eliminating existing infections and preventing new ones. The National Institute for Research in Dairying in 1970 created a 10-point control program (Constable et al., 2017). According to this program, to have profitable udders, better dairy cow health and welfare and positive treatment outcomes, the detection of udder infection should be done as early as possible (Halasa et al., 2007; Ruegg, 2017). Monitoring and preventive measurements require reliable and affordable prognostic and diagnostic methods. As discussed above, most of the diagnostic techniques are widely used, but many lack the necessary accuracy. Some are prohibitive in time and cost, while others are limited to detecting mastitis only when the cow is already severely infected.

For example, CMT has been used for a very long time as a cow side test (Luedecke et al., 1967). Still, its performance is questionable due to the variability in the execution of the test and the user's ability to read it correctly (Lam et al., 2009). Generally, CMT detects the presence of IMI 4 days after calving and isn't able to work for subclinical mastitis detection (Sargeant et al., 2001; Dingwell et al., 2003b). Compared to other diagnostic tests, the CMT has been proven less accurate (87.4-90 .8%) and more time-consuming for a large herd (Rossi et al., 2018). On the other hand, with better sensitivity and specificity, counting somatic cells in milk can be more successful. The drawback to this approach is that it requires laboratory analysis, which limits its use as a real-time detection method and is associated with higher costs per test (Labohm et al., 1998; Hillerton, 2000). Portable devices, such as the DeLaval cell counter, Porta SCC and Fossomatic are useful for on-farm SCM evaluation (Leslie et al., 2011; Ferronatto et al., 2018). Even though these

portable systems are easy to use and fast, they lack sensitivity at low SCC (Viguier et al., 2009).

Errors can occur while interpreting SCC data since this is influenced by several factors such as the presence of bacteria, diurnal variation, age, stage of lactation, and milk sample (Olde Riekerink et al., 2007). Petzer et al. (2017) determined that the accuracy of SCC should be higher than 85% to identify the bacterial species associated with IMI. They also showed how SCC levels fluctuated between various mastitis microorganisms. Out of all quarters or composite milk samples that exceeded 200,000 SCC/mL, over one third were culture negative (Petzer et al., 2017). Furthermore, they found out that if the threshold to detect *S. aureus* is 150,000-200,000 cells/mL, 30.8% of all cows will remain undetected as they had lower SCC but were bacteriologically positive. By not identifying truly-infected samples, SCC methods unintentionally create adverse outcomes for both the cow and the farm. The two most frequently used diagnostic methods, CMT & SCC, can detect abnormalities in the udder but cannot specify the causative agent. The inability to identify the pathogens leads to inappropriate treatments that increase antibiotic resistance leading to the spread of antibiotic-resistant strains (Martins et al., 2019). Other methods to fight bacterial resistance to antibiotics or toxic compounds (RATC) and inflammation can be considered, such as probiotics, prebiotics and proteobiotics (Deng et al., 2016; Jiminez et al., 2017; Tarsillo and Priefer, 2020).

There are many diagnostic approaches for mastitis detection, including SCC, CMT and EC. Other techniques such as sensor-based systems, immunoassays, and specific biomarkers from PCR, nucleotide sequencing, proteomics or metabolomics are just being introduced (Adkins and Middleton, 2018). Higher diagnostic accuracy can often be

achieved if we combine multiple methods (Chakraborty et al., 2019). A comparison between two of the most common used tests is summarized in Figure 1.3. Future pen-side mastitis tests should take into consideration the need to detect cows susceptible for new or existing IMIs before calving, while at the same time being economical and user friendly. The development of better, faster, cheaper and more convenient tests will encourage appropriate interventions to prevent transmission, reduce antimicrobial resistance and minimize financial losses.

1.6 Omics investigations of mastitis

1.6.1 Application of metabolomics in periparturient diseases

The dairy farmers and milk industry's primary goal is to breed cows for high milk production and quality. However, this goal is associated with a drawback – increased incidence of periparturient diseases (Dobson et al., 2007; Sundrum, 2015). Holstein cows, which make up 93% of Canadian herds produce about 10,753 L/milk for 305 days in milk (DIM) (CDIC, 2020). During the dry period and early lactation period, cows go through physiological, immunological, metabolic, nutritional changes and adaptions that prepare them for calving and the next lactation (Drackley, 1999; Sordillo and Raphael, 2013). As mentioned, our lab has observed such changes up to 8 weeks before calving and these continue until 8 weeks postpartum. Cows that do not adapt to these changes likely present either clinical or subclinical forms of diseases (ketosis, milk fever, retained placenta, displaced abomasum, fatty liver, metritis, mastitis, or laminitis). On average, almost 50% of the dairy cows in a Canadian dairy herd present with more than one disease during this transition period (Ametaj et al., 2010; Leblanc, 2010; Eckel and Ametaj, 2016).

Many diseases in humans and cattle can be detected or diagnosed by the perturbation of just one metabolite. For example, subclinical ketosis is still defined as an increased level of ketone bodies in the blood, especially BHBA. Ketosis occurs due to deficiencies in energy intake, and it is not detectable via visible physiological changes (David Baird, 1982; Brunner et al., 2019). With the advancements in omics technologies, other kinds of chemical or protein biomarkers are being discovered that potentially offer greater sensitivity and specificity than traditional cell-based or gross property measurements (Serkova and Niemann, 2006). For example, studies from our lab have identified that inflammatory mediators such as IL-1, IL-6, IL-8, SAA & Hp are elevated at the beginning of the dry period, up to 8 weeks before calving in several periparturient diseases (Dervishi et al., 2015, 2016b; Zhang et al., 2015, 2016, 2018). These systemic findings of inflammation present another point of view regarding pathomechanisms of such diseases. In particular, these results show that post-calving disease is preceded by a systemic inflammatory insult weeks before presenting any symptoms or physiological changes. There is mounting evidence that can attribute the origin of this insult to the presence of endotoxin in the circulation that can be translocated from the rumen, reproductive tract or mammary gland, thereby initiating an immune response (Ametaj et al., 2010; Eckel and Ametaj, 2016). Furthermore, immunosuppression during the transition period can be caused by these pathogenic bacteria (i.e., *Staphylococcus aureus; Escherichia coli*). As proven on humans, this kind of immunosuppression can slow or even prevent an immune response (Thammavongsa et al., 2015; do Vale et al., 2016). One of those mechanisms causing immunosuppression is the impairment of neutrophil functions and extravasation (Loughman and Hunstad, 2011; Lau et al., 2012).

Metabolomics, which offers a route to measure an animal's chemical phenotype, is being used to understand the underlying metabolic changes associated with the transition period in dairy cattle and how it relates to disease manifestation. Metabolomics can identify potential metabolite biomarkers to find animals susceptible to several periparturient diseases. In blood, three metabolites carnitine (C0), propionyl carnitine (C3), and lysophosphatidylcholine acyl C14:0 (LPC a C14:0) were found to be able to predict which cows would be susceptible to develop one or more diseases (retained placenta, mastitis, metritis, or laminitis) up to 4 weeks before calving (Hailemariam et al., 2014). Another study showed that up to 67 metabolites were expressed differently 21 days prepartum than the day of calving (Luo et al., 2019). Many other metabolites (amino acids (AA), acylcarnitine's (AC), phosphatidylcholines (PC), LPC, and metal ions) each specific to a given disease have been identified and measured with high predictive accuracy for a number of conditions using blood (Li et al., 2014; Sun et al., 2014; Xu et al., 2015; Zhang et al., 2017b), milk (Klein et al., 2010, 2012) or urine (Zhang et al., 2017a). Biomarkers for animals at risk for developing ketosis were identified in urine (Dervishi et al., 2018a) and blood (Zhang et al., 2017a; (Hailemariam et al., 2018)). Likewise, biomarkers for those at risk to develop metritis, retained placenta or lameness were found in serum (Zheng et al., 2016; Dervishi et al., 2020), urine (Zhang et al., 2020) or milk (Zwierzchowski et al., 2020b).

1.6.2 Application of metabolomics for mastitis biomarker discovery

One of the first metabolomic studies to look at mastitis was conducted in 2005 (Eriksson et al., 2005). Using GC-MS, Eriksson et al. (2005) demonstrated that it was

possible to differentiate milk from healthy or mastitic samples chemically with what is now called an electronic nose. Other studies led by Hettinga et al. (2008, 2009) concluded that if the concentrations of specific volatile metabolites were high in milk, it meant that they were infected, and the volatiles corresponded to metabolic by-products from bacterial pathogens. NMR studies conducted by Sundekilde et al. (2013) in milk found that lactate, acetate, BHBA, butyrate, and isoleucine were in a greater concentration in high SCC samples. On the other hand, for the same samples, lactose, hippurate, and fumarate were at lower levels than in milk with low SCC levels (Sundekilde et al., 2013). A mastitis-induced experiment studied how oxylipin profiles in milk and mammary tissues changed and influenced the disease (Ryman et al., 2015). Hydroxy octadecadienoic acid (HODE) and oxo octadecadienoic acid derived from arachidonic acid and linoleic acid were higher in *S. uberis* mastitis. These results show that oxylipids are implicated in the inflammatory state within the mammary gland. Several authors have noted the increased milk concentrations of prostaglandins and thromboxane in mastitis samples (Giri et al., 1984; Atroshi et al., 1987). These pro- and anti-inflammatory oxylipids may affect the host's ability to eliminate the pathogen (Aitken et al., 2011). In another study with skimmed milk, using untargeted LC-MS, Thomas et al. (2016) identified 690 metabolites. They challenged the cows with an *S. uberis* strain and collected milk samples 0, 36, 42, 57, 81, and 312 hours after infusion. The bacterial load peak was noted at 36h, whereas most of the metabolite changes in milk occurred after 81h. They noticed increased levels of bile acids (taurochenodeoxycholic acid (C26H45NO6S), taurocholic acid (C26H45NO7S), glycocholate (C26H43NO6), glycodeoxycholate (C26H43NO5), and cholate (C24H40O5). These bile acids support antimicrobial (Hofmann and Eckmann, 2006) and anti-inflammatory activities, facilitated

through the farnesoid X receptor pathway (Calmus and Poupon, 2014), which inhibits the activation of the NF-kB signalling pathway (Zhang et al., 2008; Sipka and Bruckner, 2014). Thomas and colleagues' results revealed that high levels of bile acids in milk decreased the levels of pro-inflammatory cytokines (Thomas et al., 2016). Other authors have noticed alterations in metabolic pathways pre-and/or postpartum in SCM and CM cows compared to healthy cows. These results show extensive evidence of bacterial activities.

Several other studies have demonstrated that mastitis is preceded by alterations of metabolic pathways in the blood corresponding to inflammatory insults in the prenatal period (Dervishi et al., 2015, 2017; Zandkarimi et al., 2018). Several serum metabolites were used to distinguish SCM cows from healthy cows up to 8 weeks before their due date using targeted GC-MS. Alterations in amino acid metabolism continued up to 8 weeks postpartum. The best indicators between the two groups were valine, serine, tyrosine, and phenylalanine (Dervishi et al., 2017). Besides, distinguishing between CM cows and healthy cows could be achieved by quantifying about a half dozen metabolites, including N-methyl ethanolamine phosphate, choline, phosphorylcholine, free carnitine, trimethyl lysine, tyrosine, and proline. The most significant discriminator was 3′-sialyl lactose in serum (Zandkarimi et al., 2018). This particular saccharide was more elevated than the control group at -21 days, probably to boost innate immunity (Ten Bruggencate et al., 2014). This compound is known to protect calves against infections (Nakamura et al., 2003). Lactate was also increased in this study, which agrees with what was found by other authors (Hamann and Krömker, 1997; Davis et al., 2004; Dervishi et al., 2015). The shift in metabolite levels can be due to acute inflammation, as shown by the increased APPs (Hailemariam et al., 2014; Dervishi et al., 2015, 2017; Zandkarimi et al., 2018). Detecting

urine changes confirmed the hypothesis that SCM cows were preceded, associated and followed by alterations of urinary metabolites (Zwierzchowski et al., 2020b).

Further validation of these findings over a larger number of cows and more diverse farm management settings can help us develop a better view of the pathology of mastitis. It might also help develop more robust pen-side tests to facilitate the identification and treatment of susceptible cows to improve overall dairy herd health.

1.6.3 Analytical techniques in metabolomics

The metabolome is the set of small-molecule metabolites equal to or less than 1500 Da found within a cell or body fluid (Oliver et al., 1998). Metabolites can be either endogenous or exogenous and correspond to any "chemical that a plant or anima1 can produce, synthesize, ingest, absorb or to which it can be exposed" (Wishart et al., 2007, 2020). While metabolites are well known for their roles as the building materials of cells, metabolites have also been described as the "canaries" of the genome (Pearson, 2007). They can alert cells about changes in the actual genome, but at the same time, they are sensitive to the environment, too. As an omics technique, metabolomics is often regarded as "the lesser-known cousin of genomics and proteomics." Metabolomics uses analytical chemistry instruments such as MS spectrometers or NMR spectrometers to separate, identify and quantify hundreds of these small molecules at a time. Metabolomics often must be combined with advanced software to read, interpret, annotate and integrate the results with various online databases (Wishart et al., 2018). The communication between genes and the environment means that metabolomics is ideal for measuring the phenotype (Fiehn, 2002). The metabolic phenotype is often called the metabotype. Metabotyping or

phenotyping via metabolomics has many advantages over phenotyping with other omics methods. Rather than telling us what might happen in a cell or tissue (as genomics does), metabolomics tells us what is happening inside the cell or tissue (Powers and Riekeberg, 2017). This has allowed metabolomics to become an ideal platform in biomedical studies for biomarker discovery.

Metabolomics is a relatively young science. The term metabolome was used for the first time by Oliver et al. (1998), while the first metabolomics study on cattle was conducted in 2005. This was a study, which analyzed urine to monitor the use of steroids and improve control strategies (Dumas et al., 2005). To conduct a proper metabolomics study, both a suitable biological sample and a suitable analytical platform are needed. Most commonly used biological samples are biofluids such as serum, plasma, urine or milk. This is because analytical chemistry instruments handle fluids more easily than solids. The platforms used in metabolomics include a wide range of analytical instruments, including nuclear magnetic resonance (NMR) spectrometers and mass spectrometers (MS) coupled with liquid (LC), gas chromatography (GC) or capillary electrophoresis (CE). These are used to separate, identify and quantify the metabolites. The results from these chemical analyses must be combined with software programs and electronic databases to visualize, analyze and interpret the generated information (Dunn et al., 2005; Wishart et al., 2007, 2020). Metabolite analysis can be run using either targeted (known metabolites) or untargeted (neither known nor identified metabolites) approaches (Dunn et al., 2011). As reviewed by Goldansaz et al. (2017), Sun et al. (2019) and Singh et al. (2019), there are many studies conducted in the field of animal science using metabolomics. The collaboration between Drs. Ametaj and Wishart has contributed significantly to bovine

metabolomics and has revealed a great deal about metabolic changes to cattle, especially for periparturient diseases (Dervishi et al., 2017, 2020; Zhang et al., 2017b, a; c; Hailemariam et al., 2018; Zwierzchowski et al., 2020c). This has led to the implementation of their findings into freely accessible metabolome databases such as the bovine metabolome database (BMDB) (Foroutan et al., 2020) and the milk composition database (MCDB) (Foroutan et al., 2019).

Liquid chromatography-mass spectrometry (LC-MS) is an integrated analytical technique that combines compound separation via LC with the sensitive detection of compounds by MS. MS measurements allow one to measure the mass-to-charge ratio (m/z) of ions. LC-MS is considered the most powerful among current metabolomics techniques as it is able to detect a larger portion of metabolome in a shorter period of time (Kennedy et al., 2018). LC-MS instruments have shown superior sensitivity and higher throughput than NMR or GC-MS, making LC-MS the most frequently used platform for metabolomics (Kennedy et al., 2018; Wishart et al., 2020). In particular, LC-MS, compared to NMR spectroscopy or even GC-MS, is 10-100 times more sensitive with detection limits down to the low nanomolar level (nM) (Emwas et al., 2019). Liquid chromatography can be used with single or tandem MS instruments (Emwas et al., 2019). The separation of analytes is mediated by a stationary phase (a chromatography column, usually made up of specially modified small-diameter silica materials to allow separation based on hydrophobicity) and mobile phase solvents. Modern LC uses high pressure/high performance (HPLC) or ultra-high pressure/performance (UPLC) for better separation and reproducibility. Different types of metabolites are best separated using distinct types of columns or stationary phases. Polar compounds such as organic acids or amino acids are best separated using a

hydrophilic interaction liquid type of chromatography (HILIC), whereas non-polar molecules such as lipids, fatty acids, and sterols are best separated with reversed-phase liquid chromatography (RPLC) (Masson et al., 2010; Wishart et al., 2020). The most common separation technique for LC-MS is RPLC (Theodoridis et al., 2012). RPLC is run using a non-polar stationary phase and aqueous, semi-polar mobile phase, a mixture of water with acetonitrile (ACN) or methanol (MeOH) and formic acid or ammonium acetate (Wishart et al., 2020). For maximal metabolite coverage, a combination of RP with HILIC is preferred (Spagou et al., 2011), but even with this combination, it is possible that some metabolites (in urine, for example) cannot be detected (Theodoridis et al., 2008). LC-MS often combined with other ionization techniques such as positive and negative ion electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI) provides a more comprehensive view of the compounds in various biological samples (Sana et al., 2008). In Figure 1.4 is demonstrated a combination of LC-MS instruments at The Metabolomics Innovation Centre (Edmonton, AB) that were used in this experiment.

In addition to varying the type of ionization method for LC-MS, it is also possible to vary the type of a mass analyzer. Several types of mass analyzers are used in modern mass spectrometers (Dunn et al., 2005). These include low-resolution mass analyzers such as triple quadrupole (QqQ) or quadrupole-iron trap (QIT) systems as well as high-resolution mass analyzers like quadrupole-time of flight (Q-TOF), Orbitrap (Orbitrap) and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers (Zhou and Yin, 2016). TOF mass analyzers determine an ion's mass by measuring how long it takes ions to pass a defined distance. The mass-to-charge ratio of ions produced via Orbitrap is calculated by trapping the ions in a spindle-like electrode, creating an electrostatic field that orbits ions

axially and radially along the central electrode (Zubarev and Makarov, 2013). Then, the m/z ratio of ions is caused by the frequency of these oscillations. This is somewhat similar to how the FT-ICR instrument determines an ion's mass and explains why both OrbiTrap and FT-ICR instruments provide the highest mass resolution. In Orbitraps, the image current of axial motion helps in ion detection, which improves the sensitivity and cycle time of Orbitraps over FT-ICR instruments (Wood, 2019).

Triple quadrupole (QqQ) mass analyzers are essentially three single quadrupole mass spectrometers connected together. QqQ MS instruments are also called tandem mass spectrometers (MS/MS). Combining two or more mass analyzers allows one to obtain more information about a given metabolite's structure (Wishart et al., 2020). QqQ instruments are not high-resolution instruments, but they are more sensitive, more robust, and they allow one to quantify metabolites more accurately than Q-TOF or Orbitrap MS instruments. This means better specificity and a more remarkable ability to conduct targeted metabolomics. The Q1 quadrupole in a QqQ mass analyzer is used to select the parent ion since it represents the original mass-to-charge (m/z) ratio of the compound. The central, Q2, is for colliding ions with a noble gas (nitrogen, helium, or argon) in the presence of radiofrequency voltage to fragment the parent ions into low molecular weight fragment ions or product ions. In Q3, these product ions are selected based on their m/z ratio (Saitman, 2019). If a chosen ion comes from Q1, Q2 or Q3, it is called select ion monitoring (SIM). But it is possible that, at the same time, many product ions can be filtered from a precursor ion (parent ion), known as multiple reaction monitoring (MRM) operation mode. The experimental approach I chose for doing metabolomics uses LC-MS/MS (tandem mass spectrometry via a QqQ) for targeted quantitative metabolomics.

Gas chromatography-mass spectrometry (GC-MS) is another analytical technique widely used in metabolomics research (Emwas et al., 2019). As the name implies, compounds are first separated by gas chromatography and then ionized, fragmented, and identified using a single quadrupole MS analyzer according to their m/z ratio (Allen et al., 2016). GC-MS offers reliable performance focusing on separation, sensitivity, and reproducibility for volatile and thermal-stable compounds. Ketones, aldehydes, and alcohols are examples of natural volatile compounds that can be easily separated and detected by GC-MS. On the other hand, sugars, amino acids, lipids, and organic acids must be derivatized with TMS (trimethylsilane) and converted into volatile derivatives before they can be separated and detected by GC-MS (Trivedi and Iles, 2014).

One of the drawbacks of GC-MS is that the compounds to be analyzed need to be of exceptionally low molecular weight (~800 Da). Another disadvantage is that many compounds of interest in metabolomics (such as amino acids, fatty acids, organic, acids and sugars) need to be specially prepared and derivatized before running them through the GC-MS. This takes time and effort, limiting the ability to perform compound identification and quantification on low volume samples or achieve high throughput. Derivatization with an oximation reagent and trimethylsilylation (TMS), or just silylation, is done to lower the hydrogen bond formation between molecules and to volatilize compounds so that they can be converted into gas phase metabolites. This step may be disadvantageous most of the time due to the extra preparation time and the possibility of creating artifacts and errors (Little, 1999). Analytes have to be vaporized and endure column temperature from as low as 2°C to as high as 400 °C (Dunn et al., 2011). Not all compounds are stable to these temperatures. Most GC-MS studies are conducted with electron ionization (EI), but some

also use chemical ionization (CI) if the compounds are particularly delicate (Beale et al., 2018). A single quadrupole or one combined with the time of flight (QTOF) mass analyzer is usually used for GC-MS (Dunn et al., 2011).

Unlike LC, which uses a liquid as a mobile phase, GC uses a gas as the mobile phase and a solid, stationary phase that lines the capillary GC column. Depending on the interaction between compounds and the column surface as well as the column temperature, different molecules will be slowed down differently as they move through the column to the mass analyzer. This leads to a retention time or elution time that is often unique for each compound (Saitman, 2019). In most GC-MS analyses, helium is used as the mobile phase to separate the compounds in the column. Once the compounds are eluted from the column, they pass through the MS, where they are fragmented into ions using an electron beam with standard energy of 70 electron volts (Emwas, 2015; Wishart et al., 2020). The sensitivity and comprehensiveness of GC-MS can be increased if a second GC separation is applied. This is called GC x GC-MS, and it can be used to detect those metabolites that a normal GC instrument cannot (Pierce et al., 2008).

Another MS instrument used in metabolomics and proteomics is capillary electrophoresis MS (CE-MS). It is not as popular as LC-MS or GC-MS (Dunn et al., 2011). In CE-MS, an electrophoretic buffer in the liquid phase is used to separate via an electro-osmotic flow (Wishart et al., 2020). Unlike LC and GC, CE separates electrically charged/polar compounds like nucleic acids, amino acids, carboxylic acids, or peptides (Ramautar et al., 2012; Hirayama et al., 2014). CE-MS functions on a similar basis as LC-MS. Both techniques can use the MRM operating mode, both can use a combination of single quadrupole, QqQ, QTOF mass analyzers, and both can use electrospray ionization

(ESI). Unlike LC or GC, electrophoresis can separate and detect metal ions. It also requires a much smaller sample size and can measure positively charged molecules that LC or GC cannot separate. Despite these benefits, CE-MS is not as reliable as LC-MS or GC-MS and so it is not as widely used for large-scale metabolomic studies. There are currently very few CE-MS instrument providers due to their lack of sensitivity and reproducibility (Wishart et al., 2020).

Nuclear magnetic resonance (NMR) spectroscopy is another analytical platform used in metabolomics. NMR was the first tool used in metabolomics and is considered as the pioneering technology for characterizing metabolic profiles (Wishart, 2019). The first NMR metabolomics study dates back to the 1980s (Bock, 1982; Bales et al., 1984). In NMR, chemical information is obtained by putting molecules under a strong magnetic field and measuring how the spinning nuclei change while exposed to this static magnetic field and to a time varying magnetic field (a radio-frequency (RF) pulse). The RF pulse is tuned and matched to one of the NMR probes coils. Based on sample properties this tune changes from sample to sample (Bainbridge and Lindon, 2019). NMR spectra provide information on chemical shifts and coupling constants, not mass or charge (which MS instruments do). A significant advantage of NMR over MS is that it is non-destructive. The same sample can be used over and over. Furthermore, NMR does not require LC, GC or CE separation or derivatization, so sample preparation is simpler and faster (Takis et al., 2019). Furthermore, NMR can analyze a much more comprehensive selection of samples including liquids and solids through solid-state NMR (ssNMR), magic-angle sample spinning (MAS-NMR) or living samples with magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) techniques (Blondel et al., 2016; Yoon et al.,

2016). Compared to LC-MS and GC-MS, NMR can also identify inorganic metabolites and lipoprotein fragments. However, as mentioned above, the "Achilles' heel" to NMR is its lack of sensitivity. In addition, the high instrument purchase and high maintenance costs make NMR spectroscopy less appealing to many than mass spectrometers (Emwas et al., 2019). The advantages and disadvantages of MS and NMR are also summarized in Table 1.2.

The NMR phenomenon is quite complicated. It is known that odd number atomic nuclei (^{1}H, ^{13}C, ^{15}N, ^{31}P) while spinning create a local nuclear magnetic field, which under radiofrequency electromagnetic radiation will cause those spinning nuclei to line up with or opposed to the external magnetic field (Wishart, 2013). Radiofrequency (RF) waves are used to "probe" what the nuclei are doing. In particular, different nuclei will absorb those radio frequency pulses and re-emit them at different frequencies depending on their chemical environment. The emitted radio frequency signals follow well-understood rules that allow NMR spectroscopists to figure out the structure and orientation of atoms within molecules. Most NMR-based metabolomic studies are performed using one-dimensional (1D) proton (^{1}H) NMR, called 1D ^{1}H NMR, because almost every organic molecule consists of one more 1H atoms. 1D ^{1}H NMR is beneficial in metabolomics as it takes only a few minutes to collect a 1D NMR spectrum. This is made easier with ready access to public NMR databases and free or inexpensive software tools. 1D NMR is limiting if the sample is complex and has many overlapping peaks, leading to misidentification and incorrect quantification (Emwas et al., 2019). This overlap problem can be solved with multidimensional NMR experiments, such as two-dimensional (2D NMR). 2D NMR can improve spectral resolution and allow one to identify more metabolites than 1D NMR

(Emwas et al., 2013). Unfortunately, 2D NMR is slow, and it often takes hours to collect a single spectrum. As a result, 2D NMR does not permit high-throughput work and does not offer the time and cost-efficiency of 1D NMR (Emwas et al., 2019).

1.6.4 Use of statistical analysis protocols in metabolomics datasets

Metabolomics generates a lot of data. Typically, dozens of samples with hundreds to thousands of metabolite concentrations are generated in a standard metabolomics experiment. The resulting data tables are large and complex. These complex data sets can be interpreted more easily using multivariate statistical techniques such as dimensional reduction. That allows one to identify significant changes between groups and maximize the extracted information from sample analysis (Trygg et al., 2007; Wishart, 2010). These statistical techniques include 1) exploratory analysis, 2) classification and discrimination techniques, 3) regression analysis, and 4) prediction. These approaches often require a combination of computing, statistical, and machine learning methods (Yi et al., 2016).

For most kinds of exploratory analysis, multivariate statistical techniques are generally used, such as unsupervised clustering. One method in particular - principal component analysis (PCA), is considered the *"workhorse in metabolomics"* (Trygg et al., 2007). PCA extracts from a metabolomics data set the most important variables and simplifies them into groups or components. In short, the essence of the original data is clustered into groups along particular axes of a graph, called principal components. The first principal component (PC1) contains the most significant variables, the second most significant variables are arranged in the second principal component (PC2) and so on for each subsequent orthogonal component. This graphical arrangement helps researchers gain

a more detailed view of the variations between groups (Issaq et al., 2009). PCA also identifies which variables are contributing most strongly to distinguish between samples.

In most cases, just two principal components, PC1 and PC2, embody almost all of the study's variation. However, when PCA cannot identify significant clusters, other clustering or classification techniques need to be considered (Wishart, 2010). Visually PCA data can be presented as score plots or loading plots (Wu and Wang, 2015). Sample patterns that show the maximal variance or covariance are summarized into 2D or 3D scores plots, whereas the loading plot demonstrates which variables are most responsible for the separations (Xia et al., 2015). One disadvantage of PCA is its orthogonality and the fact that does not generate a very clear separation of natural phenomena (Liland, 2011).

PCA is often performed before using other techniques such as hierarchical clustering or discriminant analysis (Jolliffe, 2002). If PCA cannot distinguish between samples, another multivariate but supervised classification approach called partial least square – discriminant analysis (PLS-DA) is used (Trygg et al., 2007). Unlike unsupervised clustering techniques that try to find and correlate underlying changes and components without having a measured outcome, supervised classification techniques like PLS-DA predict an outcome based on labels (Jiang et al., 2020). In short, the result of PLS-DA is based on a predefined model (Bartel et al., 2013). PLS-DA is an extension of PLS regression that links the predictors (X) with a response (Y) (Barker and Rayens, 2003). User-assigned information or class labels gives PLS-DA the ability to understand which variables should be selected to distinguish the most between groups. In other words, PLS-DA maximizes the linear covariance between independent (X) and dependent variables (Y) (Barker and Rayens, 2003). PLS-DA is widely used in metabolomics studies as it can deal

with large variables and noisy data. PLS-DA often allows statistical discrimination between two or more classes (controls and cases) and presents it in a clear, low dimensional, and quickly interpretable scores plot (Szymańska et al., 2012; Worley et al., 2013). PLS-DA takes advantage of the fact that separation is better when classes are known.

When conducting a PLS-DA, two factors should be kept in mind: model optimization and model quality assessment should be carried out in a double cross-validation and unbiased manner (Smit et al., 2007; Westerhuis et al., 2008). Cross-validation helps researchers to eliminate overfitted data and overly optimistic results (Xi et al., 2014). Several parameters can be used to evaluate PLS-DA performance. In particular, R2, the correlation index and Q2, the quality of prediction, are quite useful. Thus, the closer R2 and Q2 are to 1, the more likely the PLS-DA classification is not over-fit or overly biased. In contrast to R2, Q2 evaluates the predictive ability of the model and looks for non-real clusters. Wishart (2010) suggests that a good fit PLS-DA model should not differ between Q2 and R2 more than 0.2-0.3.

PLS-DA is not perfect and has shown some limitations, as reviewed by Gromski et al. (2015). PLS-DA may include variables that are not of interest to the study of other factors that influence the chemical and thermal noise of the metabolic fingerprint. As a result, orthogonal signal correction (OSC) may be used as a filtering technique. OSC is a modification of PLS and has been named orthogonal-PLS or OPLS-DA (Trygg and Wold, 2002). OPLS-DA simplifies separating and interpreting variation related to Y and uncorrelated/orthogonal one, at the same time providing greater confidence for variable selection (Kim et al., 2009).

Values collected from PLS-DA can be run and selected using a technique called variable importance in projection (VIP) (Mehmood et al., 2012). Ideally, these should all be close to 1 (Yi et al., 2016). VIP analysis shows or quantifies the importance a particular variable makes to a PLS-DA model (Favilla et al., 2013). VIP scores correlate with the top predictors (X) to the (orthogonal) variance (Y). The VIP approach can also be used with OPLS-DA to improve interpretability (Galindo-Prieto et al., 2014). VIP values greater than 1.0 have a significant influence on the response, but values smaller than 1.0 can still provide some information, too (Gorrochategui et al., 2016).

To consider a variable, a metabolite or a combination of metabolites as a statistically valid biomarker, a receiver operating characteristic (ROC) curve should be generated and analyzed (Xia et al., 2015). ROC curves are useful for evaluating two-state categorical classifications (such as sick vs. healthy or diseased vs. control). ROC curves compare the true positive rates (sensitivity) against/versus false positive rates (1-specificity), plotted on a diagram. The area under the ROC curve or AUC can be used to assess the performance of a biomarker or a set of biomarkers. Based on the ROC curve's shape, an optimal cut-off point can be determined that maximizes the discrimination between two classes (Metz, 2008). The AUC is a performance measurement that graphically discriminates between types. It helps to choose the critical value at which a predictor/biomarker best distinguishes between choices. The AUC can take any value from 0.5 to 1.0. The closer to 1.0, the better the test or classifier (Šimundić, 2009; Pencina et al., 2012). To choose between two ROC curves, the p-value (probability) is often taken into consideration. As these are approximate estimations, confidence intervals (CIs) should be described for ROC curve measurements. ROC analyses are usually quoted with a 95%

confidence interval (CI) (Xia et al., 2013). More practical information will be explained under "Materials and Methods" of each chapter using the MetaboAnalyst suite (metaboanalyst.ca).

1.7 Predictive biomarkers: opportunity for the dairy industry

Given that not all dairy cows are equally susceptible to mastitis (Ruegg, 2017; Welderufael et al., 2018), there is a need to develop a pen-side test with a panel of metabolites that can distinguish between cows that are more susceptible to developing mastitis from healthy cows. MS-based metabolomics approaches are highly sensitive and high-throughput instruments that allow identification and validation of biomarkers from biological biofluids. Current challenges for the dairy industry lay on high culling rates, treatment costs and tests that only screen for mastitis in milk during lactation. Considering the existing literature and approaches, we speculate that this new experiment, focused on finding predictive biomarkers during the dry-off period for the development of SCM will bring many advantages on cow's health, dairy industry and food safety.

1.8 Hypotheses

1. Cows affected postpartum by SCM show blood metabolic changes at beginning of dry off (–8 wks prepartum) as well as at –4 wks prior to parturition that can be detected by MS-based metabolomics.
2. Cows affected postpartum by SCM show urinary metabolic changes at –8 and –4 wks prior to parturition that can be detected by MS-based metabolomics.
3. Both blood and urine metabotypes show specific biomarkers for dairy cows at risk of mastitis during the dry-off period.

1.9 Objectives

1. To identify serum metabotype fingerprints starting from –8 and –4 wks prepartum that can differentiate healthy vs. pre-SCM during the dry-off period.
2. To discover a panel of urinary metabolites starting at –8 and –4 wks prior to parturition to identify cows susceptible to SCM.
3. To identify metabolite panels of blood and urine as early potential predictive biomarkers for the risk of mastitis in dairy cows (CM or SCM).

1.10 References

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Table 1.1. Classification of mastitis pathogens Adapted from Constable et al. (2017).

Contagious	Environmental	Opportunistic
Staphylococcus aureus	*Escherichia coli*	Non-aureus Staphylococci:
Streptococcus agalactiae	*Streptococcus uberis*	*Staphylococcus simulans*
Corynebacterium bovis	*Streptococcus dysgalactiae*	*Staphylococcus chromogens*
Mycoplasma spp	*Klebsiella spp*	
	Corynebacterium pyogenes	

Table 1.2. A comparison between NMR and MS instruments (Adapted from Wishart, 2019).

	NMR	MS
Advantages	Non-destructive; highly reproducible; simple sample preparation; no chemical derivatization; inherently quantitative; robust instrument	High sensitivity (nM); huge metabolite coverage; moderately expensive instrument and care; many software resources
Disadvantages	Low sensitivity (μM); moderate metabolite coverage; expensive instrument and maintenance; few software resources	Sample destruction; moderate reproducibility; complex sample preparation; frequent chemical derivatization; not inherently quantitative; delicate instrument

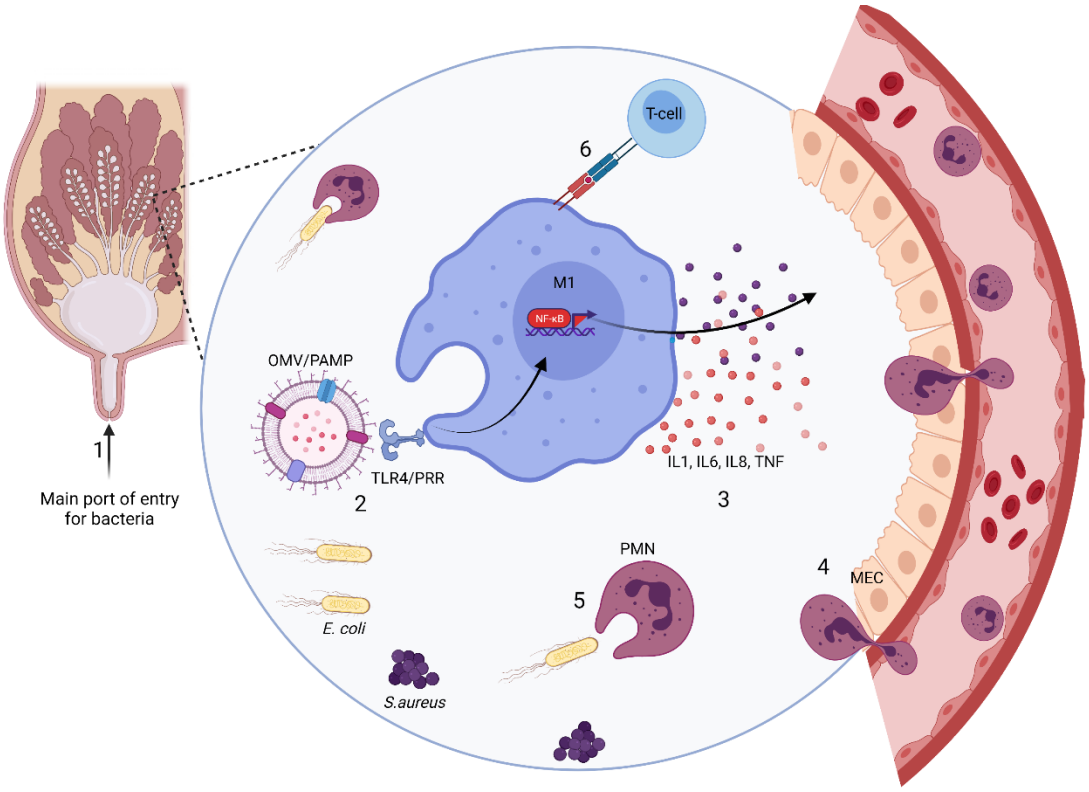

Figure 1.1 Schematic presentation of mastitis pathogenesis.
Typically, (1) once bacteria invade the teat canal and penetrate into the udder, a local immune response starts mounting from the host; (2) Bacterial by products like outer membrane vesicles (OMV) of Gram-negative pathogens act as pathogen-associated molecular patterns (PAMP), which are recognized by host pathogen recognition receptors (PRR), specifically toll-like receptor 4 (TLR4) on macrophage type 1 (M1). After this contact, (3) proinflammatory cytokines (i.e., IL1, IL6, TNF) and chemokines (IL8) are released that alert or attract other immune cells, mainly polymorphonuclear (PMN) leuckocytes, to move to the site of infection. (4) Once PMN extravasate from blood vessels have entered in the infected area through mammary epithelila cells (MEC), (5) they encounter, engulf, and kill pathogenic bacteria (i.e., *Escherichia coli* or *Staphylococccus aureus*) through phagoctysis. If inflammation persists, (6) then adaptive immunity is activated via the interaction of macrophages and lymphocytes, like T-cells.

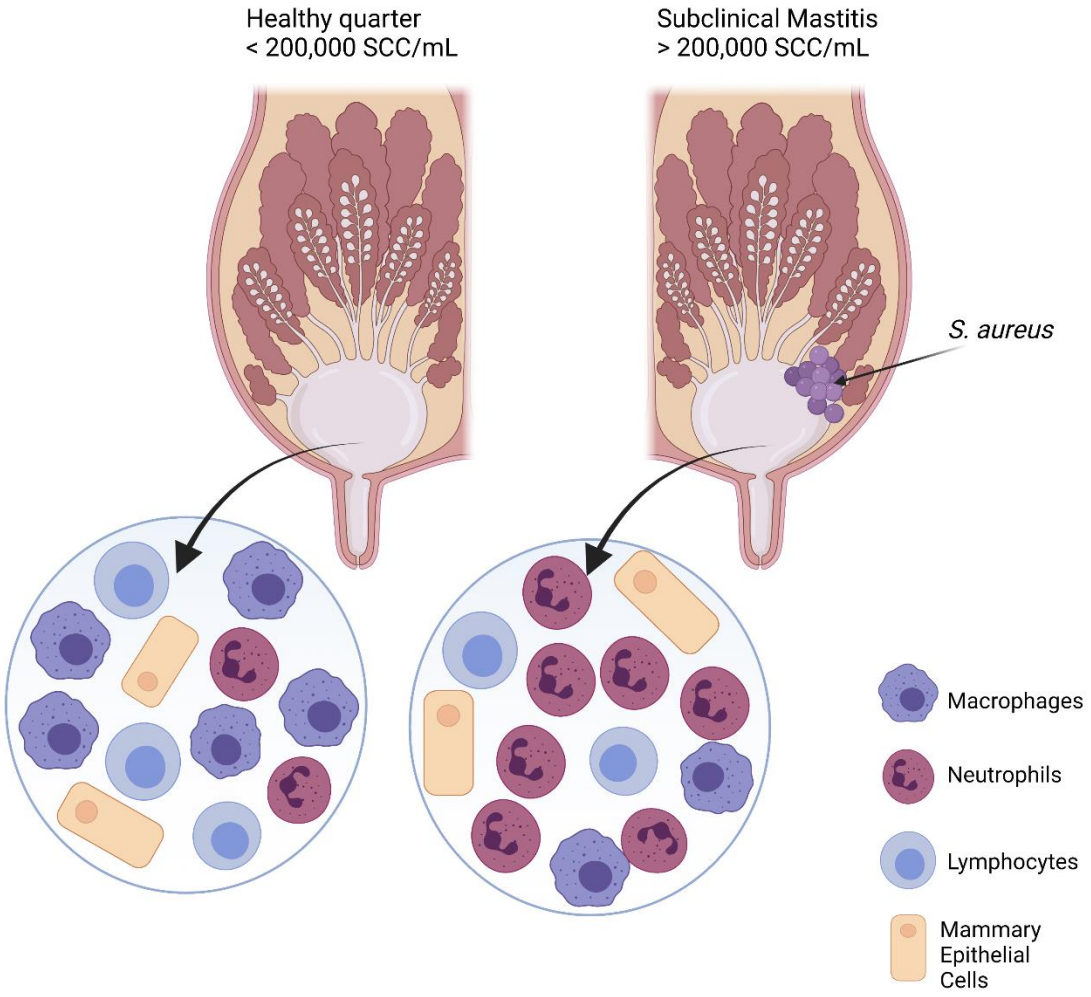

Figure 1.2 Presence of somatic cells in healthy and infected quarters of a dairy cow. A milk sample drawn from the healthy [<200,000 somatic cell count (SCC)/mL of milk] left side of the gland cistern (or sinus) of the mammary gland shows a mixture of immune cells (plus epithelail cells) dominated by macrophages and followed by a small proportion of lymphocytes, neutrophils, and epithelial cells. Whereas, a similar sample from the infected (>200,000 SCC/mL of milk) right quarter (clinical or subclinical mastitis) shows presence of pathogenic *Staphyloccoccus aureus* and immune cells dominated by with neutrophils as well as a few macrophages, lymphocytes, and epithilail cells.

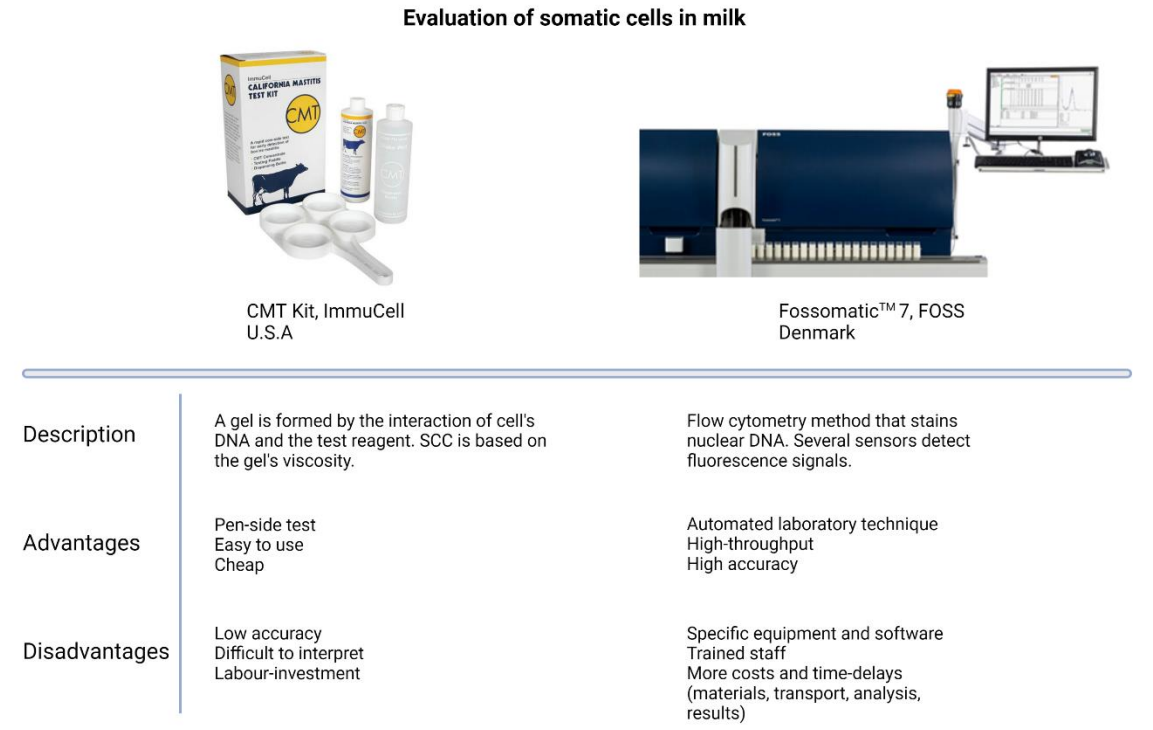

Figure 1.3 Comparison of the two most used tests to detect presence of infection in the udder of dairy cows. CMT (California Mastitis Test) kit [image courtesy of ImmuCell (U.S.A)] is a diagnostic tool that helps to quickly diagnose subclinical mastitis in dairy cows. The reagents of this test trigger a visible reaction (gel formation) when SCC is 400,000 SCC/mL of milk. The degree of gel creation indicates the severity of mastitis. Fossomatic 7 [image courtesy of FOSS (Denmark)] is used by central labs to measure somatic cells in raw milk in a few seconds. The principle of work is based on detection of fluorescence signals from milk cells through sensitive sensors and an incubation unit that are able to detect differential somatic cell count (DSCC) and SCC. DSCC is a new variable for mastitis monitoring that represents proportion of two cell populations (neutrophils and lymphocytes) in percentage.

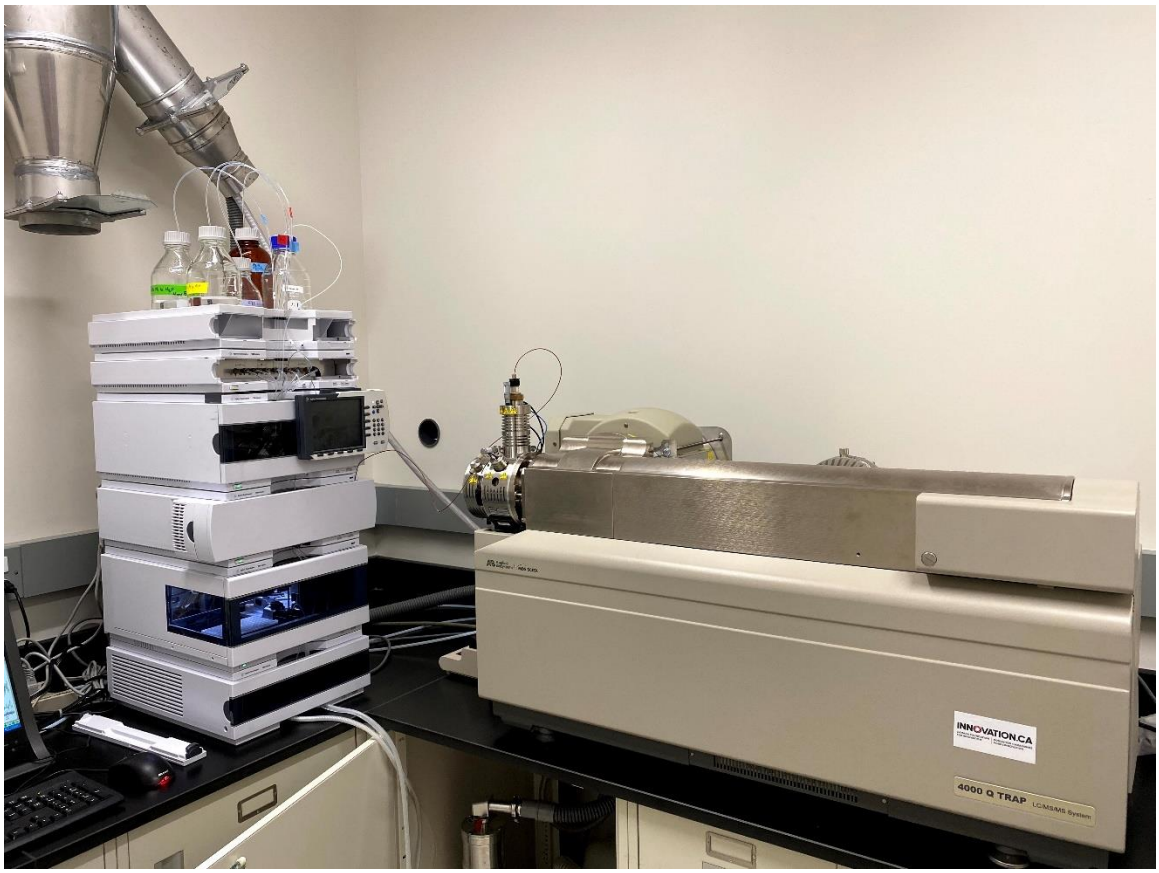

Figure 1.4 Original photograph of LC and MS instruments used to perform the FIA/LC-MS/MS analyses at The Metabolomics Innovation Centre (Edmonton, AB, Canada).

Chapter 2. Blood Metabolomic Phenotyping of Dry Cows Reveals Predictive Biomarkers for Susceptibility to Subclinical Mastitis

2.1 Abstract

Subclinical mastitis (SCM) is a very common pathology in postpartum dairy cows that negatively impacts milk yield and is associated with a high culling rate of affected cows. Early identification of susceptible cows can enable development of better preventive measurements ahead of disease occurrence. Currently, SCM is diagnosed through the measurement of somatic cell counts (SCC) in milk after calving. No screening tests are available to predict which cows may develop SCM during the dry-off period. Therefore, the objectives of this study were to identify metabolic alterations in the serum of pre-SCM cows during the dry-off period, at –8 and –4 wks before calving, through a targeted mass spectrometry (MS-based metabolomics assay). A total of 145 multiparous dairy cows were randomly selected for this nested case-control study during the drying-off period and were sampled and monitored for periparturient disease occurrence, both prepartum and postpartum. Fifteen cows, free of any disease, and 10 cows affected only by SCM postpartum served as controls (CON) and the SCM group, respectively. Cows affected by other diseases or SCM and at least one other disease were excluded from further evaluation. Results showed multiple metabolite alterations in the serum of pre-SCM cows, including several amino acids, lipids, acylcarnitines, organic acids, and glucose. Metabolic profiling of cows revealed a total of 59 and 47 metabolites that differentiated ($P \leq 0.05$) CON and pre-SCM cows at –8 and –4 wks prior to the expected date of parturition, respectively. FDR adjusted P values showed 32 and 17 metabolites ($q < 0.005$) different at both weeks.

Regression analysis indicated that a panel of 4 serum metabolites including alanine, leucine, betaine, and ornithine (AUC = 0.92; $P < 0.001$) at –8 wks as well as alanine, pyruvate, methylmalonate, and lactate (AUC = 0.92, $P < 0.01$) at –4 wks prior to parturition might serve as predictive biomarkers for SCM. More research is needed to validate these two panels of metabolite biomarkers and the development of potential pen-side tests.

2.2 Introduction

Subclinical mastitis is the most common intramammary infection (IMI) of dairy cows. It poses a significant challenge for cow's health and profitability of dairy farms (Thompson-Crispi et al., 2013; Ruegg and Petersson-Wolfe, 2018). Contagious bacteria such as *Staphylococcus aureus, Streptococcus agalactiae,* and environmental pathogens such as *Escherichia coli* are the primary cause of mammary gland infections (Zadoks et al., 2011; Dufour et al., 2019). Depending on the severity of the disease, this infection is classified as either clinical (CM) or subclinical mastitis (SCM) (Pinzón-Sánchez and Ruegg, 2011). As the name suggests, CM is the inflammation of the udder, associated with swelling, redness, and pain of the udder. Clinical mastitis also is associated with changes in milk yield and milk quality, while at the same time, cows experience systemic symptoms of the disease (Wilson et al., 1997; Kulkarni and Kaliwal, 2013). On the other hand, SCM is an asymptomatic infection mainly characterized by an influx of neutrophils in the mammary gland. Subclinical mastitis is detected only when somatic cell count (SCC) exceeds 200,000 cells per mL of milk (Schukken et al., 2003; Adkins and Middleton, 2018).

Routinely, the most frequently used methods to identify SCM cows are the California Mastitis Test (CMT), Somatic Cell Count (SCC), and electrical conductivity (EC) tests which are normally attached to milking systems in the barn (Viguier et al., 2009; Khatun et al., 2017). According to most farmers, SCC is a good indicator of udder health control strategies (Dufour et al., 2011). Counting somatic cells in milk has been more successful because it shows better sensitivity and specificity than the other SCM diagnostic tests (Rossi et al., 2018). Counting somatic cells requires laboratory analysis, limiting its use as a real-time detection method. Furthermore, it is associated with higher costs per test (Labohm et al., 1998; Hillerton, 2000). It should be noted that there are no SCM tests available during the nonlactating (dry-off) period (Hurley and Theil, 2011).

It is important to mention that in a previous study conducted by our lab, dry-off cows were found to be in a state of low-grade chronic inflammation during both –8 and –4 wks prepartum (Dervishi et al., 2015; Rollin et al., 2015). The potential reason for the chronic low-grade inflammatory state might be translocation of pathogenic bacteria, presence of bacterial endotoxins, or transport of proinflammatory cytokines from the sub-clinically infected udder to the systemic circulation (Eckel and Ametaj, 2016). Thus, the need to monitor cows during the dry-off period to identify cows at risk of SCM, is critical in developing preventative measures and better farm management strategies.

The need for new pen-side tests for SCM becomes even more important given that higher value of SCC (>200,000/mL of milk) are generally related to development of CM postpartum, but also low SCC levels have been associated with severe cases of CM (Barkema et al., 1998; Djabri et al., 2002; Rainard et al., 2018).

Recent research conducted by our lab has provided considerable information about the metabolic events around calving, particularly during the dry-off period. Previous experiments have also identified panels of metabolites in blood, urine, and milk that can be used as predictive biomarkers for the risk of developing several periparturient diseases (Hailemariam et al., 2014b, 2018; Dervishi et al., 2017, 2018b; a, 2020; Zhang et al., 2017b; c; a, 2020b; Zwierzchowski et al., 2020a). Moreover, other studies have reported data relating several milk metabolites including acetate, lactate, hippurate, butyrate or isoleucine with high SCC (Melzer et al., 2013; Sundekilde et al., 2013). However, the scope of the later study has been to identify more diagnostic biomarkers rather than predictive biomarkers of SCM. In contrast, Martins et al. (2019) indicated that milk samples negatively influence SCM detection techniques, so they suggested using other biological fluids instead. Indeed, Dervishi et al. (2017) and Zandkarimi et al. (2018) identified altered metabolic pathways in the serum of multiparous dairy cows during the dry off period. According to Dervishi et al. (2017), the best metabolites for differentiating between healthy and pre-SCM cows were valine, serine, tyrosine, and phenylalanine. Those metabolites were higher in SCM cows at both –8 and –4 wks prior to parturition (Dervishi et al., 2017). Those data suggest that a serum metabolomics test could be used to predict cows at risk of developing SCM, prior to developing subclinical or clinical mastitis postpartum.

Given that not all dairy cows are equally susceptible to mastitis (Ruegg, 2017; Welderufael et al., 2018), there is a need to develop a pen-side test with a panel of metabolites that can distinguish between cows that are more susceptible to developing mastitis from healthy controls. Our hypothesis is that serum metabolic changes might occur at the beginning (–8 wks) and in the middle (–4 wks) of the dry period in pre-subclinical

mastitis cows. We also hypothesized that a panel of screening biomarkers with high accuracy (high specificity and sensitivity) could be identified that that can be used in the future to develop reliable pen-side tests for predicting SCM. Therefore, the objectives of this study were to identify panels of serum metabolites in dairy cows, one at –8 wks and the other one at –4 wks prior to parturition that can determine, with high accuracy, the risk of cows to develop SCM postpartum during the dry-off period.

2.3 Materials and Methods

2.3.1 Animals, diets, and blood samples

In this nested case-control study, a total of 145 multiparous cows were selected from which blood samples were collected from the coccygeal vein. Cows were selected from a commercial dairy farm in the province of Alberta, Canada. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and conducted following the Canadian Council's guidelines on Animal Care (CCAC, 2009).

Cows were selected based on their expected date of calving. Sampling occurred at the beginning of the dry-off period and in the middle, respectively, at –8 wks (55-58 days) and –4 wks (27-30 days) before parturition. Blood samples were collected before the morning feed, between 07:00-08:00, using 10 mL vacutainer tubes (clot activator and serum separator tube; Becton Dickinson, Franklin Lakes, NJ). The blood samples were allowed to clot in ice. All metabolomic analyses were conducted at The Metabolomics Innovation Centre (University of Alberta, Edmonton, AB, Canada). Blood samples were centrifuged at 4,000 rpm for 15 minutes (Rotanta 460 R centrifuge, Hettich Zentrifugan,

Tuttlingen, Germany) to collect serum samples. The separated serum was aspirated into sterile tubes using a transfer pipette (Fisher Scientific, Toronto, ON, Canada). Two hundred µL of serum were transferred into aliquots and stored at –80℃ to be run later on LC-MS/MS.

Cows sampled prepartum presented various disease conditions including mastitis, metritis, retained placenta, laminitis, displaced abomasum, milk fever, and ketosis postpartum. Health records for the periparturient diseases were gathered from the farm's database. Data was collected for the number of culled cows or those that were removed by the veterinarian's decision. Positive SCM cases were considered only for those cows having two or more consecutive weeks with milk SCC equal to or higher than 200,000 cells/mL. By this judgement, 15 dairy cows were considered healthy (CON), whereas only 10 cows were free of other diseases and had SCC levels classified as pre-SCM. Body condition score (BCS) was measured for both groups during the sampling weeks. The feed ingredients, on a dry matter basis, offered to cows pre- and post-partum is presented in Table 2.1 and 2.2.

2.3.2 FIA/LC - MS/MS compound identification and quantification

2.3.2.1 Sample preparation

Samples were thawed on ice and vortexed before analysis. For the analysis of biogenic amines, amino acids, lipids, acylcarnitines, and glucose, 10 µL each of flow injection analysis (FIA) running buffer and LC internal standards (ISTD) were loaded into a 96-well filter plate, except for the first well, which acted as a double blank. From the 2nd to the 14th well of the filter plate, three phosphate-buffer saline (PBS) "zero-point" control

samples, seven calibration curve standards and three quality control samples (QC) were added. Thawed serum samples were then added to the remaining wells. For samples and standards, a total of 10 µL was added to the respective wells. The plate was then incubated and dried under a flow of nitrogen [Zanntek Analytical Evaporator (Glas-Col, Terre Haute, IN, USA)] for 30 min. After being dried, 50 µL of 5 % phenylisothiocyanate (PITC) solution was added to each well, and the plate was incubated at room temperature for 20 min. The plate was then dried again for 90 minutes under a flow of nitrogen. Extraction of the metabolites was accomplished by adding 300 µL methanol, containing 5 mM ammonium acetate. The plate was then placed on a shaker, shaking at 330 rpm for 30 min and then centrifuged for 5 min at 500 rpm (50 x g), [Sorvall Evolution RC Superspeed Centrifuge (Fisher Scientific, Toronto, ON, Canada)], into the lower 96 deep-well plate. For the analysis of amino acids and biogenic amines, the extract was diluted with water 1:1, and 10 µL was injected into the column. For the analysis of acylcarnitines, lipids, and glucose, 150 µL of the extract was diluted with 400 µL of FIA running buffer, and 20 µL was injected in the column.

Protein precipitation was first conducted for the analysis of organic acids. In 1.5 mL Eppendorf tubes, 10 µL of an internal standard (ISTD) mixture solution, 50 µL of the samples (three phosphate-buffered saline [PBS] blank samples, seven calibration standards, three quality control samples and serum samples) and 150 µL ice-cold methanol were added. 3:1 methanol: water was used in place of methanol for the blanks, calibration standards, and quality control (QC) samples. Tubes then were vortexed and placed at -20℃ overnight. Samples were centrifuged at 13,000 rpm for 15 min before use. Following centrifugation, 50 µL of the samples were pipetted into the 96-deep well plate's wells.

Twenty-five microliters of each of the following three solutions: 1) 3-nitrophenylhydrazine (250 mM in 50 % aqueous methanol), 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (150 mM in methanol) and pyridine (7.5% in 75% aqueous methanol) were added to each well. The whole plate was then shaken at 450 rpm (41 x g) for 2 h at room temperature to complete the derivatization reaction. After the reaction, 350 µL of water and 50 µL MeOH were added to each well to dilute and stabilize the solution for LC-MS/MS analysis.

2.3.2.2 FIA/LC – MS/MS method

Identification of metabolites in serum samples was done through a targeted metabolomics approach using a TMIC Prime kit in Agilent 1100 series liquid chromatographic system (LC) (Agilent, Palo Alto, CA, USA) equipped with an Agilent reversed-phase Zorbax Eclipse XDB C18 column (3.0 mm × 100 mm, 3.5 µM particle size, 80 Å pore size) with a Phenomenex (Torrance, CA, USA) SecurityGuard C18 pre-column (4.0 mm × 3.0 mm) coupled with AB SCIEX QTRAP® 4000 mass spectrometer (Sciex Canada, Concord, ON, Canada). LC/MS grade formic acid and HPLC grade water were purchased from Fisher Scientific (Ottawa, ON, Canada). Ammonium acetate, phenylisothiocyanate (PITC) and HPLC grade acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LC-MS assay workflow was controlled through the Analyst® 1.6.2 software (Sciex Canada, Concord, ON, Canada).

The LC parameters used to analyze amino acids and biogenic amines were as follows: mobile phase A 0.2% (v/v) formic acid in water, and mobile phase B 0.2% (v/v) formic acid in acetonitrile. The gradient profile was as follows: t = 0 min, 0% B; t = 0.5

min, 0% B; t = 5.5 min, 95% B; t = 6.5 min, 95% B; t = 7.0 min, 0% B; and t = 9.5 min, 0% B. The column oven was set at 50 °C. The flow rate was 500 µL/min, and the sample injection volume was 10 µL.

For the analysis of organic acids by LC-MS/MS, the solvents used were (A) 0.01% (v/v) formic acid in water and (B) 0.01% (v/v) formic acid in methanol. The column oven temperature was set to 40 °C. The flow rate was 300 µL/min, and the sample injection volume was 10 µL. The mass spectrometer was assigned to a negative electrospray ionization mode with scheduled MRM scanning.

2.3.3. Statistical analysis

Univariate analyses were conducted using Wilcoxon rank-sum test from the emmeans package in R (v4.0.2) with a significance level of $P \leq 0.05$. The adjusted *P* values were performed using the false discovery rate (FDR) method and a value of $q < 0.005$. Multivariate statistical and biomarker analyses were run using MetaboAnalyst (v4.0) following specific guidelines described in reference protocols (Xia et al., 2009; Chong et al., 2019). Data normalization of all metabolite concentrations was done using data transformation and scaling options to create a Gaussian distribution (a bell-shaped curve). Metabolites with >50% of missing values were removed from further analysis. Half of the minimum concentration value was imputed in those with <50% of missing values.

To perform a standard cross-sectional 2-group analysis, we compared healthy cows (CON) and the pre-SCM group at each time point, –8 wks and –4 wks prepartum. Multivariate statistical analyses, such as principal component analysis (PCA) and partial least-squares–discriminant analysis (PLS-DA) were conducted via MetaboAnalyst (v4.0).

Metabolic pathway analysis and identification of biomarker profiles were also performed following Chong et al.'s (2019) guidelines with MetaboAnalyst. PCA and PLS-DA were used to distinguish the CON and SCM cows using Score plots. Model validation was carried out using a cross-validation test, and a 2000 set permutation test was implemented to validate the reliability of the model (Xia and Wishart, 2011). In addition, the most influential compounds were ranked using Variable Importance in Projection (VIP) plots. Metabolites with $P < 0.05$ and VIP scores > 1 were the most discriminatory between the groups.

Metabolite set enrichment analysis (MSEA) was used to identify perturbed metabolic pathways, as implemented in MetaboAnalyst (v4.0). Metabolite sets obtained from quantitative enrichment pathway analysis were considered statistically significant if the Holm corrected *P* value was < 0.05. The quality of the biomarker sets was determined using the receiver-operating characteristic (ROC) curve as generated by Monte-Carlo cross-validation (MCCV). ROC curves are often assessed using a single metric known as the area under the ROC curve (AUROC), which indicates a test's accuracy for correctly distinguishing one group from another, such as pre-SCM cows from CON cows. A general guide for assessing the utility of a biomarker set based on its AUROC is: 0.9~1.0 = excellent; 0.8~0.9 = good; 0.7~0.8 = fair; 0.6~0.7 = poor; 0.5~0.6 = fail (Xia et al., 2015). Supervised classification method, such as Support Vector Machine (SVM) and logistic regression analyses for several significant metabolites were also performed to train the model.

2.4 Results

Cows were selected based on the development of SCM postpartum. The distinction between pre-SCM and CON was made possible by measuring SCC postpartum in the milk. We grouped cows into those that presented SCM against the CON group (Tables 2.3, 2.4). A cut-off value of > 200,000 SCC/mL of milk is a good indicator of an infected udder and for the diagnosis of SCM (Sargeant et al., 2001; Schukken et al., 2003). Of the recorded data, just 10 cows were diagnosed to be affected by subclinical mastitis only (SCM, n=10); free of other diseases, and 15 cows that were ascertained to be completely free of periparturient diseases (CON, n=15). Control and pre-SCM cows had an average BCS of 3.78 and 3.95 and 3.70 and 3.92 at –8 and –4 wks, respectively. In this study, a total of 126 metabolites were identified and quantified by FIA/LC-MS/MS from the samples collected prior to disease diagnosis. From this set, we found 59 metabolites at –8 wks and 47 metabolites at 4 wks prepartum that were different between the two groups ($P \leq 0.05$). On the other hand, FDR adjusted P values ($q < 0.005$) showed 36 metabolites to be different at –8 wks and 17 compounds at –4 wks.

The univariate mean ± SEM concentration values, P values, and direction of change of these metabolites are provided in respective tables (Tables 2.3, 2.4), for both –8 and at –4 wks prepartum. Multivariate analysis, using both PCA and PLS-DA, showed a clear separation between pre-SCM (n=10) vs CON (n=15) with respect to serum metabolites, at –8 wks prior to calving (Figures 2.1a, 2.1b). The VIP plot (Figure 2.1.c), for the PLS-DA, ranks the top metabolites responsible for the differentiation between the two groups of cows, highlighting several glycerophospholipids and one sphingomyelin (SM), which were lower in the pre-SCM group than the CON group. At the same time, 4 metabolites, namely

leucine, phenylalanine, ornithine, and choline were found to be higher in pre-SCM cows. Permutation testing with 2000 repetitions yielded a $P = 0.0075$, which shows that the PLS-DA separation was statistically significant.

The top 5 metabolites found from the PLS-DA VIP plot showed an excellent performance in their respective ROC curve model (Figure 2.2a; AUC=1; 95% CI: 1-1; $P = 0.002$). While overall, the multivariate exploratory analysis presented the best AUROC for the first 10 most important features with zero samples classified to the wrong group (Figure 2b). In support of building a significant panel of biomarkers, we selected specific variables from the univariate analysis (Table 2.3, 2.4), the VIP plot (Figure 2.1c) and the metabolic pathways (Table 2.5) to build a default linear Support Vector Machine (SVM) model (AUC = 0.92; P = 0.001; Figure 2.2c) and a logistic regression model (AUC = 0.81; $P = 0.05$; Figure 2.2d). To prevent optimistic and over-fitting results, 10-fold cross-validation (CV) was used to evaluate performance of each model.

Unsupervised multivariate analysis such as PCA managed to satisfactorily separate the two groups of cows at –4 wks before calving, whereas PLS-DA yielded excellent separation (Figure 2.3a; 2.3b). Figure 2.3c and 2.3d show those metabolites that had the most impact in separating these two groups. Among these, methylmalonate, trimethylamine N-oxide, lactate, pyruvate, and eight ACs were higher in the serum of those cows that eventually developed SCM. This time, we selected the top 25 important features from VIP to display other variables apart from several glycerophospholipids similar to –8 wks group. Indeed, these new metabolites were able to differentiate between both classes, even during pathway analysis. For example, as seen on the VIP plot (Figure 2.3c), pyruvate and lactate were found to be higher in pre-SCM cows than CON cows at –4 weeks.

The top scoring metabolites from the PLS-DA contributed the most to the separation of clusters. In particular, PC aa 38:0, PC aa 40:0, C9, PC aa 36:0, and PC aa 32:2 (Figure 2.3c), demonstrated excellent cluster separation between the –4 wks pre-SCM group and the CON group (Figure 2.4a; $P = 0.001$). In general, the best multivariate ROC curves, based on the cross-validation performance, were achieved from a model with 10 features, which generated a zero-confusion matrix (Figure 2.4b). Specific metabolites (methylmalonate, lactate, pyruvate, and alanine) that showed outstanding performance throughout the analysis, produced two AUCs using two different algorithms, respectively, the default linear SVM model (AUC = 0.92; $P = 0.01$; Figure 2.4c) and logistic regression model (AUC = 0.81; $P = 0.04$; Figure 2.4c).

Figure 2.5 displays the results of the quantitative enrichment analysis performed via MetaboAnalyst using various summary plots. At the same time, Table 2.5 presents the significant components of the metabolic pathways involved in the onset and progression of subclinical mastitis (Holm $P \leq 0.05$). Metabolites involved in betaine, methionine, glycine, and serine metabolism were significantly higher in pre-SCM at –8 wks before parturition. Pathway analysis also indicated that up to 4 wks before parturition, other perturbed metabolic sets, such as Glucose-Alanine cycle and Seleno-Amino Acid metabolism were affected in cows susceptible to SCM.

The most discriminatory metabolites between pre-SCM and CON at –8 wks (Figure 2.1c) and at –4 wks (Figure 2.3c) were PCs and LPCs, which have small concentration compared to other metabolites in serum. The rationale for selecting the specific metabolites (Figure 2.2c,d; Figure 2.4c,d) for our predictive biomarker model is due to the fact that

these are more abundant and easily measured in a convenient pen-side test. However, a lab-based test could look at PCs and LPCs for high-throughput blood analysis.

2.5 Discussion

We hypothesized that cows affected postpartum by subclinical mastitis (SCM) might show serum metabolic changes starting from –8 and –4 wks prior to calving. If this is proved, a panel of serum biomarkers could be constructed for predicting SCM. Indeed, the results of this study indicated that multiple serum metabolites were altered between pre-SCM and CON cows during the two dry-off time-points measured. As a result, two specific metabolite panels were constructed. Given that cows were in a state of pre-SCM, it is assumed that systemic metabolite-changes were triggered from local immune responses in the mammary gland, to support the host in the fight against mammary infection. Our data showed that in pre-SCM cows, at –8 wks prepartum, there was a total of 10 PCs, 10 LPCs, 10 SMs, 6 ACs, 17 AAs, and 6 OAs that differentiated pre-SCM from the CON cows. At –4 wks prepartum, there was a total of 12 AAs, 3 OAs, 1 glucose/hexose, 2 LPCs, 10 PCs, 6 SMs, and 13 ACs that differentiated healthy cows from the pre-SCM ones. Identifying and understanding these metabolic changes is essential to predict the risk of cows developing SCM at the earliest, prior to diagnosis of SCM infection.

2.5.1 Serum lipid alterations and related metabolites in pre-SCM cows

An important finding of the present study was the large number of PC species (10 out of 10 identified and measured) that differentiated the pre-SCM cows from the healthy

ones. All serum PCs were lowered in pre-SCM versus CON cows. These PCs were mostly long-chain fatty acid species with C:32-C:40 carbon atoms. Most of the PCs found in the blood are produced in the liver and secreted as part of lipoprotein fractions. It should be noted that PCs, triacylglycerols (TAGs), and cholesterol are required for the assembly of the lipoprotein particles in the liver (Feingold and Grunfeld, 2000). If any of these three lipid components is suppressed, then lipoprotein synthesis is downregulated. Suppression of lipid secretion from the liver is associated with an accumulation of lipids in hepatocytes and the development of hepatic steatosis. Indeed, in a study conducted by Minuti et al. (2015), intramammary infusion of LPS was accompanied with systemic inflammation and accumulation of TAGs in the liver. Previously, we demonstrated that pre-SCM cows, starting from –8 wks and –4 wks prepartum, were in a state of chronic low-grade systemic inflammation (Dervishi et al., 2015). Phosphatidylcholines have been proven to have anti-inflammatory activities, too (Jung et al., 2013). Given that concentrations of PCs in the blood were lowered in pre-SCM cows, this might support mounting of a low-grade inflammatory response in those cows and potentially predispose them to fatty liver.

Alterations in ACs were found at both time points in the study. Many of the short and mid-chain ACs were increased both at –8 wks and –4 wks prepartum in pre-SCM cows, whereas several of the long-chain ACs were lowered in the serum of pre-SCM cows. Acylcarnitines function as carriers of fatty acyl-CoA from the cytosol into the mitochondria (McFadden, 2020). Incomplete fatty acid oxidation leads to the accumulation of ACs in the cytosol. Acylcarnitines are considered biomarkers of lipid-induced mitochondrial dysfunction (Schooneman et al., 2013). Altered AC levels have been reported in several human pathologies including obesity, diabetes, and cardiovascular disease (Li et al., 2019).

Moreover, high ACs are correlated with the increased presence of pro-inflammatory mediators such as TLR (toll-like receptor), TNF, and IL-8 (Interleukin) (Rutkowsky et al., 2014). This is in line with our previously reported findings of increased blood ACs in pre-ketotic and pre-metritic cows and the fact that those cows were in a state of chronic low-grade inflammatory status, during the pre-calving period (Hailemariam et al., 2014a; Zhang et al., 2017a; b). These data suggest malfunctioning of mitochondrial β-oxidation associated with accumulation of ACs in the blood, which commonly are excreted from the kidneys out of the host.

Multiple serum SM species were found downregulated at both –8 wks and –4 wks prepartum in pre-SCM cows. Sphingomyelins belong to a large class of bioactive lipids known as sphingolipids. They are essential and critical regulators of cell membrane and multiple activities including immunity, inflammation, cellular growth, proliferation, apoptosis, metabolism, and related pathologies (Hannun and Obeid, 2008). Sphingomyelin synthase is the enzyme that converts ceramide to sphingomyelin and a phosphocholine headgroup (Carroll et al., 2015). Most importantly, SMs have been shown to regulate neutrophil migration toward the infection site as well as the phagocytosis process. Sphingolipids have been associated with enhanced oxidative burst in neutrophils (Nakamura et al., 1994). Also, SM degradation to ceramide regulates neutrophil chemotaxis as well as superoxide generation and degranulation in the phagosome necessary for killing of pathogenic bacteria (Feldhaus et al., 2002; Sitrin et al., 2010). Ceramides also are precursors to sphingosine-1-phosphatase (S1P) which promotes neutrophil recruitment to the pro-inflammatory site. The higher chemotaxis of neutrophils is triggered by increasing concentrations of IL-8 and ICAM-1 expression in epithelial cells (MacEyka and

Spiegel, 2014). Considering that SMs were lower in pre-SCM cows in our study, it suggests that neutrophil migration and their killing capacity were impaired, increasing host susceptibility to infections.

A total of 8 LPCs at –8 wks and 2 other LPCs at –4 wks were all downregulated in pre-SCM cows. The length of fatty acid attached to the LPC that were downregulated in pre-SCM cows included 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 26:0, 26:1, 28:0 and 28:1 at –8 wks prepartum and 20:3 and 28:1 at –4 wks prepartum. The length of fatty acid attached to the LPC is very important for physiological functions of the LPC molecule as will be discussed below. It should be noted that plasma LPCs are catabolites of PC, which are produced by the action of secretory phospholipase A2 ($sPLA_2$), after removal of a fatty acid (Fuchs and Schiller, 2012). Lysophosphatidylcholines in plasma are bound mainly to albumin and to a lesser extent to lipoproteins (Switzer and Eder, 1965). Yan et al. (2004), in a series of experiments with mice aiming at using LPC species as therapeutic intervention against sepsis induced by *E. coli* or cecal ligation and puncture (CLP), showed that 18:0 LPC only (authors tested several other species of LPC) was able to markedly enhance *in vivo* elimination of bacteria introduced by CLP or *E. coli* injection. Lysophosphatidylcholine also increased the *in vitro* bactericidal activity of neutrophils, which correlated with increased production of H_2O_2 in neutrophils that had ingested *E. coli*. Moreover, the same authors showed that 18:0 LPC effectively inhibited LPS-induced lethality and the release of TNF from neutrophils in response to LPS. Overall, it is obvious that LPCs play important roles in host protection against bacterial infection. Therefore, decreased concentrations of various species of LPCs might have negatively affected the mounting of an efficient host response to udder infection during the dry off period.

2.5.2 Serum amino acid changes in pre-SCM cows

Branched-chain amino acids (BCAA; Leu, Ile, Val) were higher in the serum of pre-SCM vs CON ones. These data are in line with a previous study from our lab that showed elevated serum concentrations of these AAs prior to and during the occurrence of SCM (Dervishi et al., 2017). As reviewed by Calder (2006), BCAAs are essential for protein synthesis in immune cells. These amino acids support the production of cytokines, immunoglobulins, and acute phase proteins. They also help in the synthesis of Glu, an essential metabolite for the function of immune cells. Recently, it was reported that a high concentration of BCAAs contributes to the development of pro-inflammatory responses (Zhenyukh et al., 2017). High plasma BCAAs were shown to activate NF-kB and mTOR, oxidative stress, and aided in the migration of peripheral blood mononuclear cells (PBMC) (Zhenyukh et al., 2017). These authors reported a higher expression of CD40L and ICAM-1 receptors, expressed on PBMCs that trigger their activation and migration into systemic circulation. This might explain the increased presence of somatic cells in the udder in pre-SCM cows which are necessary to mount an immune response. Neutrophils were proven to have better phagocytic activity under supplementation with BCAA in both animal and human subjects (Nakamura et al., 2004). Adding dietary Leu or its metabolic by-product, beta-hydroxy-beta-methyl butyrate (HMB), to calves increased the killing capabilities of granulocytes and monocytes (Wójcik et al., 2020). Therefore, increased serum BCAAs might be beneficial to the host to mount a pro-inflammatory response.

Arginine was increased at –8 wks in pre-SCM cows; however, there was no difference between the two groups of cows regarding the arginine level at –4 wks prepartum. Additionally, ornithine a by-product of arginine was elevated in the serum of

pre-SCM cows at both –8 and –4 wks prepartum. Arginine, besides its role in protein synthesis, is also the precursor for synthesis of urea, ornithine, nitric oxide (NO), polyamines, proline, creatine, glutamate, and agmatine (Reviewed by Satriano, 2004). Arginine is also involved in the process of inflammation. Two pathways of arginine metabolism related to inflammation have been described including its conversion to NO and its catabolism to urea and ornithine. Conversion of arginine to large amounts of NO is the early phase response to inflammatory insult. Nitric oxide is known for its antimicrobial activities towards certain pathogens (Stuehr and Nathan, 1989; De Groote and Fang, 1995). Additionally, conversion of arginine to ornithine and urea starts in a later phase of the inflammatory response that involves healing and repair activities (Satriano, 2004). Results of our study showed that ornithine was higher in pre-SCM cows at both –8 wks and –4 wks prepartum. It is known that ornithine is converted to polyamines (putrescine, spermidine, and spermine), which have proliferative activities and to proline, an important component of extracellular matrix. Putrescine, spermidine, and spermine are cationic molecules required for cell growth and homeostasis (Tabor and Tabor, 1983; Pegg et al., 2003). As we have already pointed out in our discussion, pre-SCM cows at –8 wks and –4 wks prepartum were found to be in a chronic low-grade inflammatory state (Dervishi et al., 2015). The data from the current study are in line with that study showing increased arginine and ornithine, both playing roles during inflammatory conditions as proinflammatory compounds.

The concentration of alanine in the serum of pre-SCM cows was lowered at both time points in this study (at –8 wks and –4 wks) prepartum. Alanine is the second most abundant amino acid in the systemic circulation, after glutamine, which makes it very

accessible to immune cells (Matheson et al., 2015). Given that alanine is a glucogenic amino acid and given that pre-SCM cows potentially were in a state of chronic low-grade inflammatory state (Dervishi et al., 2015), it is possible that most of alanine in the systemic circulation is taken up by the liver and used to mount an acute phase response (Druml et al., 2001). In addition, alanine serves as a glucose precursor and provides the necessary energy for immune cells (Newsholme and Newsholme, 1989; Li et al., 2007). In dairy cows, it was reported the alanine release was enhanced in blood neutrophils (Garcia et al., 2016). These PMN (polymorphonuclear) cells were isolated from early and mid-lactation cows and supplemented in vitro with amino acids. The authors observed changes in the expression of genes related to nutrient metabolism and lowered TNF in the media. Studies conducted by Ron-Harel et al. (2019) showed a large increase in the alanine transporters in naïve $CD4^+$ T cells, during their activation process. The same authors also found that alanine is essential for protein synthesis during the first 24 h of T cell activation. Alanine also is very important for restimulation of memory $CD8^+$ T cells and for dueling protein synthesis. Therefore, lowered concentrations of alanine in the systemic circulation might reflect extensive utilization of alanine by the liver and immune cells.

Besides the already discussed AAs, there were also several other metabolites including glycine, trans-hydroxyproline, aspartate and methionine-sulfoxide that were lowered at –8 wks prepartum in pre-SCM cows. Additionally, several other AAs and their catabolites were lowered in pre-SCM cows at –4 wks prepartum including isoleucine, aspartate, serine, proline, methionine, histidine, ornithine, lysine, methionine-sulfoxide, and acetyl-ornithine. The question is what would be the reason that these 12 serum AAs decreased in the systemic circulation in pre-SCM cows? In a review, Lang et al. (2007)

indicates that during chronic inflammatory conditions as, for example, during LPS-induced inflammation or sepsis there is a significant decrease in the synthesis of proteins in the muscle and decreased muscle mass. Besides, there is a decrease in the plasma concentration of most AAs as well as a reduction of AAs from the systemic circulation (Druml et al., 2001). Hasselgren and Fischer, (1999) in their review article indicated that inflammatory mediators and particularly TNF increase proteolysis of skeletal muscle and stimulate the uptake of AAs by the liver. Overall, the lower serum AAs in pre-SCM cows might be related to presence of chronic low-grade inflammatory state in those cows and the potential negative impact of proinflammatory cytokines on muscle protein synthesis and their use for mounting of an acute phase response.

2.5.3 Alterations in blood methyl-donor compounds

The concentration of choline in the serum of pre-SCM cows was higher than the CON counterparts at –8 wks prepartum only. There were no differences between the pre-SCM cows and CON cows with regard to choline concentrations at –4 wks prepartum. Choline is an essential nutrient that is obtained mainly from the diet but can be synthesized also by the liver via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (Zeisel and Blusztajn, 1994; Reo et al., 2002). Choline has multiple functions including serving as a methyl donor in the one-carbon metabolism important in methylation of DNA, RNA, and proteins (Finkelstein, 1990). There is mounting evidence pinpointing an important role of choline in regulation of immune functions. Indeed, a study conducted by Garcia et al., (2018) reported that the phagocytic and killing capacity of neutrophils *in vitro* decreased with rising doses of choline. On the other hand, the proliferation of lymphocytes

was increased linearly with the increased dose of choline in the medium. The same authors also reported that, increasing doses of choline in the medium increased expression of genes involved in the synthesis of choline metabolites including betaine, phosphatidylcholine, and acetylcholine. Another study by Vailati-Riboni et al. (2017) proved that feeding rumen protected choline to peripartal dairy cows enhanced the killing capability of monocytes but not neutrophils. Choline has shown, *in vitro,* to improve the antioxidant balance and lower inflammation by targeting and downregulating various genes related to the inflammatory response in neonatal Holstein neutrophils (Abdelmegeid et al., 2017). Increased choline in the serum of pre-SCM cows might serve the host to keep the inflammation under control.

Serum betaine was found to be higher in pre-SCM cows versus CON cows. Betaine is a by-product of choline metabolism that provides one-carbon units in the conversion of homocysteine (Hcy) to methionine and production of the methyl donor S-adenosylmethionine (Zeisel, 1981). Earlier studies have shown that betaine treatment inhibits NLRP3 inflammasome-related proteins including NLRP3 and caspase-1 and the pro-inflammatory cytokine IL-1 in elderly people (Go et al., 2007). Additionally, betaine was shown to decrease the activation of NF-kB, in elderly people (Go et al., 2005). By suppressing NF-kB, betaine also blocks the expression of genes involved in inflammation, such as IL-1, COX-2, and iNOS (Monaco et al., 2004; Go et al., 2005). It is obvious that production and release of betaine in large quantities in the blood serves the host to keep the inflammatory response under control.

Two other methyl donor metabolites measured and quantified in the serum were glycine and serine. Both of them were lowered in pre-SCM cows at different time points prepartum with glycine lowered only at –8 wks and serine at –4 wks. Glycine is a non-

essential AA with multiple physiological functions. Moreover, glycine is a bioactive AA that contributes in the regulation of gene expression (Luka et al., 2002), protein configuration and activity (Martínez-Chantar et al., 2008), and glutathione synthesis (Durkin and Friedberg, 1952). Besides its physiological functions, mounting evidence indicates that glycine plays significant roles in regulation of immune responses. For example, glycine has been shown to suppress ROS formation by inhibiting the activation of macrophages (Zhong et al., 1999). This lowers the activation of transcription factors inhibiting the production of proinflammatory cytokines. Glycine also has been proved to inhibit activation of NF-kB in various disease models in rats (Mauriz et al., 2001). Another study found that glycine decreases TNF and expression of IL-1 but at the same time increases IL-10 in monocytes (Spittler et al., 1999).

A recent study conducted by He et al. (2019) demonstrated that mice infected with the Gram-negative bacterium *Pasteurella multocida* had lower blood serine levels. Moreover, supplementation with serine increased the survival rate among infected mice and decreased colonization of *P. multocida* in the lungs of infected mice. The most interesting finding was that the intranasal supplementation of serine decreased both macrophage and neutrophil-mediated inflammatory responses by decreasing the secretion of IL-1, IL-17, IFN-γ, and TNF. It is obvious that lowered glycine and serine in the serum of pre-SCM cows might support a proinflammatory response of the host during this pre-clinical mastitis conditions.

2.5.4 Changes in carbohydrate and organic acids in the blood of pre-SCM cows

The concentration of glucose in the serum of pre-SCM cows was higher at both time points measured in this study. Besides its major role as an energy provider for various cells in the body, there is mounting evidence that glucose plays significant roles in immune cell functions. It has been shown that neutrophils use large amounts of glucose through glycolysis (Mowat and Baum, 1971). They also store glucose in the form of glycogen and use it during the process of phagocytosis by releasing glucose (Scott, 1968; Borregaard and Herlin, 1982). Additionally, more recent research has shown that glucose uptake and a metabolic shift from oxidative phosphorylation to glycolysis is crucial for neutrophils and M1 macrophages during bacterial infections and the production of lactate (Loftus and Finlay, 2016; Faas and de Vos, 2020). Neutrophils are the most predominant cells in the mammary gland during both subclinical and clinical mastitis. They are mostly glycolytic cells that produce ROS through the cytosolic enzyme NOX (nicotinamide adenine dinucleotide phosphate-oxidase). This process is essential for pathogen killing and the regulation of inflammation. A recent study reported that induction of inflammation by administration of LPS is associated with increased glucose uptake by neutrophils, higher production of ROS, and downregulation of genes related to the TCA cycle (Khatib-Massalha et al., 2020). Therefore, increased blood glucose in pre-SCM cows might reflect the host response to support the activity of immune cells during the dry off period.

In the pre-SCM cohort of cows, the concentration of lactate exhibited a similar trend seen for glucose, being higher in pre-SCM cows compared to CON at –4 wks prepartum. This finding is in line with a previous report from our lab of higher concentrations of lactate in pre-SCM cows, at both –8 and –4 wks prepartum (Dervishi et

al., 2015). Lactate has been shown to support phagocytic activity and as an extracellular trap for the formation of neutrophils (Borregaard and Herlin, 1982; Awasthi et al., 2019). Higher blood lactate levels have also been reported in mice treated intraperitoneally with LPS, deriving mainly from neutrophils expediting glycolysis and producing more lactate (Khatib-Massalha et al., 2020). Interestingly, exogenous administration of lactate in mice was reported to stimulate extravasation of neutrophils from the bone marrow into the blood circulation as well as to increase vascular permeability by decreasing the expression of VE-cadherin receptors (Khatib-Massalha et al., 2020). In dairy cows, D-lactate was reported to increase neutrophil adhesion to the endothelial cells through increased expression of neutrophil extracellular traps (NET) and CD11b/ICAM-1 (Alarcón et al., 2017). The latter proteins are cell surface receptors that facilitate migration of neutrophils to the site of inflammation. These data suggest that elevated blood glucose and lactate might be a host response to support activities of immune cells during the dry off period.

Another altered metabolite related to metabolism of carbohydrates and aerobic glycolysis was pyruvate. Pyruvate was found elevated in pre-SCM cows at both time periods studied. Pyruvate participates in several metabolic pathways including glycolysis, intracellular and mitochondrial amino acid turnover, and the TCA cycle. It has also been shown to play a role in neutrophil functions (Mathioudakis et al., 2011). This intermediate of glucose metabolism has been shown to have anti-inflammatory and antioxidant properties, by inhibiting TNF and NF-κB expression in animal models of endotoxemia (Das, 2006). Similar findings were reported from LPS-stimulated canine PBMCs. In this study, authors observed downregulation of mRNA expression for IL-6 and TNF, with enhanced expression of IL-10 (Yu et al., 2010). In a recent study, Zwaag et al. (2020)

demonstrated mitigation of the inflammation induced by LPS treatment by increasing concentrations of both pyruvate and lactate in the blood. Both metabolites induced production of anti-inflammatory IL-10 in PBMCs and diminished secretion of proinflammatory cytokines IL-1 and IL-6 (Zwaag et al., 2020). These results suggest that pyruvate might be one of the metabolites that lowers the anti-inflammatory response to keep down the over-response of the host to inflammatory stimuli.

Another important finding of this study was higher blood trimethylamine-N-oxide (TMAO), which differentiated the pre-SCM cows from healthy cows. This is a molecule that derives from trimethylamine (TMA), produced when gut microbiota metabolizes choline, and choline-containing compounds, such as betaine, and carnitine (Zeisel and Warrier, 2017). Once in the liver, TMA is converted to TMAO. TMAO has been shown to induce pathological changes to endothelial cells that increase the adhesion of monocytes and leukocytes to blood vessels (Seldin et al., 2016; Ma et al., 2017). A growing body of evidence has shown that human subjects with high blood TMAO are in a state of systemic inflammation (Missailidis et al., 2016; Rohrmann et al., 2016). On the other hand, Chan et al. (2019) suggested TMAO to be a danger-associated molecular pattern (DAMP) that interacts with the host's PRR (pattern-recognition receptors), activating the innate immune response (Chan et al., 2019). It seems that TMAO is a metabolite that supports the mounting of an inflammatory response and supports migration of immune cells to the site of infection.

2.6 Conclusions

In summary, the results from this study indicate that pre-SCM cows are undergoing alterations in the serum metabolites during the dry-off period, more particularly at –8 and –4 wks prior to diagnosis of SCM. Many of the differences between pre-SCM and CON cows were related to lipid, amino acid, carbohydrate, and organic acid metabolism. Specifically, multiple species of PCs, LPCs, and SMs were lowered in pre-SCM cows, whereas ACs were higher. Essential AAs, such as BCAA and nutrients such as choline and betaine were higher in the blood of pre-SCM cows, whereas glycine, serine, and alanine were lower. Several metabolites linked in the carbohydrate metabolism including glucose, lactate, and pyruvate were elevated. Overall, these changes might be related to the presence of chronic low-grade inflammatory state in pre-SCM cows. We speculate that alterations in metabolites with pro-inflammatory properties, such as BCAA, alanine, glucose and lactate, among others, are happening to support the inflammatory response and fight those stimuli. On the other hand, compounds such as PCs, glycine, serine, choline, and betaine might play a role in keeping the inflammatory response under control or resolving it. Multivariate analysis (PCA, PLS-DA) demonstrated two clearly separated clusters of healthy and pre-SCM cows. These analyses allowed the development of two panels of metabolites with high accuracy to serve as potential predictive biomarkers for identifying cows at risk of SCM during the dry-off period. Finally, all the panels of biomarkers identified need to be validated in a larger cohort of dairy cows prior to developing pen-side tests.

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Table 2.1. Ingredients of the prepartum diet for the dry off cows.

Ingredient	Weight/cow (kg)	DM (%)	Final DMI (kg)[1]
Hay	5.50	85.14%	4.68
Oats	5.75	36.20%	2.08
Corn	8.84	30.30%	2.68
Protein	2.00	93.00%	1.86
Ground Barley	0.75	97.26%	0.66
Minerals	0.42	97.26%	0.41
Total	23.36	53.17%	12.37

[1]Dry Matter Intake (DMI) is calculated based on the DM% over the offered amount (kg) of feed. Daily DMI is formulated to 2% of cow's body weight.

Table 2.2. Feed ingredients of cows during early lactation.

Ingredient	Weight/cow (kg)	DM (%)	Final DMI (kg)
Hay dairy	2.50	88.50	2.21
Grass silage	10.75	31.80	3.42
Oats	5.99	36.20	2.17
Barley-Dakota	11.50	40.00	4.80
Corn	13.52	31.50	4.26
Whey	2.75	17.00	0.47
Protein	4.75	93.30	4.43
Energy dairy	4.25	88.00	3.74
Ground Barley	1.75	88.00	1.54
Mineral & Fat	1.26	97.26	1.23
Total	59.02	47.56	28.07

Table 2.3. Concentration of serum metabolites (MEAN ± SEM) in pre-subclinical mastitis cows only (pre-SCM, n=10) and healthy controls (CON, n=15) cows at –8 wks before parturition, as identified by LC-MS/MS.

	MEAN ± SEM				
Metabolites (μM)	Pre-SCM[1] (n=10)	CON[2] (n=15)	*P* value	Fold change	SCM/ CON
Creatinine	72.2 ± 4.65	74.4 ± 4.09	0.1	0.97	down
Glycine	317 ± 22.8	372 ± 20	0.005	0.85	down
Alanine	215 ± 15.7	266 ± 13.8	0.004	0.81	down
Serine	76.2 ± 7.76	86.8 ± 6.83	0.09	0.88	down
Proline	98.7 ± 9.07	111.1 ± 8.42	0.2	0.89	down
Valine	250 ± 26.3	202 ± 23.2	0.03	1.24	up
Threonine	108.2 ± 8.94	93.6 ± 7.87	0.15	1.16	up
Taurine	82.3 ± 9.43	71.7 ± 8.3	0.5	1.15	up
trans-Hydroxyproline	10.9 ± 0.671	12 ± 0.591	0.03	0.91	down
Leucine	248 ± 21.2	183 ± 18.7	0.002	1.36	up
Isoleucine	137 ± 11.24	111 ± 9.89	0.03	1.23	up
Asparagine	27.4 ± 2.67	32.1 ± 2.35	0.04	0.85	down
Aspartic acid	7.57 ± 0.951	7.74 ± 0.836	0.7	0.98	down
Glutamine	281 ± 15.4	263 ± 13.6	0.3	1.07	up
Glutamic acid	70 ± 5.43	65.3 ± 4.78	0.4	1.07	up
Methionine	22.6 ± 1.75	20.5 ± 1.54	0.2	1.10	up
Histidine	56.8 ± 4.13	51.7 ± 3.63	0.1	1.10	up
alpha-Aminoadipic acid	2.79 ± 0.387	1.74 ± 0.34	0.02	1.60	up
Phenylalanine	56.5 ± 3.36	46.8 ± 2.95	0.01	1.21	up
Methionine-sulfoxide	1.8 ± 0.241	2.35 ± 0.212	0.02	0.77	down
Arginine	149 ± 9.19	123 ± 8.09	0.05	1.21	up
Acetyl-ornithine	4.58 ± 0.609	4.17 ± 0.535	0.4	1.10	up
Citrulline	91.1 ± 8.57	87.6 ± 7.53	0.6	1.04	up
Serotonin	12.01 ± 2.32	7.44 ± 2.04	0.1	1.61	up
Tyrosine	69.8 ± 6.39	66.1 ± 5.62	0.4	1.06	up
Asymmetric dimethyl arginine	0.875 ± 0.0686	0.647 ± 0.0603	0.002	1.35	up
Total dimethylarginine	1.89 ± 0.174	1.85 ± 0.153	0.9	1.02	up
Tryptophan	42.4 ± 2.86	44.8 ± 2.52	0.2	0.95	down
Kynurenine	7.21 ± 1.108	7.79 ± 0.974	0.2	0.93	down
Carnosine	14 ± 2.27	24.3 ± 1.99	0.001	0.58	down
Ornithine	62.6 ± 6.02	49.2 ± 5.58	0.001	1.27	up
Lysine	88.5 ± 8.09	72.1 ± 7.12	0.01	1.23	up
Spermidine	0.354 ± 0.0514	0.325 ± 0.0452	0.6	1.09	up
Creatine	228 ± 12.1	241 ± 10.6	0.08	0.95	down

Betaine	154.1 ± 20.6	76.6 ± 19.1	<0.001	2.01	up
Choline	15.3 ± 1.85	10.3 ± 1.63	0.01	1.49	up
Trimethylamine N-oxide	47.3 ± 15.7	38.7 ± 13.8	0.9	1.22	up
Methyl histidine	8.33 ± 0.892	11.32 ± 0.784	0.06	0.74	down
Lactic acid	2270 ± 330	1895 ± 290	0.1	1.20	up
beta-Hydroxybutyric acid	730 ± 148	781 ± 131	0.3	0.93	down
alpha-Ketoglutaric acid	25.3 ± 3.91	24.6 ± 3.44	0.7	1.03	up
Citric acid	218 ± 26.5	267 ± 23.3	0.02	0.82	down
Butyric acid	7.07 ± 3.45	13.92 ± 3.03	0.01	0.51	down
Propionic acid	16.2 ± 7.29	29.6 ± 6.41	0.05	0.55	down
Succinic acid	1.58 ± 0.148	1.62 ± 0.13	0.4	0.98	down
Fumaric acid	1.23 ± 0.36	1.84 ± 0.316	0.04	0.67	down
Pyruvic acid	77.3 ± 7.9	62.9 ± 6.95	0.03	1.23	up
Isobutyric acid	3.41 ± 0.89	4.72 ± 0.782	0.1	0.72	down
Hippuric acid	57.5 ± 4.79	64.4 ± 4.21	0.05	0.89	down
Methylmalonic acid	0.569 ± 0.0736	0.547 ± 0.0647	0.9	1.04	up
Indole acetic acid	0.455 ± 0.0988	0.555 ± 0.0869	0.1	0.82	down
Uric acid	30 ± 5.21	37.9 ± 4.58	0.1	0.79	down
Glucose	3602 ± 144	3462 ± 127	0.8	1.04	up
LYSOC14:0	0.994 ± 0.1045	1.327 ± 0.0919	<0.001	0.75	down
LYSOC16:1	1.37 ± 0.153	1.71 ± 0.135	0.004	0.80	down
LYSOC16:0	27 ± 2.93	29.5 ± 2.58	0.05	0.92	down
LYSOC17:0	1.58 ± 0.172	1.65 ± 0.151	0.1	0.96	down
LYSOC18:2	30.2 ± 3.65	41.9 ± 3.21	<0.001	0.72	down
LYSOC18:1	13.4 ± 1.67	18.3 ± 1.47	<0.001	0.73	down
LYSOC18:0	17 ± 1.92	19.6 ± 1.68	0.01	0.87	down
LYSOC20:4	2.33 ± 0.262	2.6 ± 0.231	0.1	0.90	down
LYSOC20:3	3.23 ± 0.376	3.66 ± 0.331	0.1	0.88	down
LYSOC24:0	0.0979 ± 0.01118	0.1123 ± 0.00983	0.1	0.87	down
LYSOC26:1	0.0462 ± 0.0078	0.0628 ± 0.00686	0.003	0.74	down
LYSOC26:0	0.12 ± 0.0323	0.161 ± 0.0284	0.02	0.75	down
LYSOC28:1	0.298 ± 0.0445	0.519 ± 0.0391	<0.001	0.57	down
LYSOC28:0	0.234 ± 0.0346	0.373 ± 0.0305	<0.001	0.63	down
PC32:2AA	8.69 ± 1.25	14.93 ± 1.1	<0.001	0.58	down
PC36:0AE	2.24 ± 0.27	4.03 ± 0.237	<0.001	0.56	down

PC36:6AA	3.08 ± 0.336	4.08 ± 0.295	<0.001	0.75	down
PC36:0AA	11.5 ± 1.79	25.4 ± 1.57	<0.001	0.45	down
PC38:6AA	2.95 ± 0.291	4.82 ± 0.256	<0.001	0.61	down
PC38:0AA	1.82 ± 0.287	4.02 ± 0.252	<0.001	0.45	down
PC40:6AE	1.89 ± 0.191	2.61 ± 0.168	<0.001	0.72	down
PC40:6AA	1.89 ± 0.191	2.61 ± 0.168	<0.001	0.72	down
PC40:2AA	0.918 ± 0.14	2.062 ± 0.123	<0.001	0.45	down
PC40:1AA	0.312 ± 0.0317	0.495 ± 0.0279	<0.001	0.63	down
16:1SM	14.5 ± 1.2	17.8 ± 1.06	<0.001	0.81	down
16:0SM	128 ± 12.2	160 ± 10.8	<0.001	0.80	down
18:1SM	22.6 ± 1.91	29.3 ± 1.68	<0.001	0.77	down
18:0SM	20.6 ± 1.88	27.7 ± 1.66	<0.001	0.74	down
20:2SM	2.46 ± 0.258	3.52 ± 0.227	<0.001	0.70	down
14:1SMOH	11.6 ± 1.18	14.2 ± 1.04	0.002	0.82	down
16:1SMOH	13.7 ± 1.26	17.4 ± 1.11	<0.001	0.79	down
22:2SMOH	10.9 ± 1.042	14.7 ± 0.917	<0.001	0.74	down
22:1SMOH	21.4 ± 2.56	30.8 ± 2.26	<0.001	0.69	down
24:1SMOH	2.54 ± 0.204	3.35 ± 0.179	<0.001	0.76	down
C0	4.23 ± 0.443	3.04 ± 0.389	0.1	1.39	up
C2	1.74 ± 0.15	1.46 ± 0.132	0.8	1.19	up
C3	0.191 ± 0.0175	0.178 ± 0.0154	0.2	1.07	up
C3OH	0.0184 ± 0.00159	0.0178 ± 0.0014	0.5	1.03	up
C3:1	0.0292 ± 0.00278	0.0301 ± 0.00245	0.6	0.97	down
C4	0.093 ± 0.00793	0.0796 ± 0.00697	0.2	1.17	up
C4OH	0.0219 ± 0.00315	0.0328 ± 0.00277	0.001	0.67	down
C4:1	0.0155 ± 0.00153	0.0164 ± 0.00134	0.4	0.95	down
C5	0.0617 ± 0.00617	0.0648 ± 0.00542	0.5	0.95	down
C5OH	0.065 ± 0.00942	0.0629 ± 0.00828	0.6	1.03	up
C5:1	0.0159 ± 0.00131	0.0172 ± 0.00115	0.08	0.92	down
C5:1DC	0.0159 ± 0.00186	0.0189 ± 0.00164	0.01	0.84	down
C5MDC	0.0189 ± 0.000967	0.0203 ± 0.000851	0.09	0.93	down
C6	0.0411 ± 0.00578	0.0516 ± 0.00509	0.3	0.80	down

C5DC/C6OH	0.0106 ± 0.001101	0.0118 ± 0.000968	0.05	0.90	down
C6:1	0.0239 ± 0.00224	0.0296 ± 0.00197	0.006	0.81	down
C8	0.0184 ± 0.00192	0.0114 ± 0.00169	0.009	1.61	up
C9	0.00766 ± 0.000669	0.00777 ± 0.000589	0.2	0.99	down
C10	0.0481 ± 0.00471	0.0467 ± 0.00414	0.4	1.03	up
C10:1	0.093 ± 0.00774	0.0989 ± 0.0068	0.4	0.94	down
C10:2	0.0246 ± 0.00258	0.0236 ± 0.00227	0.9	1.04	up
C12	0.027 ± 0.00204	0.0216 ± 0.0018	0.1	1.25	up
C12:1	0.0476 ± 0.003	0.053 ± 0.00264	0.3	0.90	down
C12DC	0.018 ± 0.00181	0.0182 ± 0.00159	0.4	0.99	down
C14	0.0252 ± 0.00314	0.0182 ± 0.00276	0.2	1.38	up
C14:1	0.0448 ± 0.00883	0.0677 ± 0.00777	0.1	0.66	down
C14:1OH	0.00859 ± 0.000792	0.00985 ± 0.000697	0.01	0.87	down
C14:2	0.00996 ± 0.000707	0.00856 ± 0.000622	0.4	1.16	up
C14:2OH	0.01086 ± 0.00103	0.00964 ± 0.00091	0.7	1.13	up
C16	0.0188 ± 0.00183	0.0168 ± 0.00161	0.7	1.12	up
C16OH	0.00798 ± 0.000888	0.0075 ± 0.000781	0.6	1.06	up
C16:1	0.0188 ± 0.000993	0.0184 ± 0.000873	0.7	1.02	up
C16:1OH	0.0134 ± 0.0012	0.0141 ± 0.00105	0.3	0.95	down
C16:2	0.00732 ± 0.000679	0.00698 ± 0.000597	0.9	1.05	up
C16:2OH	0.00668 ± 0.000728	0.00731 ± 0.00064	0.1	0.91	down
C18	0.0408 ± 0.00456	0.0343 ± 0.00401	0.7	1.19	up
C18:1	0.0151 ± 0.00139	0.0146 ± 0.00122	0.8	1.03	up

C18:1OH	0.00863 ± 0.000569	0.00774 ± 0.0005	0.4	1.11	up
C18:2	0.00685 ± 0.000629	0.00694 ± 0.000554	0.6	0.99	down

[1]pre-SCM = SCM = cows that were sampled before being classified as SCM
[2]CON = healthy cows

Table 2.4. Concentration of serum metabolites (MEAN ± SEM) in pre-subclinical mastitis cows only (pre-SCM, n=10) and healthy controls (CON, n=15) cows at –4 wks before parturition, as identified by LC-MS/MS.

	MEAN ± SEM				
Metabolites (μM)	Pre-SCM[1] (n=10)	CON[2] (n=15)	*P* value	Fold change	SCM/ CON
Creatinine	80.3 ± 5.1	88.8 ± 4.6	0.1	0.90	down
Glycine	267 ± 11.6	287 ± 10.5	0.1	0.93	down
Alanine	201 ± 11.6	249 ± 10.5	<0.001	0.81	down
Serine	75 ± 4.17	81.8 ± 3.77	0.03	0.92	down
Proline	82.6 ± 5.4	99.4 ± 4.88	0.002	0.83	down
Valine	275 ± 12.9	311 ± 11.6	0.001	0.88	down
Threonine	103 ± 7.42	102 ± 6.7	0.8	1.01	up
Taurine	66.2 ± 7.6	75 ± 6.86	0.08	0.88	down
trans-Hydroxyproline	12.5 ± 1.33	14.4 ± 1.2	0.06	0.87	down
Leucine	241 ± 16.5	250 ± 14.9	0.1	0.96	down
Isoleucine	137 ± 5.73	151 ± 5.17	0.005	0.91	down
Asparagine	27.3 ± 1.88	31.9 ± 1.7	0.01	0.86	down
Aspartic acid	7.05 ± 1.22	7.95 ± 1.1	0.3	0.89	down
Glutamine	324 ± 14.9	341 ± 13.5	0.1	0.95	down
Glutamic acid	63.3 ± 6.55	67.6 ± 5.91	0.5	0.94	down
Methionine	27.2 ± 1.38	31.3 ± 1.25	<0.001	0.87	down
Histidine	67.5 ± 2.76	73.9 ± 2.5	0.005	0.91	down
alpha-Aminoadipic acid	2.83 ± 0.505	2.2 ± 0.456	0.4	1.29	up
Phenylalanine	65.4 ± 2.33	66.7 ± 2.1	0.06	0.98	down
Methionine-sulfoxide	2.39 ± 0.222	2.99 ± 0.2	<0.001	0.80	down
Arginine	154 ± 7.42	157 ± 6.7	0.1	0.98	down
Acetyl-ornithine	2.86 ± 0.463	4.05 ± 0.418	0.01	0.71	down
Citrulline	84.6 ± 6.83	89.7 ± 6.17	0.3	0.94	down
Serotonin	5.94 ± 3.21	5.95 ± 2.9	0.5	1.00	down
Tyrosine	67 ± 5.13	66.1 ± 4.64	0.4	1.01	up
Asymmetric dimethylarginine	0.962 ± 0.076	1.092 ± 0.0686	0.1	0.88	down
Total dimethylarginine	1.97 ± 0.17	2.23 ± 0.154	0.3	0.88	down
Tryptophan	45.7 ± 2.24	47.1 ± 2.03	0.1	0.97	down

Kynurenine	7.16 ± 0.759	6.51 ± 0.686	0.5	1.10	up
Carnosine	10.4 ± 1.7	13.5 ± 1.53	0.08	0.77	down
Ornithine	59.9 ± 3.44	65.6 ± 3.1	0.03	0.91	down
Lysine	91.2 ± 8.47	107.2 ± 7.65	0.04	0.85	down
Spermidine	0.419 ± 0.0553	0.351 ± 0.05	0.7	1.19	up
Creatine	233 ± 12.2	244 ± 11	0.2	0.95	down
Betaine	155 ± 14.3	162 ± 13	0.2	0.96	down
Choline	10.6 ± 1.93	13.1 ± 1.74	0.1	0.81	down
Trimethylamine N-oxide	49.8 ± 14.8	19.3 ± 13.4	0.1	2.58	up
Methyl histidine	11.1 ± 1.7	14.2 ± 1.53	0.07	0.78	down
Lactic acid	2107 ± 409	1166 ± 370	0.03	1.81	up
beta-Hydroxybutyric acid	630 ± 100.3	618 ± 90.6	0.7	1.02	up
alpha-Ketoglutaric acid	19.5 ± 1.9	16.3 ± 1.72	0.2	1.20	up
Citric acid	310 ± 40.2	289 ± 36.3	0.7	1.07	up
Butyric acid	5.42 ± 0.986	6.76 ± 0.891	0.06	0.80	down
Propionic acid	19.4 ± 3.11	18.1 ± 2.81	0.8	1.07	up
Succinic acid	1.33 ± 0.0757	1.09 ± 0.0684	0.1	1.22	up
Fumaric acid	1.137 ± 0.188	0.912 ± 0.17	0.4	1.25	up
Pyruvic acid	82.7 ± 7.58	71.7 ± 6.85	0.03	1.15	up
Isobutyric acid	4.49 ± 0.642	4.84 ± 0.58	0.4	0.93	down
Hippuric acid	67.7 ± 6.24	70 ± 5.64	0.6	0.97	down
Methylmalonic acid	0.545 ± 0.0762	0.285 ± 0.0688	0.01	1.91	up
Indole acetic acid	0.39 ± 0.0578	0.382 ± 0.0522	0.6	1.02	up
Uric acid	26.8 ± 9.88	35.2 ± 8.93	0.3	0.76	down
Glucose	4928 ± 99.5	4045 ± 89.9	0.03	1.22	up
LYSOC14:0	0.794 ± 0.0798	0.854 ± 0.0721	0.2	0.93	down
LYSOC16:1	1.08 ± 0.14	1.11 ± 0.126	0.5	0.97	down
LYSOC16:0	16.4 ± 1.74	17.5 ± 1.57	0.1	0.94	down
LYSOC17:0	1.4 ± 0.127	1.29 ± 0.115	0.9	1.09	up
LYSOC18:2	19.1 ± 2.17	20.5 ± 1.96	0.2	0.93	down
LYSOC18:1	9.62 ± 1.29	10.98 ± 1.16	0.1	0.88	down
LYSOC18:0	15.8 ± 1.43	16.1 ± 1.29	0.2	0.98	down
LYSOC20:4	1.93 ± 0.262	1.75 ± 0.237	0.8	1.10	up
LYSOC20:3	2.97 ± 0.28	3.41 ± 0.253	0.03	0.87	down
LYSOC24:0	0.111 ± 0.00934	0.12 ± 0.00843	0.4	0.93	down
LYSOC26:1	0.0387 ± 0.00579	0.0369 ± 0.00523	0.9	1.05	up

LYSOC26:0	0.1028 ± 0.0113	0.0938 ± 0.0102	0.5	1.10	up
LYSOC28:1	0.243 ± 0.0312	0.35 ± 0.0282	0.001	0.69	down
LYSOC28:0	0.246 ± 0.0213	0.251 ± 0.0192	0.3	0.98	down
PC32:2AA	7.63 ± 0.844	12.45 ± 0.762	<0.001	0.61	down
PC36:0AE	2.22 ± 0.219	3.48 ± 0.198	<0.001	0.64	down
PC36:6AA	2.53 ± 0.265	3.87 ± 0.24	<0.001	0.65	down
PC36:0AA	7.65 ± 0.807	14.3 ± 0.729	<0.001	0.53	down
PC38:6AA	2.21 ± 0.217	3.55 ± 0.196	<0.001	0.62	down
PC38:0AA	0.985 ± 0.106	1.944 ± 0.096	<0.001	0.51	down
PC40:6AE	0.719 ± 0.0704	1.147 ± 0.0636	<0.001	0.63	down
PC40:6AA	1.46 ± 0.194	2.43 ± 0.175	<0.001	0.60	down
PC40:2AA	0.543 ± 0.0602	1.02 ± 0.0544	<0.001	0.53	down
PC40:1AA	0.256 ± 0.0278	0.415 ± 0.0251	<0.001	0.62	down
16:1SM	10.2 ± 1.073	11.5 ± 0.969	0.1	0.89	down
16:0SM	88 ± 9.99	104 ± 9.02	0.06	0.85	down
18:1SM	17 ± 1.39	19.1 ± 1.25	0.02	0.89	down
18:0SM	14.4 ± 1.51	18.1 ± 1.36	0.01	0.80	down
20:2SM	2.41 ± 0.196	2.81 ± 0.177	0.006	0.86	down
14:1SMOH	8.67 ± 1.042	9.55 ± 0.941	0.2	0.91	down
16:1SMOH	9.1 ± 0.963	10.5 ± 0.87	0.08	0.87	down
22:2SMOH	7.5 ± 0.881	10.2 ± 0.795	0.007	0.74	down
22:1SMOH	13.1 ± 1.76	18.3 ± 1.59	0.008	0.72	down
24:1SMOH	1.93 ± 0.22	2.44 ± 0.199	0.03	0.79	down
C0	5.1 ± 0.541	4.88 ± 0.488	0.5	1.05	up
C2	1.7 ± 0.223	1.99 ± 0.202	0.1	0.85	down
C3	0.203 ± 0.0173	0.191 ± 0.0157	0.6	1.06	up
C3OH	0.0254 ± 0.0026	0.0215 ± 0.00235	0.6	1.18	up
C3:1	0.0233 ± 0.0023	0.0217 ± 0.00208	0.4	1.07	up
C4	0.123 ± 0.00729	0.116 ± 0.00658	0.4	1.06	up
C4OH	0.0317 ± 0.00354	0.0266 ± 0.0032	0.3	1.19	up
C4:1	0.0198 ± 0.00229	0.0138 ± 0.00206	0.02	1.43	up
C5	0.074 ± 0.00673	0.0756 ± 0.00608	0.4	0.98	down

C5OH	0.0664 ± 0.0117	0.0518 ± 0.0105	0.2	1.28	up
C5:1	0.0217 ± 0.00205	0.0134 ± 0.00185	0.01	1.62	up
C5:1DC	0.0156 ± 0.00167	0.0114 ± 0.00151	0.03	1.37	up
C5MDC	0.017 ± 0.00159	0.0153 ± 0.00143	0.2	1.11	up
C6	0.0342 ± 0.00413	0.0328 ± 0.00373	0.6	1.04	up
C5DC/C6OH	0.01717 ± 0.00351	0.00681 ± 0.00317	0.04	2.52	up
C6:1	0.0214 ± 0.00297	0.0212 ± 0.00268	0.8	1.01	up
C8	0.0133 ± 0.00118	0.0111 ± 0.00106	0.1	1.20	up
C9	0.0222 ± 0.00547	0.0047 ± 0.00494	0.02	4.72	up
C10	0.0448 ± 0.00465	0.035 ± 0.0042	0.2	1.28	up
C10:1	0.129 ± 0.0184	0.117 ± 0.0166	0.6	1.10	up
C10:2	0.0255 ± 0.00302	0.0178 ± 0.00273	0.05	1.43	up
C12	0.0271 ± 0.00252	0.0199 ± 0.00228	0.02	1.36	up
C12:1	0.057 ± 0.00723	0.054 ± 0.00653	0.9	1.06	up
C12DC	0.0103 ± 0.000992	0.0095 ± 0.000896	0.5	1.08	up
C14	0.0129 ± 0.00117	0.0139 ± 0.00106	0.3	0.93	down
C14:1	0.0363 ± 0.00652	0.0532 ± 0.00589	0.008	0.68	down
C14:1OH	0.00776 ± 0.000576	0.00738 ± 0.00052	0.4	1.05	up
C14:2	0.00849 ± 0.001064	0.0083 ± 0.000961	0.7	1.02	up
C14:2OH	0.00867 ± 0.000547	0.00693 ± 0.000494	0.04	1.25	up
C16	0.0207 ± 0.00159	0.0254 ± 0.00144	0.01	0.81	down
C16OH	0.00538 ± 0.000716	0.00662 ± 0.000647	0.1	0.81	down
C16:1	0.0161 ± 0.00118	0.017 ± 0.00106	0.2	0.95	down

C16:1OH	0.00883 ± 0.000583	0.01033 ± 0.00052	0.01	0.85	down
C16:2	0.00611 ± 0.000575	0.00583 ± 0.00052	0.7	1.05	down
C16:2OH	0.00709 ± 0.000683	0.007 ± 0.000617	0.5	1.01	up
C18	0.0222 ± 0.00324	0.0303 ± 0.00293	0.02	0.73	down
C18:1	0.0113 ± 0.00207	0.0179 ± 0.00187	0.008	0.63	down
C18:1OH	0.01052 ± 0.00142	0.00982 ± 0.00128	0.8	1.07	up
C18:2	0.00602 ± 0.000733	0.00548 ± 0.000662	0.8	1.10	up

[1]pre-SCM = SCM = cows that were sampled before being classified as SCM
[2]CON = healthy cows

Table 2.5. Significant metabolic pathways identified from quantitative enrichment analysis and involved prior to occurrence of subclinical mastitis at –8 and –4 wks before calving.

Metabolic pathways	Total compounds	Hits	Significant metabolites	Holm p-value
Glycine and Serine metabolism[a]	59	12	Betaine; Ornithine; Glycine; L-Alanine; Pyruvic acid; Creatine; L-Serine; L-Arginine; L-Threonine; L-Methionine; L-Glutamic acid; Oxoglutaric acid	0.004
Methionine metabolism[a]	43	7	Betaine; Choline; Glycine; Methionine sulfoxide; L-Serine; L-Methionine; Spermidine	0.01
Betaine metabolism[a]	21	3	Betaine; Choline; Methionine	0.02
Glucose-Alanine Cycle[b]	13	5	D-Glucose; L-Glutamic acid; L-Alanine; Oxoglutaric acid; Pyruvic acid	0.03
Selenoamino Acid metabolism[b]	28	2	L-Alanine; L-Serine	0.05

[a]Significant metabolic pathway at –8 weeks before parturition
[b]Significant metabolic pathway at –4 weeks before parturition

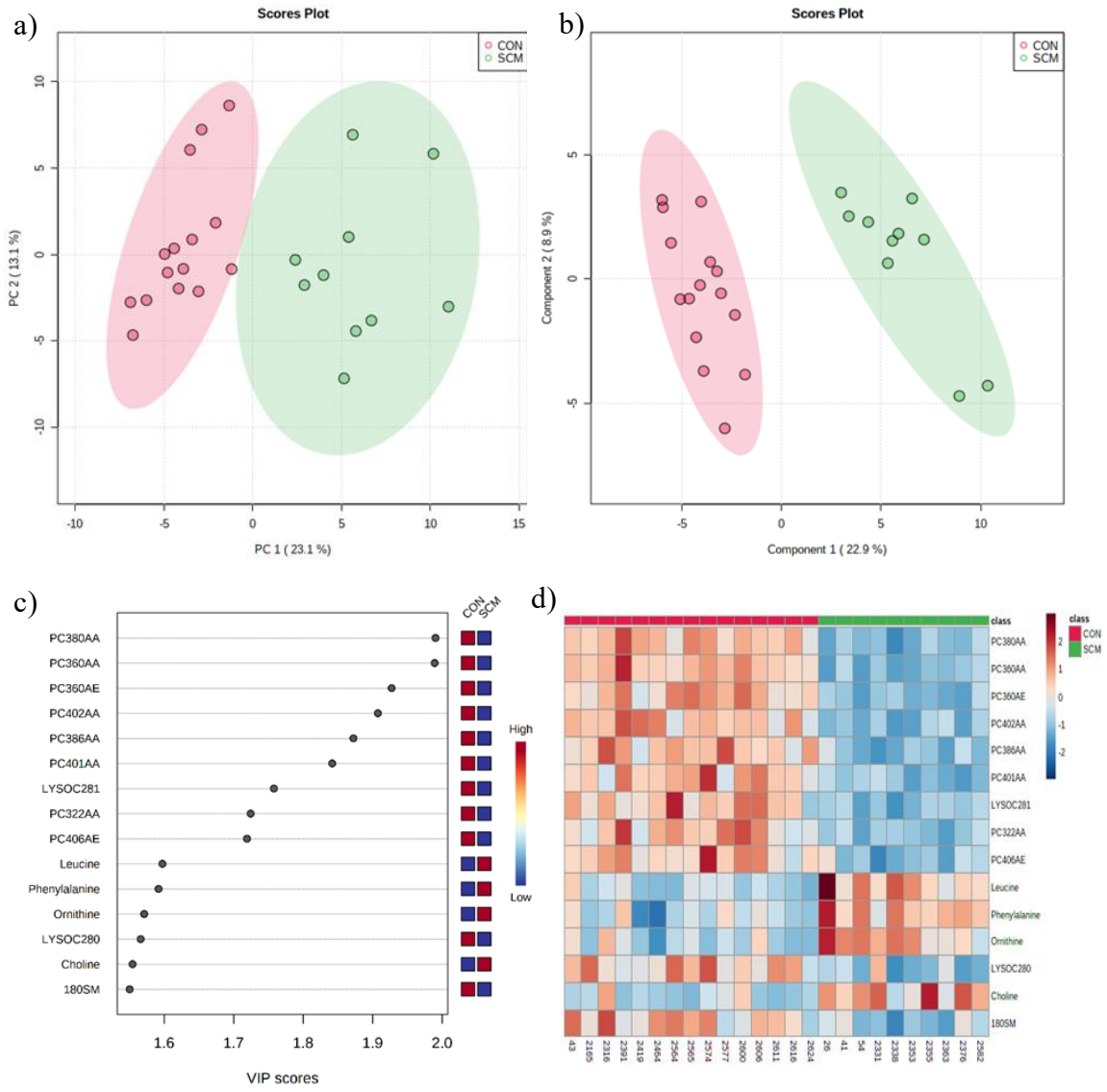


Figure 2.1. a) Principal Component Analysis (PCA) and **b)** Partial Least Square – Discriminant Analysis (PLS-DA; permutation test: $P < 0.05$*)* showing two separate clusters for CON and pre-SCM cows at –8 weeks before parturition; **c)** Metabolites ranked by Variable Importance in Projection (VIP) and **d)** Heatmap of both, samples and features based on PLS-DA, to further investigate the identified variables.

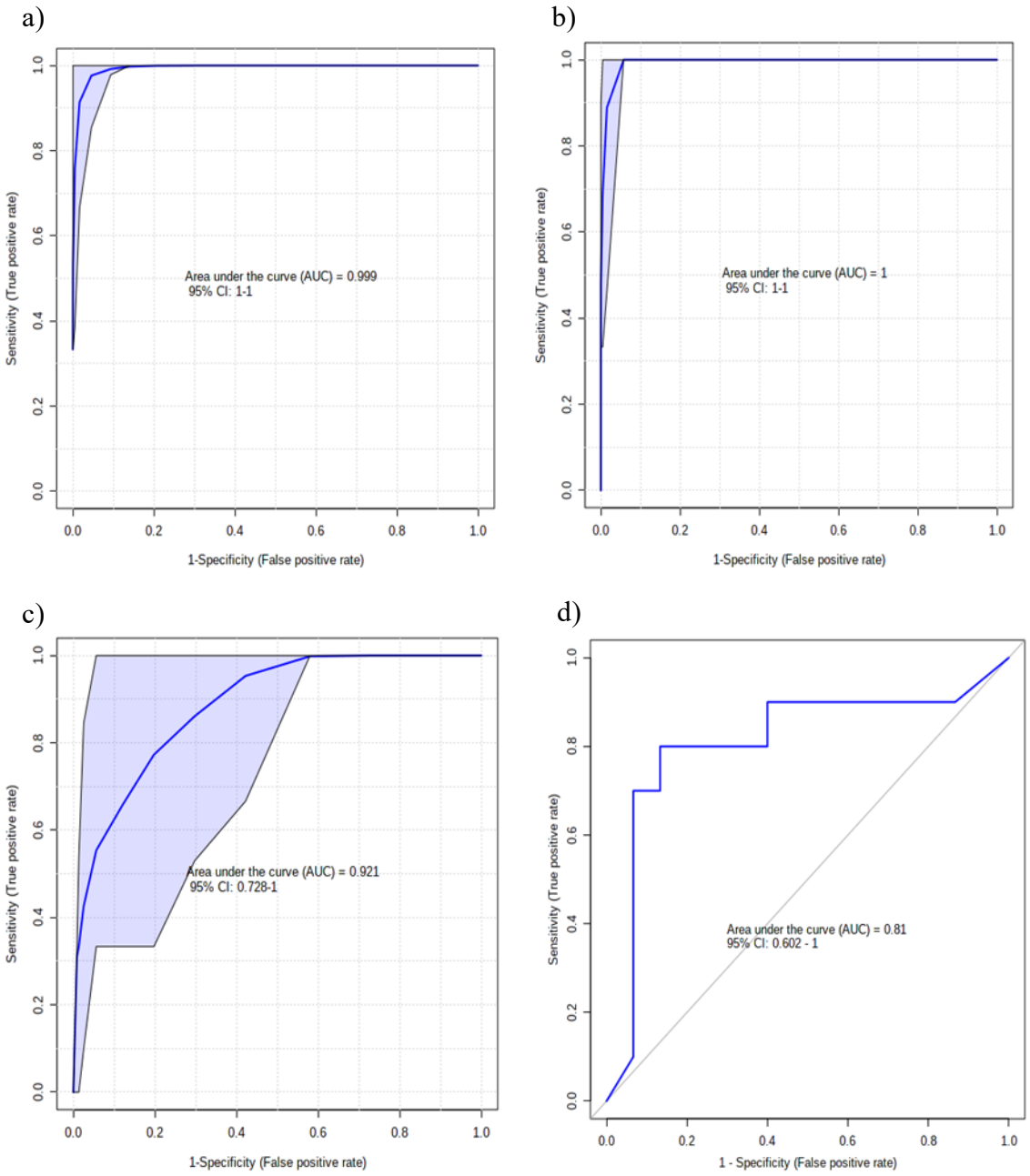


Figure 2.2. a) AUC of the top 5 VIP PLS-DA metabolites (PC aaC38:0, PC aa C36:0, PC ae C36:0, PC aa C40:2, PC aa C38:6); **b)** AUC of model's classification; **c)** default linear SVM AUC of Leu, Betaine, Ala and Orn (AUC = 0.92; $P = 0.001$) and **d)** ROC plot with 10-fold CV for logistic regression of these 4 amino acids (AUC = 0.81; $P = 0.05$).

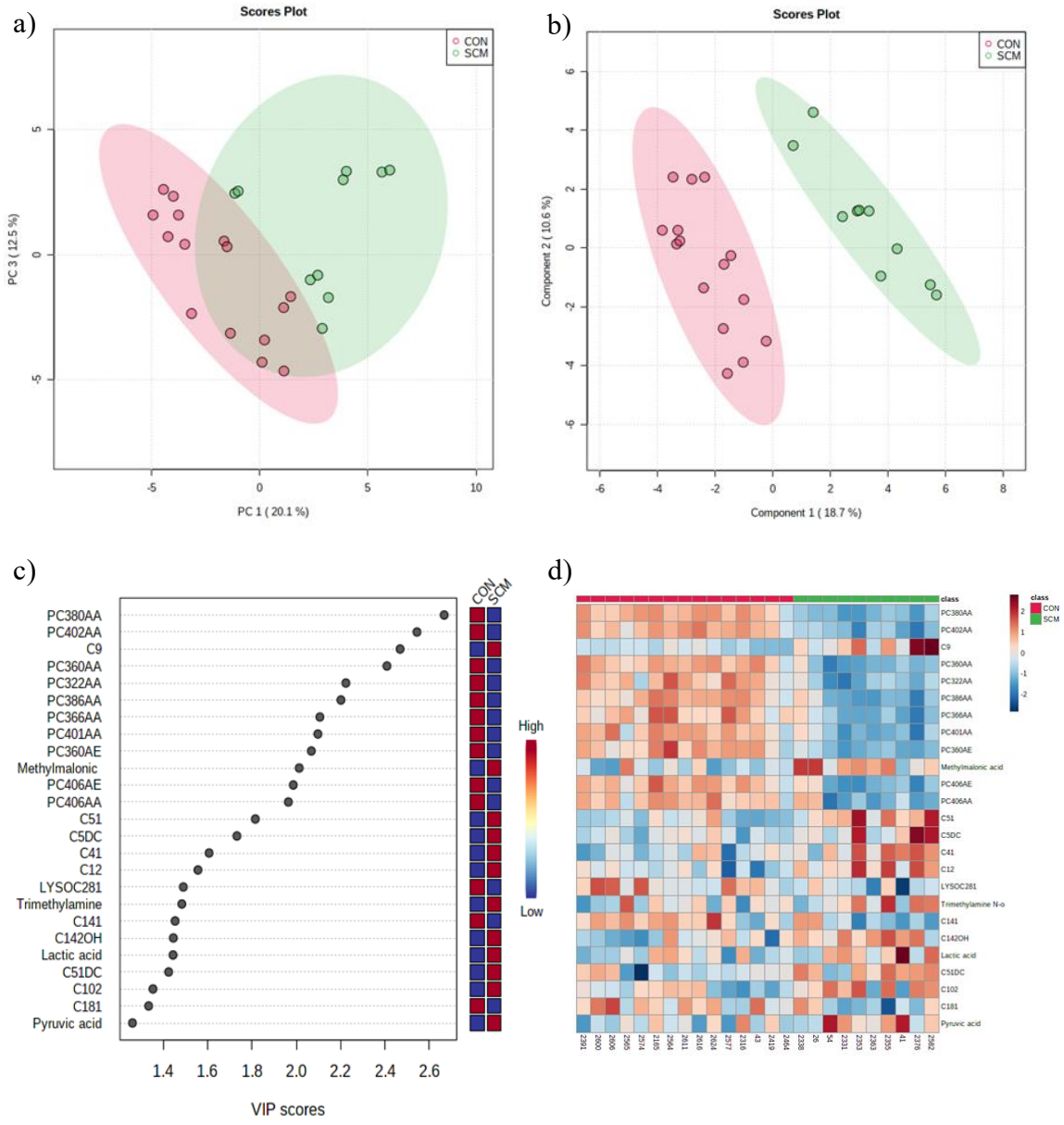


Figure 2.3. a) Principal Component Analysis (PCA) and **b)** PLS-DA (permutation test: $P < 0.05$) of 15 CON and 10 pre-SCM cows; **c)** VIP plot of top 25 important features and **d)** Heatmap of both, samples and features based on PLS-DA to further investigate the identified variables.

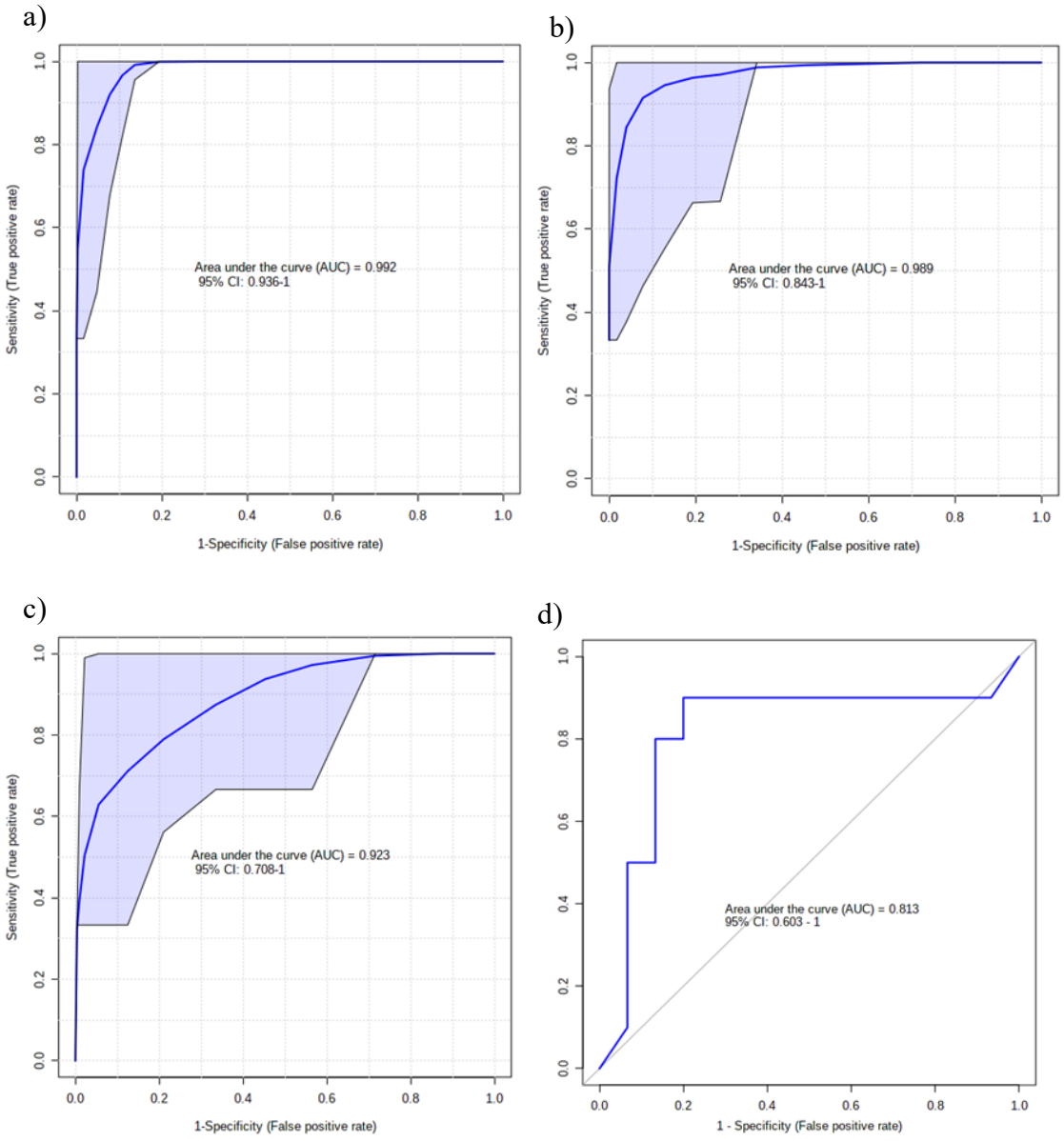
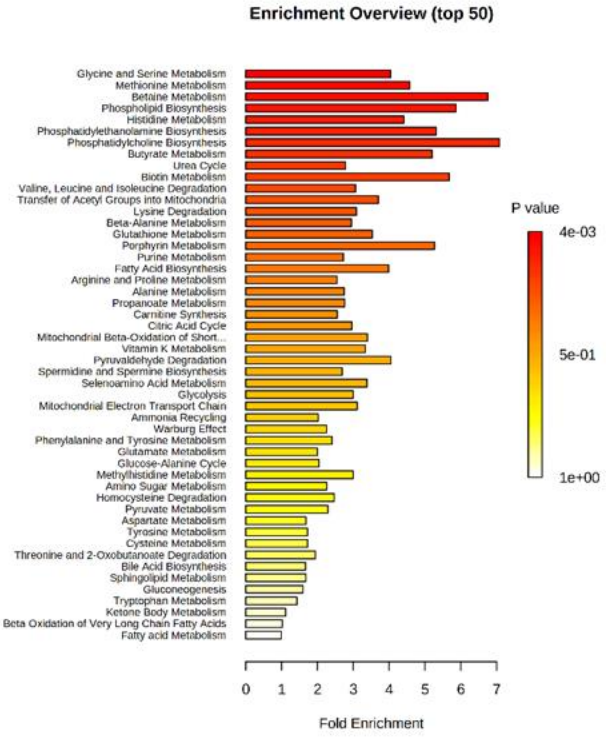


Figure 2.4. a) AUC of the top 5 VIP PLS-DA metabolites and **b)** AUC of model's classification; **c)** default linear SVM AUC model (AUC = 0.92; $P = 0.01$); **d)** ROC plot with 10-fold CV for logistic regression algorithm (AUC = 0.81, $P = 0.04$).

a)



b)

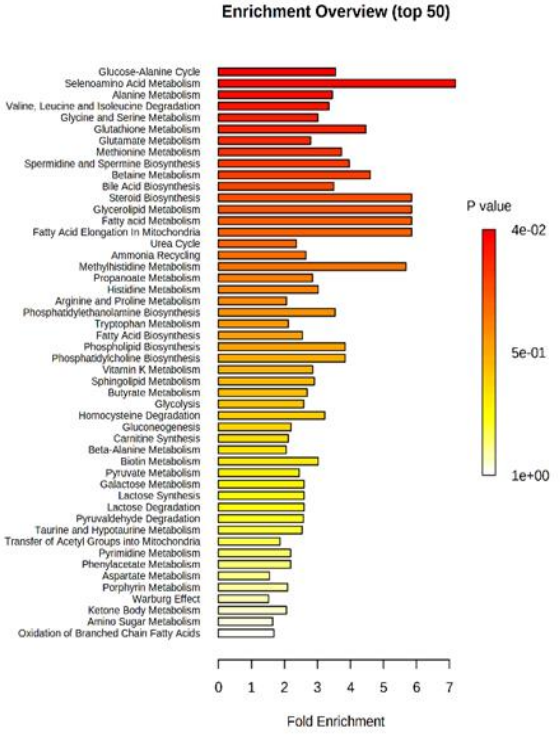


Figure 2.5. Summary plots for quantitative enrichment analysis at **a)** –8 weeks and **b)** –4 weeks before calving.

Chapter 3. Urinary Metabotyping Identifies Predictive Biomarkers of Subclinical Mastitis in Dairy Cows

3.1 Abstract

Mastitis is one of the most important infectious diseases of dairy cows. It is associated with a considerable loss regarding milk yield and the culling of dairy cows. Early identification of cows at risk of mastitis can enable the development of better preventive measures prior to disease occurrence. Subclinical mastitis is currently diagnosed through measurement of somatic cell count in the milk after calving. Currently, there are no predictive tests available. Therefore, the objective of this study was to identify metabolic alterations in the urine of pre-SCM cows at –8 wks and –4 wks before calving, through a LC-MS based targeted metabolomic assay. A total of 145 multiparous dairy cows were included in this nested case-control study at the beginning of dry off period and were sampled and monitored for periparturient disease occurrence both prepartum and postpartum. Only 15 cows were free of disease and served as healthy controls, and ten cows were affected only by SCM. Cows affected by other conditions or SCM with at least one other disease were excluded from further evaluation. Results showed multiple metabolite alterations in the urine of pre-SCM cows including changes in acylcarnitines, amino acids, and organic acids. Metabotyping of cows revealed 27 metabolites that differentiated pre-SCM from healthy CON cows at both –8 wks and –4 wks prior to the expected day of parturition. At $q < 0.005$ only 4 metabolites were altered from each week. Biomarker analysis indicated that a panel of 4 serum metabolites including asymmetric dimethylarginine (ADMA), proline, leucine, and homovanillate (AUC = 0.88; $P = 0.02$) at

–8 wks and ADMA, methylmalonate, citrate, and spermidine (AUC = 0.88, $P = 0.03$) at –4 wks before parturition might serve as predictive urinary biomarkers for the risk of SCM. Overall, our data showed that starting from –8 wks and –4 wks prepartum cows susceptible to SCM can be identified by metabolite testing. More research is needed to validate the two panels of metabolites identified.

3.2 Introduction

Bovine mastitis, which is characterized by the inflammation of the udder, is a significant concern for the dairy industry. Mastitis is a multifactorial disease. A combination of bacterial prevalence and poor management practices related to farm hygiene, dry cow therapy or automated milking machines contribute to the udder's inflammation (Ferrero et al., 2014; Adkins and Middleton, 2018). It is estimated that farm losses associated with mastitis in Canada are $660 per case, and in North America, it causes around US$2.5 billion annually (Viguier et al., 2009; Aghamohammadi et al., 2018). Inflammation of the mammary gland is presented either as clinical or subclinical mastitis. Subclinical mastitis (SCM) is an asymptomatic inflammation of the udder characterized by the influx of cellular elements, mostly polymorphonuclear neutrophils into the mammary gland. In contrast, clinical mastitis (CM) is distinguished by external changes of the udder (swollen, hot) and appearance of milk (discoloration, thickness), accompanied by systemic signs in the cow (fever, reduced feed intake) (Adkins and Middleton, 2018). Udder infections are associated with lower milk production in both current and following lactations, and lower future reproductive performance and conception rates, which leads to

the early culling of dairy cows (Ahmadzadeh et al., 2009; Ruegg, 2017). Indeed, mastitis is the second most common reason for dairy cows being culled in Canada (CDIC, 2020).

The traditional method to diagnose subclinical mastitis is counting of immune cells (mainly neutrophils) in the milk, known as the somatic cell count (SCC) test. This test is conducted shortly after calving and for the entire duration of the milking period. However, high incidence of new intramammary infections (IMI) during the dry period have been recently observed (De Prado-Taranilla et al., 2020). The presence of subclinical IMI during dry off may persist as such or present itself as an acute or subclinical case of mastitis after calving (Bradley and Green, 2004). Our lab has reported that cows diagnosed as infected after parturition were in a systemic chronic inflammatory state during the dry-off period (Dervishi et al., 2015). In another study, we also reported that SCM cows had multiple alterations in their serum and urinary metabolic signatures that differentiated them from healthy dairy cows (Dervishi et al., 2017; Zwierzchowski et al., 2020a).

The application of metabolomics and microbiomics approaches to the study of the pathobiology of periparturient diseases of dairy cows has increased during the last decade (Ametaj, 2015). Most of the studies conducted have used postpartum biofluids such as milk or blood for diagnostic purposes (Martins et al., 2019). There is very little data available concerning metabolite fingerprinting of the urine in dairy cows for the purpose of identifying biomarkers for mastitis or for predicting cows that would be susceptible to mastitis. In a previous study conducted by our lab, we used urine samples to determine metabolic profiles around calving cows that were later affected by SCM postpartum. Our data showed presence of urinary alterations of a variety of metabolites including

acylcarnitines (ACs), phosphatidylcholines (PCs), biogenic amines (BAs), and amino acids (AAs) starting from –8 wks until +8 wks around calving (Zwierzchowski et al., 2020a).

Recently, Donadeu et al. (2020) reported that UK farmers preferred test results for their cows within 24 hours from sampling time. At the same time, the demand and the necessity for lab-on-chip or pen-side tests are increasing. Consequently, our lab has been working on identifying metabolites that distinguish healthy cows from pre-SCM cows. In this study, we used a metabolomics approach to study urine samples from one dairy farm in Alberta to verify and validate previously identified metabolites and whether we can use those metabolites for screening purposes during the non-lactating period as a pen-side test to predict the risk of SCM. We hypothesized that starting from –8 wks and –4 wks prepartum those cows susceptible to SCM would show detectable urinary metabolite alterations that can be used to identify cows that are susceptible to SCM prior to its diagnosis postpartum. Therefore, the objective of this study was to identify a panel of urinary metabolites that would predict at-risk SCM cows as early as –8 wks and –4 wks prepartum.

3.3 Materials and Methods

3.3.1 Animals, diets and urine samples

In this nested case-control study, a total of 145 multiparous cows were selected to collect urine samples. Cows were chosen from a commercial dairy farm in the province of Alberta, Canada. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and conducted following the Canadian Council's guidelines on Animal Care (CCAC, 2009).

Cows were selected based on their expected date of calving. They were sampled at the beginning of the dry off period and in the middle, respectively, at –8 wks (55 – 58 days) and –4 wks (27 – 30 days) before parturition. Urine samples were collected before the morning feed, between 07:00 – 08:00, using 50 mL sterile specimen tubes (Fisher Scientific, Toronto, ON, Canada). All urinary metabolite analyses were conducted at The Metabolomics Innovation Centre (University of Alberta, Edmonton, AB, Canada). All samples were stored at –80℃ until the analysis was performed.

The cows sampled prepartum presented with many different conditions including mastitis, metritis, retained placenta, laminitis, displaced abomasum, milk fever, and ketosis postpartum. Health records for the periparturient diseases were gathered from the farm's database. All diseases were diagnosed by a herd veterinarian that visited the herd on a weekly basis. For SCM, positive cases were defined as those cows that had two or more consecutive weeks with milk SCCs equal to or higher than 200,000 cells/mL of milk. Healthy or control (CON) cows were considered only those that did not present any health issues throughout the dry period and after calving (up to 4 wks postpartum) and which had SCCs lower than 200,000 cells/mL of milk. By this judgement, 15 dairy cows out of 145 were considered healthy (CON), whereas only 10 cows had SCC levels classified as subclinical mastitis and were free of other diseases. The body condition score (BCS) was measured for both groups during the two sampling weeks (–8 wks and –4 wks prepartum). Feed ingredients, on a dry matter basis, are presented in Tables 3.1 & 3.2.

3.3.2 FIA/LC – MS/MS compound identification and quantification

3.3.2.1 Sample preparation

Urinary samples were thawed on ice and vortexed before analysis. For the analysis of biogenic amines, amino acids, lipids, acylcarnitines, and hexose (mainly glucose), 10 µL of urine each of flow injection analysis (FIA) running buffer and LC internal standards (ISTD) were loaded in a 96-well filter plate. The first 14 wells were used for quality control and standardization. Specifically, one blank, three zero samples, seven standards and three quality control (QC) samples were placed in those 14 wells. Thawed samples were then added to the remaining 82 wells. For samples and standards, a total of 10 µL was added to each of the respective wells. The 96-well plate was then incubated and dried under a flow of nitrogen [Zanntek Analytical Evaporator (Glas-Col, Terre Haute, IN, USA)], for 30 min. After being dried, 50 µL of 5% phenylisothiocyanate (PITC) solution was added to each well, and the plate was incubated at room temperature for 20 min. The plate was then dried again for 90 min under a flow of nitrogen. Extraction of the metabolites was accomplished by adding 300 µL methanol, containing 5 mM ammonium acetate. The plate was placed for shaking at 330 rpm for 30 min and then centrifuged for 5 min at 500 rpm, [Sorvall Evolution RC Superspeed Centrifuge (Fisher Scientific, Toronto, ON, Canada)], into the lower 96 deep-well plate. For the analysis of amino acids and biogenic amines, the extract was diluted with water 1:1, and 10 µL was injected into the LC column. For the analysis of acylcarnitines, lipids, and hexose compounds, 150 µL of the extract was diluted with 400 µL of FIA running buffer, and 20 µL was injected in the LC column.

Twenty-five microliters of each of the following three solutions: 3-nitrophenylhydrazine (3-NPH) (250 mM in 50 % aqueous methanol), 1-Ethyl-3-(3-

dimethyl aminopropyl) carbodiimide (150 mM in methanol), and pyridine (7.5% in 75% aqueous methanol) were added to each well. The whole plate was then shaken on a shaker at 450 rpm for 2 h at room temperature to complete the derivatization reaction. After the reaction, 350 µL of HPLC water and 50 mL of 2 mg/mL butylated hydroxytoluene (BHT) were added to each sample well to dilute and stabilize the solution for LC-MS/MS analyses.

3.3.2.2 FIA/LC – MS/MS method

Identification of urine metabolites was done through a targeted metabolomics approach using the TMIC Prime assay (in-house developed) using an Agilent 1100 series liquid chromatographic system (LC) (Agilent, Palo Alto, CA, USA) equipped with an Agilent reversed-phase Zorbax Eclipse XDB C18 column (3.0 mm × 100 mm, 3.5 µM particle size, 80 Å pore size) with a Phenomenex (Torrance, CA, USA) SecurityGuard C18 pre-column (4.0 mm × 3.0 mm), coupled with AB SCIEX QTRAP® 4000 mass spectrometer (Sciex Canada, ON, Canada). LC/MS grade formic acid and HPLC grade water were purchased from Fisher Scientific (Ottawa, ON, Canada). Ammonium acetate, phenylisothiocyanate (PITC) and HPLC grade acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LC-MS assay workflow was controlled through the Analyst® 1.6.2 software (Sciex Canada, ON, Canada).

The HPLC parameters used to analyze amino acids and biogenic amines were as follows: mobile phase A 0.2% (v/v) formic acid in HPLC grade water, and mobile phase B 0.2% (v/v) formic acid in acetonitrile. The gradient profile for this HPLC solvent run was as follows: t = 0 min, 0% B; t = 0.5 min, 0% B; t = 5.5 min, 95% B; t = 6.5 min, 95% B; t = 7.0 min, 0% B; and t = 9.5 min, 0% B. The column oven temperature was set at 50

°C. The flow rate was 500 µL/min, and the sample injection volume was 10 µL. The mass spectrometer was set to positive electrospray ionization (ESI) mode with a scheduled multiple reaction monitoring (MRM) scan.

For FIA-MS/MS analysis, the HPLC autosampler was connected directly to the MS ion source by red PEEK tubing. The FIA running buffer was used as the mobile phase. The flow rate was programmed as: t=0 min, 30 µL/min; t=1.6 min, 30 µL/min; t=2.4 min, 200 µL/min; t=2.8 min, 200 µL/min; t=3.0 min, 30 µL/min. The sample injection volume was 20 µL. The mass spectrometer was set to the positive ESI mode with MRM scanning to analyze lipids and acylcarnitines and the negative ESI mode to detect glucose/hexose.

For the analysis of organic acids by LC-MS/MS, the solvents used were (A) 0.01% (v/v) formic acid in water and (B) 0.01% (v/v) formic acid in methanol. The gradient profile was as follows: t=0 min, 30% B; t=2.0 min, 50% B; t=12.5 min, 95% B; t=12.51 min, 100% B; t=13.5 min, 100% B; t=13.6 min, 30% B, and maintained at 30% B for 4.4 min. The column oven temperature was set to 40 °C. The flow rate was 300 µL/min, and the sample injection volume was 10 µL. The mass spectrometer was set to the negative ESI mode with scheduled MRM scanning.

3.3.3 Statistical analysis

Univariate analyses were performed using Wilcoxon rank-sum test from the emmeans package in R (v4.0.2) with a significance level of $P \leq 0.05$. The adjusted P values were performed using the false discovery rate (FDR) method and a value of $q < 0.005$. Metabolomic data, such as multivariate statistical analysis and biomarker analysis, were performed using MetaboAnalyst (v4.0), following published guidelines (Xia et al., 2009;

Chong et al., 2019). The data were normalized using creatinine and transformed and scaled to create a Gaussian distribution. Multivariate statistical analyses between the two groups of cows, SCM and CON, were conducted via unsupervised and supervised methods such as principal component analysis (PCA) and partial least squared discriminant analysis (PLS-DA). The most influential compounds from the PLS-DA were ranked using variable importance in projection (VIP) plots. Typically, metabolites with $P < 0.05$ and VIP scores > 1 are the strongest discriminators between the groups. Model validation was carried out using a cross-validation test, and a permutation test with 2000 repetitions was implemented to assess the reliability of the PLS-DA model (Xia and Wishart, 2011).

Identification of biomarker profiles and metabolite set enrichment analyses (MSEA) were performed using MetaboAnalyst (v4.0). Perturbed metabolic pathways identified from MSEA were considered statistically significant if the Holm corrected P value was < 0.05. The quality of the biomarker sets was determined using receiver-operating characteristic (ROC) curves generated by Monte-Carlo cross-validation (MCCV). A permutation test with 1000 repetitions was performed for the validation of these ROC curves. We picked the top metabolites for biomarker analysis and calculated the area under the curves (AUCs) for those biomarker panels for both prepartum time points. Supervised classification method, such as Support Vector Machine (SVM) and logistic regression analyses for several significant metabolites were also performed to train the model.

3.4 Results

Results of this study showed that urinary metabolites of pre-SCM cows were different from healthy ones starting from –8 wks and –4 wks before parturition. A total of 82 urinary metabolites were identified and measured in the urine of dairy cows in this study. Results showed that 27 metabolites differentiated the only pre-SCM and CON groups at each week, –8 and –4 wks prepartum (Table 3.3, 3.4). FDR adjusted *P* values presented only 4 metabolites from each week within the threshold of $q < 0.005$. Control cows had a BCS of 3.78 at –8 wks and 3.95 at –4 wks and pre-SCM cows had BCS values of 3.70 and 3.92 at –8 wks and –4 wks, respectively.

Multivariate statistical methods including PCA and PLS-DA were performed to cluster and discriminate the two groups of cows. PCA analysis did not show a clear separation between CON and pre-SCM cows at –8 wks prior to parturition; however, a clear separation of the two groups of cows was shown by the PLS-DA analysis (Figures 3.1a, 3.1b). To measure the importance of the variables from the PLS-DA, we considered the VIP scores. The top 15 most important metabolites that distinguished cows that developed SCM from the healthy ones at parturition are presented in the VIP plot (Figure 3.1c). Asymmetric dimethylarginine (ADMA) was the top metabolite with the highest VIP score, at 2.2. Homovanillic acid (HVA) is the second most important metabolite and reported here for the first time to be associated with pre-SCM. Both ADMA and HVA were consistently increased in the urine at both –8 wks and –4 wks prepartum.

Figure 3.2 shows the performance of urinary metabolites identified as potential biomarkers for SCM. The top 5 metabolites from the VIP plot showed good performance parameters with an AUC = 0.88, $P = 0.02$ (Figure 3.2a). Overall, the multivariate

exploratory analysis presented the best area under the ROC (AUROC) curve for the top ten prominent features (Figure 3.2b). To build top performing panel of predictive biomarkers, we selected specific metabolites easily accessible to be validated. A default linear support vector machine (SVM) model and a logistic regression algorithm were constructed for each prepartum sampling period. Both presented a permutation test value of $P \leq 0.05$ out of 1000 repeats (Figures 3.2c, 3.2d).

On the other hand, at –4 wks before calving, our multivariate analysis showed better separation of pre-SCM from CON cows (Figures 3.3a, 3.3b). Figure 3.3c and 3.3d show the metabolites that had the most impact in separating these two groups. According to the VIP plot, 6 acylcarnitines (ACs) were lower in pre-SCM cows at –4 weeks, whereas 2 organic acids (OAs), including alpha-ketoglutaric and citric acid had high VIP score of > 1.5 in pre-SCM. The first five metabolites of the VIP plot generated an AUC = 0.95 (P = 0.009), and again the model with the best classification was that which included all 10 high scoring metabolites, based on cross-validation (Figures 3.4a, 3.4b). For linear SVM and regression analysis, we selected 4 metabolites that produced the best results throughout the model validation analysis (Figures 3.4c, 3.4d). The linear SVM model produced a highly significant result (P = 0.03), whereas the logistic regression model gave a sufficiently significant result (P = 0.05).

Figure 3.5 displays the results from the quantitative enrichment analysis (QEA), done using MSEA in MetaboAnalyst 4.0. No significantly perturbed metabolic pathways were found at week –8 prior to parturition with a Holm value of $P < 0.05$. Whereas, at –4 wks prior to calving, only one pathway was statistically significant (Holm's $P < 0.05$), which was the pathway associated with spermidine and spermine biosynthesis. Urinary

metabolites such as spermine, methionine, spermidine, ornithine, and putrescine were found to be significantly different between the two groups of cows (Holm $P = 0.02$) at –4 wks, whereas no significant metabolic pathways were found at –8 wks before calving.

3.5 Discussion

This study evaluated the hypothesis that dairy cows susceptible to SCM will show urinary metabotypes that are different from healthy CON cows, both at –8 wks and –4 wks prepartum, prior to diagnosis of SCM postpartum. Indeed, the data showed that pre-SCM cows displayed urinary metabotypes that differentiated them from the healthy cows starting from –8 wks and –4 wks prepartum. The distinction between pre-SCM and healthy CON cows was made based on the SCC in the milk collected pre- and post-partum. A cut-off value of > 200,000 SCC/mL of milk was selected for diagnosing SCM cows and a SCC value of < 200,000 for diagnosing healthy cows (Adkins and Middleton, 2018).

3.5.1 Urinary lipid alterations in pre-SCM cows

The main species of lipid metabolites that differentiated the pre-SCM cows from CON cows were urinary ACs, more specifically, short chain ACs (C3-C5 carbons). Acylcarnitines serve as carriers of long-chain fatty acids into the mitochondria for β-oxidation (Tarasenko et al., 2018). Similar findings were reported previously in the urine of pre-SCM and pre-ketotic cows by our lab (Zwierzchowski et al., 2020a; Zhang et al., 2021). Elevated ACs have been considered as biomarkers for the activation of the immune system (Rutkowsky et al., 2014). They are produced as remnants of incomplete

mitochondrial fatty acid oxidation and given that they are toxic compounds they need to be excreted from the body (Mirzoyan et al., 2017). A study conducted by Minuti et al. (2015) demonstrated increased blood ACs in dairy cows infused with intramammary lipopolysaccharide (LPS). The same authors reported that administration of LPS was associated with suppression of apolipoprotein B genes, acetyl-CoA acyltransferase-2 (ACAA2), and hydroxymethylglutaryl-CoA synthase (HMGCS2), which are related to β-oxidation in hepatocytes, leading to the accumulation of ACs in the systemic circulation. Indeed, our lab has previously reported that pre-SCM cows were experiencing a chronic low-grade inflammatory state during the dry off period and the week the disease was first detected (Dervishi et al., 2015).

Intriguingly, human subjects with high urinary ACs were reported to have been in a state of methylmalonic acidemia (MMAemia) (Chalmers et al., 1984). The pre-SCM cows in our study were found to have increased levels of methylmalonate in the urine as well as in the blood circulation, especially at –4 wks prior to parturition. Typically, MMAemia is a condition related to genetic errors or due to vitamin B12 deficiency in humans and cattle (Barton and Elliot, 1977; Baumgartner et al., 2014). Methylmalonate is a by-product of BCAA catabolism. It may be related to defects in the methylmalonyl-CoA mutase (MUT) or in the synthesis of vitamin B12 that prevents it from entering the Krebs cycle. Under this condition, the body cannot metabolise amino acids, such as valine, isoleucine, threonine, methionine or fatty acids and accumulates methylmalonic acid in the systemic circulation (Fowler et al., 2008).

3.5.2. Changes in urinary amino acids in pre-SCM cows

Urinary BCAAs including valine, leucine, and isoleucine were found to be greater in pre-SCM group compared with healthy cows, at both prepartum time points in our study. Our lab has reported similar findings in a previous study involving pre-SCM and pre-lame cows (Zhang et al., 2020a; Zwierzchowski et al., 2020a). Of note, BCAAs were higher in the serum of the same cows at –8 wks prior to parturition. This suggests that for some unknown reason BCAAs are not fully utilized by the host but excreted in the urine. A similar pathogenesis with that of diabetic kidney disease (DKD) in humans may be speculated (Hinden et al., 2021). Zhenyukh et al. (2017) demonstrated that BCAAs increase the production of ROS through both activation of NADPH oxidase and from mitochondria, and activation of Akt-mTOR signal in cultured PBMCs. BCAAs also stimulate the NF-kB pathway in those cells, which results in release of proinflammatory cytokines including IL-6, TNF, intracellular adhesion molecule-1 (ICAM-1) (or CD40L), and the migration of PBMCs. These authors suggested that high concentration of BCAA might contribute to the proinflammatory and oxidative stress in various diseases. We suspect that high BCAA in the blood and urine of pre-SCM cows in our study, might have contributed to a systemic inflammatory status, as previously reported by our lab in pre-SCM cows (Dervishi et al., 2015).

A potential reason why BCAA and several other AA species were excreted in the urine and not reabsorbed back into the systemic circulation could be due to increased proteocatabolism in the skeletal muscles to support the inflammatory response (Dervishi et al., 2018a). This may have led to an over abundance of BCAA that were too high for resorptive capacity or due to low presence of electrolytes that could pair with BCAA.

During chronic inflammatory states the release of proinflammatory cytokines like TNF or translocation of LPS into the systemic circulation trigger a loss of skeletal muscle protein as a result of increased muscle proteolysis and decreased muscle protein synthesis (Svanberg et al., 2000; Nystrom et al., 2009). In support of this speculation is our finding that two urinary AAs, including His and methyl-His, were increased in pre-SCM cows at –4 wks prior to parturition. Those two AAs have been linked to increased muscle protein proteolysis (Zhou et al., 2017b; Koshikawa et al., 2020). Humans with increased proteolysis and urinary excretion of His have been found to have higher concentrations of IL-6, C-reactive protein (CRP), and reactive oxygen species (ROS) (Koshikawa et al., 2020). A similar finding was reported also in rodents (Zhang et al., 2009). Histidine has anti-inflammatory effects in response to LPS challenge, including the scavenging of ROS, and the inhibition of the secretion of IL-8 and NF-κB (Wade and Tucker, 1998; Son et al., 2005). However, high urinary excretion of His and methyl-His might be a host response to favor an inflammatory response to a potential subclinical bacterial infection of the udder during the dry off period.

Another important finding included elevated urinary concentrations of arginine (at –8 wks prepartum) and two of its close functional components ADMA and TDMA (total dimethylarginine) (at both –8 and –4 wks prepartum), in the urine of pre-SCM cows. These results align with our previously reported findings in pre-SCM and pre-lameness cows (Eckel et al., 2020; Zwierzchowski et al., 2020). Elevated concentration of arginine was also found in the serum of our pre-SCM cows, at –4 wks prepartum. Arginine is an essential AA, important for production of nitric oxide (NO), polyamines, proline, and the stimulation of the immune system (Satriano, 2004). Catabolism of arginine has been shown to increase

during inflammatory conditions (Flynn et al., 2002). Interestingly, LPS-induced inflammation of the mammary epithelial cells is lowered by arginine, reducing the release of IL-1β, IL-6, TNF and enhancing mTOR signaling (Wu et al., 2016). Additionally, blood infusion of arginine in cows during early lactation is associated with decreased TNF and Hp and increased levels of IgM and total protein concentration as well as improved antioxidant capacity (Zhao et al., 2018; Ding et al., 2020). In a sheep study, LPS-induced inflammation was shown to be associated with elevated removal of arginine from the liver and spleen (McNeil et al., 2016; Coleman et al., 2020). Increased urinary excretion of arginine might support a proinflammatory status to counteract potential subclinical mastitis in the pre-SCM cows.

Importantly, ADMA has received particular attention recently because it inhibits the activity of nitric oxide synthase (NOS) (Tsikas, 2017; Tsikas et al., 2018). The latter is the enzyme that converts arginine to NO and L-citrulline. ADMA is produced during the process of asymmetric demethylation of guanidine group of arginine residues in selected proteins and is released by proteolysis. Elevated concentrations of free ADMA in the systemic circulation are considered a risk factor for morbidity and mortality in humans (Zhou et al., 2017a). The risk has been related to inhibition of NO secretion, which is a crucial mediator in host defense and one of the major killing mechanisms for macrophages (Zhao et al., 1997). Excretion of ADMA and TDMA in the urine might be a host response to eliminate this compound that inhibits the killing activity of immune cells.

Our results showed that choline and betaine were increased in the urine of pre-SCM cows at –8 wks prepartum. However, only urinary choline was higher in those cows at –4 wks prior to parturition. Choline is an amine and an essential nutrient that participates in

acetylcholine synthesis and methyl group donor (in the liver and kidneys) when metabolized to betaine (Aoyama et al., 2004). Choline also is used for synthesis of phosphatidylcholine (Fullerton et al., 2006). Snider et al. (2018) looked at the role of choline on murine macrophages and demonstrated that polarization of primary bone marrow macrophages with LPS was associated with an increased rate of choline uptake. Choline uptake also has been shown to contribute to macrophage-mediated IL-1β-dependent inflammation (Sanchez-Lopez et al., 2019). Both choline and betaine has been shown to have anti-inflammatory activity. For example, Parrish et al. (2008) reported that intraperitoneal treatment with choline (at 50 mg/kg), prior to LPS administration in mice lowered circulatory concentrations of systemic TNF. Moreover, the same authors demonstrated that choline suppressed TNF release from human macrophages.

Betaine is part of the one carbon metabolism pathway and serves as a methyl group donor (Williams and Schalinske, 2007). Betaine also has been shown to suppress the NF-kB pathway and the associated genes including TNF, vascular cell adhesion molecule-1, intracellular cell adhesion molecule-1, inducible nitric oxide synthase, and cyclooxygenase-2 (Go et al., 2005). It seems odd that the host is excreting in the urine anti-inflammatory compounds; although it should be noted that both choline and betaine are in higher concentrations in the serum of the same cows at –8 wks prepartum. These results support our previously stated hypothesis that the host metabolic response (i.e., urinary excretion of choline and betaine) is supporting a proinflammatory response against presence of a potential subclinical infection in the udder.

3.5.3. Changes in the urinary carbohydrate and organic acid species in pre-SCM cows

At the beginning of the dry off period (–8 wks prepartum), there was a higher concentration of glucose in the urine of pre- SCM cows than at –4 wks prepartum. There was also a tendency for serum glucose to be higher in the pre-SCM cows at –8 wks prepartum. Although glucose plays important functions as energy substrate for the host, it has been reported that high concentrations of glucose have been associated with impaired immune functions and, consequently, higher susceptibility to bacterial infections. In a study conducted by Kim et al. (2019) the authors reported impaired functions of NK cell activity in type 2 diabetes patients compared to healthy controls and prediabetic patience. In a recent study, Kuwabara et al. (2018) demonstrated a deficiency (impairment) of neutrophil migration to the site of inflammation (lungs) in diabetic (hyperglycemic) rats. Moreover, Saito et al. (2013) showed that chronic hyperglycemia caused an increase in basal ROS production of neutrophils and increased susceptibility to infection related to lowered neutrophil reactions. Overall, given that high blood glucose might impair immune responses, and more particularly neutrophil and NK functions, the host seems to increase urinary excretion to prevent glucose-related impairment of immune responses.

To the best of our knowledge this is the first time that urinary homovanillic acid (HVA) is reported to be higher in pre-SCM cows. Homovanillic acid is a downstream metabolite of tyrosine. This acid was increased at both sampling timepoints but presented higher forecasting abilities at –8 wks. Elevated HVA is an established urine biomarker for several tumours, metabolic, and neurological disorders in humans (Hrdlička et al., 2021). It is a catecholamine-derived metabolite, especially of dopamine. These are considered coping hormones in stressful situations and significant activators of lipolysis and

glycogenolysis for energy support (O'Neill, 2019). Homovanillic acid in humans has been found to activate the immune system and its low levels correlate with survival of the host (Bonifačić et al., 2017). The reason why HVA is increased in the urine of pre-SCM cows is not understood presently, which is an indication of the broad scope of mastitis pathomechanisms.

An important finding from this study was a major difference in the urinary concentration of citrate between the pre-SCM cows (21-fold higher) and CON cows, at –4 wks prepartum. Citrate is a key metabolite of the Krebs cycle. Citrate is produced in mitochondria and then moves from the mitochondria to the macrophages' cytosol, which is essential for the pro-inflammatory response (Infantino et al., 2014). Cytosolic citrate under normal conditions is converted into acetyl-CoA and oxaloacetate. Acetyl-CoA is used for fatty acid synthesis, whereas oxaloacetate helps generate ROS, important in fighting pathogenic bacteria. There is growing research pinpointing the importance of citrate in regulating immune cell response. Citrate has been linked to the production of several important proinflammatory mediators in macrophages including NO, ROS, and prostaglandin E_2 (PGE_2) (Infantino et al., 2013). Indeed, inhibition of the citrate carrier protein (CIC) leads to a marked decrease in production of NO, ROS, and PGE_2 (Infantino et al., 2011). Moreover, treatment of macrophages with LPS has been shown to increase mitochondrial CIC in LPS-induced macrophages (Infantino et al., 2011). An important question for our study is why the host (pre-SCM cow) is excreting extremely high amounts of citrate in the urine? The answer to this is not known yet. However, this finding is in line with our hypothesis that pre-SCM cows are trying to mount an inflammatory response and given that citrate has inhibitory effects on proinflammatory mediators, then, it is excreted

in very significant amounts in the urine to maintain the inflammatory response under limited ranges.

Noticeable changes between the two groups of cows also occurred in the concentrations of urinary polyamines. For example, spermidine and putrescine were higher in pre-SCM versus CON cows at –8 wks prepartum. Additionally, urinary spermine was higher in the urine of pre-SCM cows at –4 wks prior to parturition. The polyamines spermidine, putrescine, and spermine are polycations derived from ornithine and play essential physiological roles (Løvaas and Carlin, 1991). They engage in the synthesis of DNA and proteins. Moreover, they participate in proliferation and differentiation of cells (Igarashi and Kashiwagi, 2000). They also act as scavengers of ROS and protect DNA, proteins, and lipids from oxidative injury (Chattopadhyay et al., 2003). Polyamines have been postulated to have anti-inflammatory and anti-oxidant properties (Løvaas and Carlin, 1991). It has been suggested that they exert anti-inflammatory effects by their direct action on lymphocytes (Theoharides, 1980). Lagishetty and Naik (2008), assessed the *in vivo* effects of polyamines on acute, subacute, and chronic inflammation. They reported significant anti-inflammatory activity in acute, sub-acute, and chronic models of inflammation. It can be concluded that polyamines, which also have anti-inflammatory activities are excreted in the urine in higher amounts in the pre-SCM cows than CON cows. These data suggest that the host is excreting multiple metabolites in the urine with anti-inflammatory activity. This supports our postulate that pre-SCM cows are trying to mount an inflammatory response; however, they are excreting multiple metabolites that potentially have been released into the systemic circulation to also keep the inflammatory response under control.

3.6 Conclusions

In conclusion, pre-SCM cows experienced altered concentration of urinary metabolites related to lipid, amino acid, carbohydrate, and organic acid metabolism. Of interest was the higher excretion of ACs and urinary clearance of several AAs, such as valine, leucine, isoleucine, histidine, methyl-histidine as well as arginine, choline and betaine might have contributed to a systemic inflammatory response from the presence of a subclinical infection in the udder. Other metabolites including ADMA, TDMA, glucose, and citrate are removed from the host due to their inhibitory effect on immune cells that limit the cow's response against the disease agent(s). Overall, our data suggest that several metabolites are excreted in the urine to decrease the inflammatory response, whereas other metabolites are maintained to support it. Multivariate analysis showed a clear separation of the two groups at –4 wks prior to parturition but not at –8 wks prepartum. Two potential panels of urinary metabolites, specific for predicting SCM, were constructed with very good accuracy. These metabolites included ADMA, MMA, spermidine, and citrate at –8 wks and ADMA, leucine, proline, and HVA at –4 wks to predict the risk of occurrence of SCM starting from the dry off period.

3.7 Acknowledgments

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Table 3.1. Ingredients of the prepartum diet for the dry off cows.

Ingredient	Weight/cow (kg)	DM (%)	Final DMI (kg)[1]
Hay	5.50	85.14%	4.68
Oats	5.75	36.20%	2.08
Corn	8.84	30.30%	2.68
Protein	2.00	93.00%	1.86
Ground Barley	0.75	97.26%	0.66
Minerals	0.42	97.26%	0.41
Total	23.36	53.17%	12.37

[1]Dry Matter Intake (DMI) is calculated based on the DM% over the offered amount (kg) of feed. Daily DMI is formulated to 2% of cow's body weight

Table 3.2. Feed ingredients on a dry-mater basis for cows during early lactation.

Ingredient	Weight/cow (kg)	DM (%)	Final DMI (kg)
Hay dairy	2.50	88.50	2.21
Grass silage	10.75	31.80	3.42
Oats	5.99	36.20	2.17
Barley-Dakota	11.50	40.00	4.80
Corn	13.52	31.50	4.26
Whey	2.75	17.00	0.47
Protein	4.75	93.30	4.43
Energy dairy	4.25	88.00	3.74
Ground Barley	1.75	88.00	1.54
Mineral & Fat	1.26	97.26	1.23
Total	59.02	47.56	28.07

Table 3.3. Concentration of urinary metabolites (MEAN ± SEM) in pre-subclinical mastitis cows (pre-SCM, n=10) and healthy controls (CON, n=15) at –8 weeks prior to parturition as identified by LC-MS/MS.

	MEAN ± SEM				
Metabolites (μM)	Pre-SCM[1] (n=10)	CON[2] (n=15)	P value	Fold change	SCM/ CON
Creatinine	13903 ± 1679	8732 ± 1477	0.01	1.59	up
Glycine	302 ± 169	196 ± 149	0.78	1.54	up
Alanine	265 ± 60	171 ± 52.8	0.48	1.55	up
Serine	103 ± 17.3	56 ± 15.2	0.11	1.84	up
Histamine	0.1315 ± 0.0244	0.0915 ± 0.0214	0.28	1.44	up
Proline	6.14 ± 1.56	5.54 ± 1.37	0.42	1.11	up
Valine	18.48 ± 3.41	8.04 ± 3	0.02	2.30	up
Threonine	84.4 ± 18.3	46.4 ± 16.1	0.2	1.82	up
Taurine	655 ± 211	420 ± 186	0.55	1.56	up
Putrescine	0.724 ± 0.144	0.299 ± 0.127	0.01	2.42	up
trans-Hydroxyproline	1.69 ± 0.598	1 ± 0.526	0.86	1.69	up
Leucine	12.4 ± 1.94	6.3 ± 1.7	0.01	1.97	up
Isoleucine	19.34 ± 6.6	7.83 ± 5.8	0.28	2.47	up
Asparagine	13.1 ± 1.79	8.47 ± 1.57	0.05	1.55	up
Aspartic acid	174 ± 36.8	127 ± 32.4	0.43	1.37	up
Glutamine	347 ± 75.1	154 ± 66	0.15	2.25	up
Glutamic acid	107 ± 31.2	80 ± 27.5	0.64	1.34	up
Methionine	3.33 ± 0.29	2.7 ± 0.255	0.06	1.23	up
Histidine	108.2 ± 24.6	53.1 ± 21.6	0.17	2.04	up
alpha-Aminoadipic acid	135 ± 26.6	72 ± 23.4	0.25	1.88	up
Phenylalanine	16.38 ± 2.72	9.87 ± 2.4	0.09	1.66	up
Methionine-sulfoxide	3.81 ± 0.948	1.86 ± 0.834	0.17	2.05	up
Arginine	15.46 ± 2.05	8.15 ± 1.8	0.01	1.90	up
Acetyl-ornithine	76.1 ± 12.5	44 ± 11	0.08	1.73	up
Citrulline	8.54 ± 2.36	3.5 ± 2.07	0.07	2.44	up
Serotonin	1.79 ± 0.297	1.37 ± 0.262	0.44	1.31	up
Tyrosine	27.4 ± 5	16.4 ± 4.4	0.11	1.67	up
Asymmetric dimethylarginine	9.48 ± 1.21	2.61 ± 1.07	<0.001	3.63	up
Total dimethylarginine	33.8 ± 4.03	18.3 ± 3.55	0.007	1.85	up
Tryptophan	36 ± 8.65	19.3 ± 7.61	0.28	1.87	up
Kynurenine	1.585 ± 0.449	0.967 ± 0.395	0.71	1.64	up
Carnosine	21.7 ± 4.39	15.5 ± 3.86	0.4	1.40	up
Ornithine	25.8 ± 4.44	14 ± 3.91	0.07	1.84	up
Lysine	82.1 ± 14.1	43.4 ± 12.4	0.08	1.89	up
Spermidine	0.2248 ± 0.0871	0.0746 ± 0.0766	0.05	3.01	up

Spermine	0.0868 ± 0.0171	0.0791 ± 0.015	0.43	1.10	up
Sarcosine	4.69 ± 1.17	1.27 ± 1.03	0.17	3.69	up
Tyramine	0.183 ± 0.042	0.111 ± 0.0501	0.26	1.65	up
Creatine	5098 ± 663	2354 ± 583	0.001	2.17	up
Betaine	265.2 ± 67.6	74.9 ± 59.4	0.02	3.54	up
Choline	92.8 ± 21.5	32.1 ± 18.9	0.007	2.89	up
Trimethylamine N-oxide	6150 ± 1895	3677 ± 1667	0.38	1.67	up
Methylhistidine	198 ± 52.3	370 ± 59.4	0.05	0.54	down
Lactic acid	125 ± 53.9	112 ± 47.4	0.93	1.12	up
beta-Hydroxybutyric acid	400 ± 471	416 ± 415	0.47	0.96	down
alpha-Ketoglutaric acid	25.7 ± 45.1	34.8 ± 39.7	0.47	0.74	down
Citric acid	856 ± 801	778 ± 684	0.84	1.10	up
Butyric acid	28.4 ± 14.7	29.5 ± 12.9	0.35	0.96	down
p-hydroxyhippuric acid	36.7 ± 14.1	37.9 ± 12.4	0.93	0.97	down
Succinic acid	30.3 ± 9.86	20.6 ± 8.67	0.69	1.47	up
Pyruvic acid	8.71 ± 1.79	6.33 ± 1.58	0.16	1.38	up
Isobutyric acid	7.23 ± 1.66	5.56 ± 1.46	0.59	1.30	up
Hippuric acid	14438 ± 2073	13225 ± 1823	0.65	1.09	up
Methylmalonic acid	29.4 ± 6.68	17.9 ± 5.87	0.31	1.64	up
Homovanillic acid	14.67 ± 1.46	8.83 ± 1.28	<0.001	1.66	up
Indole acetic acid	67.5 ± 20.7	51.3 ± 18.2	0.96	1.32	up
Uric acid	5014 ± 883	4279 ± 776	0.44	1.17	up
Glucose	3369 ± 462	1955 ± 406	0.002	1.72	up
C0	2.516 ± 0.386	0.893 ± 0.339	0.01	2.82	up
C2	0.714 ± 0.0915	0.305 ± 0.0805	0.001	2.34	up
C3:1	0.0319 ± 0.00428	0.0258 ± 0.00376	0.02	1.24	up
C3	0.0402 ± 0.00722	0.037 ± 0.00635	0.33	1.09	up
C4:1	0.0871 ± 0.00819	0.0631 ± 0.00721	0.04	1.38	up
C4	0.58 ± 0.1096	0.129 ± 0.0964	0.002	4.50	up
C3OH	0.0855 ± 0.0092	0.0642 ± 0.00809	0.11	1.33	up
C5:1	0.251 ± 0.0226	0.147 ± 0.0199	0.001	1.71	up
C5	0.1598 ± 0.0273	0.0929 ± 0.024	0.18	1.72	up

C4OH	0.0898 ± 0.00984	0.0653 ± 0.00865	0.05	1.38	up
C6:1	0.057 ± 0.0122	0.0817 ± 0.0108	0.27	0.70	down
C6	0.072 ± 0.0134	0.0872 ± 0.0118	0.77	0.83	down
C5OH	0.1372 ± 0.0131	0.0849 ± 0.0115	0.002	1.62	up
C5:1DC	0.0438 ± 0.00413	0.0377 ± 0.00363	0.23	1.16	up
C5DC	0.0469 ± 0.00595	0.0323 ± 0.00523	0.03	1.45	up
C8	0.0556 ± 0.00556	0.0356 ± 0.00489	0.003	1.56	up
C5MDC	0.0514 ± 0.00366	0.0466 ± 0.00322	0.02	1.10	up
C9	0.147 ± 0.023	0.106 ± 0.0203	0.21	1.39	up
C7DC	0.0437 ± 0.00899	0.0385 ± 0.00791	0.5	1.14	up
C10:2	0.0578 ± 0.00732	0.0437 ± 0.00644	0.19	1.32	up
C10:1	0.171 ± 0.0177	0.15 ± 0.0156	0.15	1.14	up
C10	0.135 ± 0.0125	0.104 ± 0.011	0.01	1.30	up
C12:1	0.14 ± 0.0387	0.127 ± 0.034	0.43	1.10	up
C12	0.1037 ± 0.00933	0.0943 ± 0.0082	0.01	1.10	up

[1]pre-SCM = SCM = cows that were sampled before being classified as SCM
[2]CON = healthy cows

Table 3.4. Metabolite concentration of urine metabolites MEAN ± SEM in pre-subclinical mastitis (pre-SCM, n=10) and healthy controls (CON, n=15) at –4 weeks prior to parturition as identified by LC-MS/MS.

	MEAN ± SEM				
Metabolites (μM)	Pre-SCM[1] (n=10)	CON[2] (n=15)	*P* value	Fold change	SCM/ CON
Creatinine	14300 ± 1521	10569 ± 1140	0.01	1.35	up
Glycine	105 ± 28.3	67.5 ± 21.2	0.1	1.56	up
Alanine	97.5 ± 11.7	82.5 ± 8.8	0.23	1.18	up
Serine	77 ± 10.39	69 ± 7.79	0.1	1.12	up
Histamine	0.0994 ± 0.0154	0.0642 ± 0.0116	0.12	1.55	up
Proline	3.63 ± 0.531	3.94 ± 0.398	0.44	0.92	down
Valine	11.1 ± 1.185	10.3 ± 0.888	0.28	1.08	up
Threonine	70.7 ± 10.08	50.9 ± 7.56	0.01	1.39	up
Taurine	439 ± 136	395 ± 102	0.71	1.11	up
Putrescine	0.915 ± 0.503	1.167 ± 0.377	0.84	0.78	down
trans-Hydroxyproline	1.22 ± 0.425	1.92 ± 0.318	0.3	0.64	down
Leucine	9.1 ± 0.99	9.13 ± 0.742	0.38	1.00	up
Isoleucine	7.72 ± 0.745	5.98 ± 0.558	0.007	1.29	up
Asparagine	13.46 ± 1.48	9.67 ± 1.11	0.01	1.39	up
Aspartic acid	190 ± 31.7	131 ± 23.7	0.17	1.45	up
Glutamine	285 ± 46.8	206 ± 35.1	0.04	1.38	up
Glutamic acid	78.6 ± 11.7	53.6 ± 8.8	0.09	1.47	up
Methionine	3.34 ± 0.252	3.25 ± 0.189	0.4	1.03	up
Histidine	76.6 ± 9.85	60.4 ± 7.38	0.05	1.27	up
alpha-Aminoadipic acid	79.9 ± 14.5	72.9 ± 10.8	0.31	1.10	up
Phenylalanine	13.1 ± 1.08	10.4 ± 0.81	0.03	1.26	up
Methionine-sulfoxide	3.12 ± 0.478	3.13 ± 0.358	0.83	1.00	up
Arginine	11.3 ± 1.316	10.1 ± 0.986	0.48	1.12	up
Acetyl-ornithine	57 ± 6.82	47.4 ± 5.11	0.08	1.20	up
Citrulline	3.47 ± 1.265	6.94 ± 0.948	0.17	0.50	down
Serotonin	1.66 ± 0.202	1.33 ± 0.151	0.13	1.25	up
Tyrosine	19.4 ± 2.38	20 ± 1.78	0.8	0.97	down
Asymmetric dimethylarginine	8.39 ± 1.024	6.26 ± 0.768	0.02	1.34	up
Total dimethylarginine	36.2 ± 3.36	26.4 ± 2.52	0.008	1.37	up
Tryptophan	19.8 ± 3.17	17.8 ± 2.38	0.58	1.11	up
Kynurenine	0.735 ± 0.0835	0.696 ± 0.0625	0.94	1.06	up
Carnosine	14.7 ± 1.61	11.1 ± 1.21	0.03	1.32	up
Ornithine	16.7 ± 2.1	15.5 ± 1.58	0.6	1.08	up
Lysine	59.7 ± 5.68	48.5 ± 4.26	0.07	1.23	up

Spermidine	0.0772 ± 0.0256	0.1113 ± 0.0192	0.36	0.69	down
Spermine	0.1219 ± 0.0113	0.0596 ± 0.015	0.008	2.05	up
Sarcosine	3.04 ± 2.02	6.92 ± 1.51	0.04	0.44	down
Tyramine	0.133 ± 0.0237	0.113 ± 0.0188	0.82	1.18	up
Creatine	5737 ± 1558	6460 ± 1168	0.69	0.89	down
Betaine	134 ± 95.7	364 ± 71.7	0.06	0.37	down
Choline	18 ± 12.98	56.9 ± 9.73	0.05	0.32	down
Trimethylamine N-oxide	5083 ± 1353	1338 ± 1014	0.03	3.80	up
Methylhistidine	373 ± 33.7	246 ± 25.2	0.001	1.52	up
Lactic acid	256 ± 83.6	107 ± 62.6	0.13	2.39	up
beta-Hydroxybutyric acid	116 ± 60.2	135 ± 45.1	0.6	0.86	down
alpha-Ketoglutaric acid	129 ± 66.6	17.8 ± 49.9	0.07	7.27	up
Citric acid	1911 ± 723	91 ± 542	0.04	21.00	up
Butyric acid	11.59 ± 2.18	7.43 ± 1.63	0.09	1.56	up
p-hydroxyhippuric acid	44.9 ± 17.2	53.9 ± 12.9	0.96	0.83	down
Succinic acid	42.3 ± 14.7	17.9 ± 11	0.22	2.36	up
Pyruvic acid	21.87 ± 6.85	7.63 ± 5.13	0.08	2.87	up
Isobutyric acid	5.14 ± 0.753	2.71 ± 0.564	0.009	1.90	up
Hippuric acid	20896 ± 3944	17503 ± 2955	0.32	1.19	up
Methylmalonic acid	25.2 ± 4.18	10.5 ± 3.13	0.001	2.40	up
Homovanillic acid	13.55 ± 1.88	8.01 ± 1.41	0.03	1.69	up
Indole acetic acid	58.5 ± 9.75	36.4 ± 7.3	0.06	1.61	up
Uric acid	4707 ± 748	3036 ± 561	0.05	1.55	up
Glucose	387 ± 555	973 ± 416	0.35	0.40	down
C0	1.77 ± 0.167	1.15 ± 0.125	0.01	1.54	up
C2	0.685 ± 0.1239	0.685 ± 0.0928	0.75	1.00	up
C3:1	0.0501 ± 0.00367	0.047 ± 0.00275	0.9	1.07	up
C3	0.0492 ± 0.00432	0.0573 ± 0.00324	0.17	0.86	down
C4:1	0.0729 ± 0.0089	0.0767 ± 0.00667	0.86	0.95	down
C4	0.484 ± 0.0904	0.423 ± 0.0677	0.24	1.14	up
C3OH	0.0695 ± 0.00729	0.0714 ± 0.00546	0.83	0.97	down
C5:1	0.259 ± 0.0338	0.152 ± 0.0253	0.005	1.70	up

C5	0.155 ± 0.025	0.154 ± 0.0187	0.88	1.01	up
C4OH	0.0785 ± 0.00747	0.0739 ± 0.0056	0.52	1.06	down
C6:1	0.0691 ± 0.0081	0.0841 ± 0.00607	0.28	0.82	down
C6	0.0857 ± 0.0145	0.1026 ± 0.0108	0.61	0.84	down
C5OH	0.14 ± 0.0152	0.109 ± 0.0114	0.02	1.28	up
C5:1DC	0.0453 ± 0.00418	0.0349 ± 0.00313	0.07	1.30	up
C5DC	0.048 ± 0.00412	0.0282 ± 0.00309	<0.001	1.70	up
C8	0.0483 ± 0.00476	0.0428 ± 0.00357	0.33	1.13	up
C5MDC	0.0483 ± 0.0046	0.0482 ± 0.00345	0.79	1.00	up
C9	0.152 ± 0.0188	0.11 ± 0.0141	0.04	1.38	up
C7DC	0.0488 ± 0.00611	0.0281 ± 0.00458	0.005	1.74	up
C10:2	0.0466 ± 0.00574	0.0514 ± 0.0043	0.96	0.91	down
C10:1	0.204 ± 0.0213	0.176 ± 0.016	0.23	1.16	up
C10	0.12 ± 0.0151	0.136 ± 0.0113	0.84	0.88	down
C12:1	0.083 ± 0.0315	0.229 ± 0.0236	0.002	0.36	down
C12	0.0444 ± 0.0184	0.091 ± 0.0138	0.04	0.49	up

[1]pre-SCM = SCM = cows that were sampled before being classified as SCM
[2]CON = healthy cows

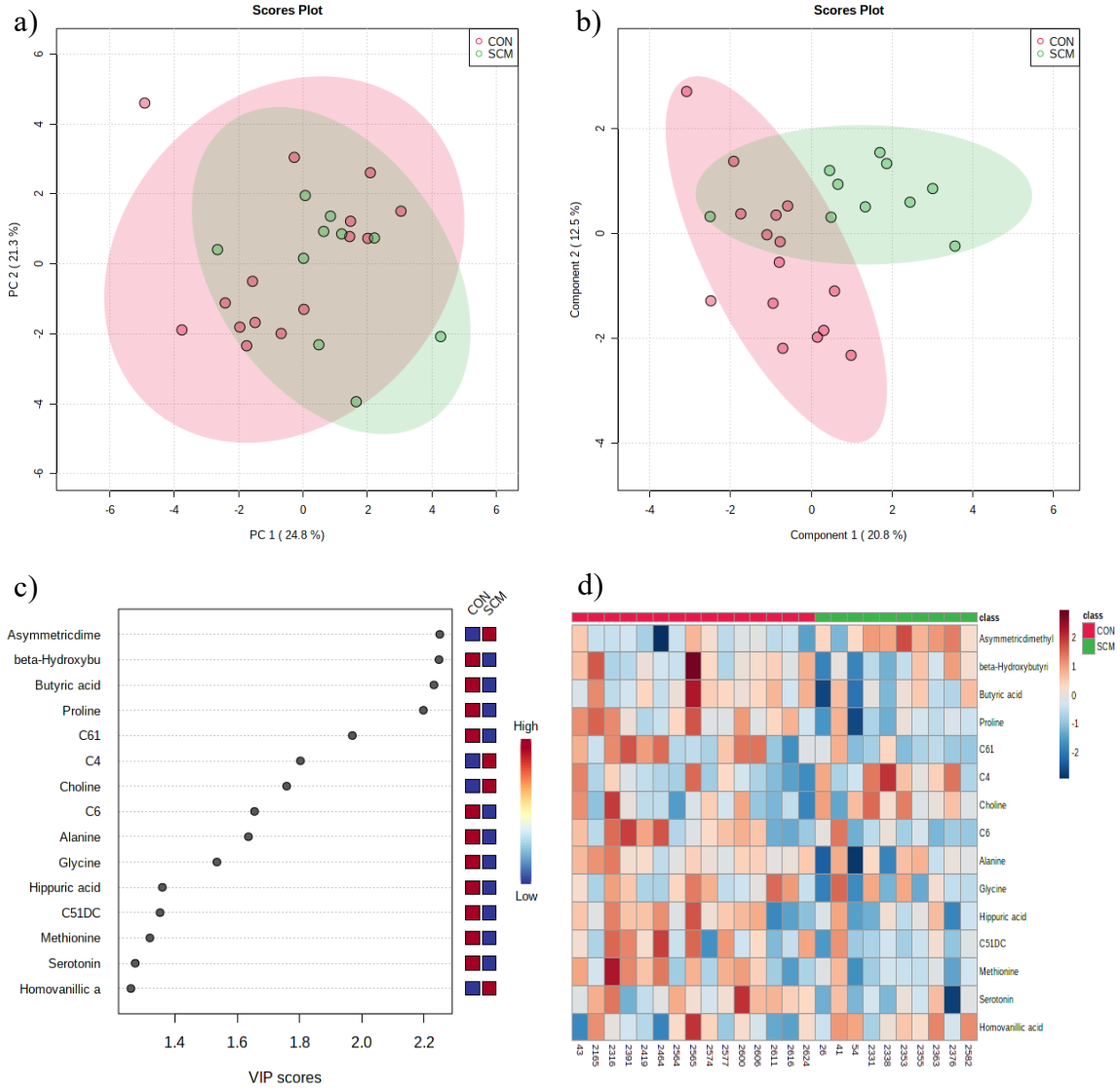


Figure 3.1. a) Principal Component Analysis (PCA) and **b)** Partial Least Square – discriminant analysis (PLS-DA, $P > 0.05$), showing the classification for CON and pre-SCM metabolites at –8 weeks before parturition; **c)** Metabolites ranked by variable importance in projection (VIP), and **d)** Heatmap of both, samples and features based on PLS-DA, to further investigate the identified variables.

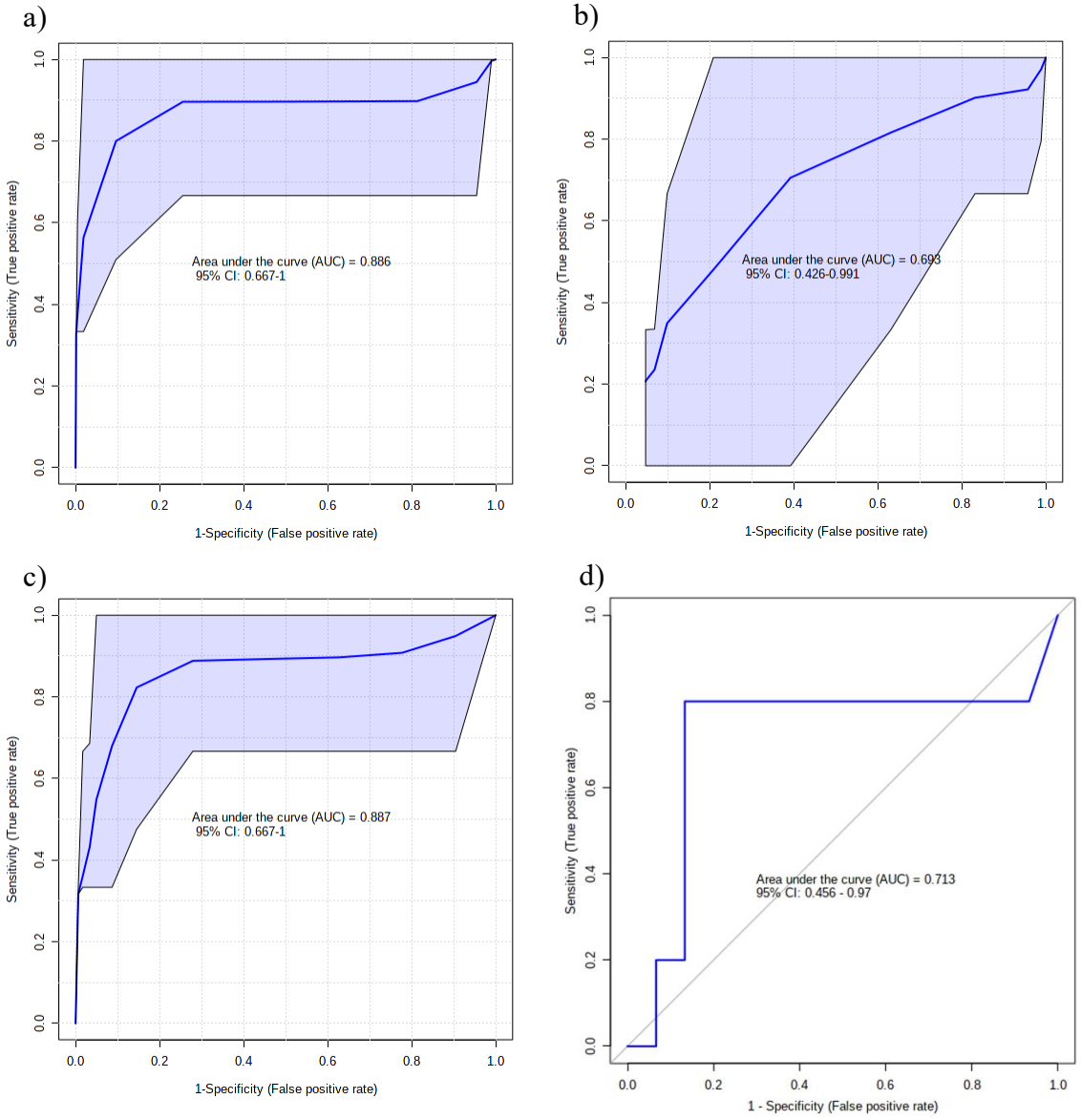


Figure 3.2. a) AUC of the top 5 metabolites identified from the VIP plot; **b)** AUC of the best biomarker model through automated important feature identification, 10 in this case; **c)** The linear SVM model of a specific panel of biomarkers, ADMA, Pro, Leu, HVA (AUC = 0.88, $P = 0.02$), and **d)** ROC plot with 10-fold CV for logistic regression model of these 4 amino acids (AUC = 0.71, $P = 0.04$).

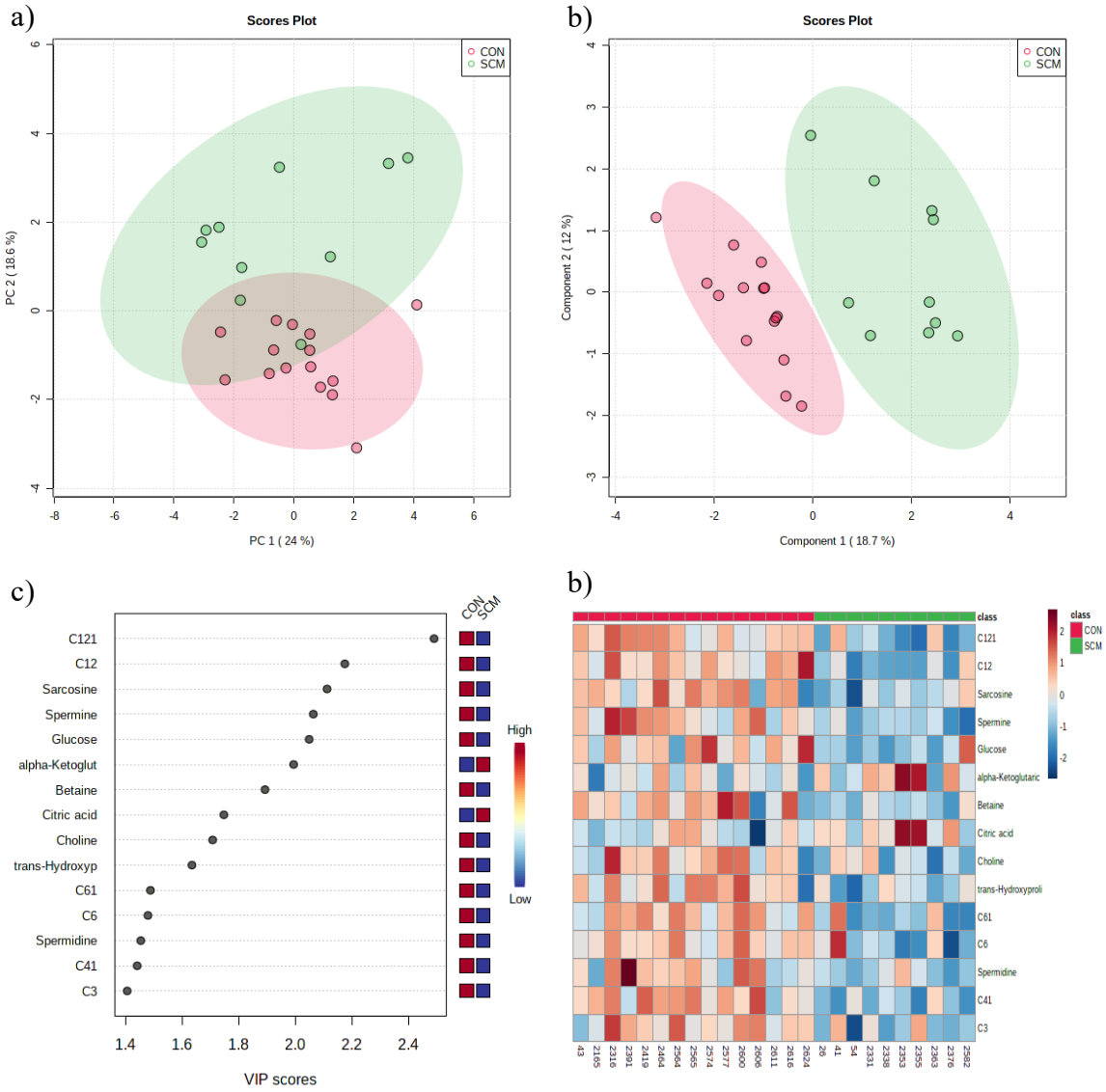


Figure 3.3. a) PCA and **b)** clear separation of PLS-DA ($P > 0.05$) of 15 CON and 10 SCM; **c)** VIP plot of top 15 important features (metabolites), and **d)** Heatmap of both, samples and features based on PLS-DA to further investigate the classification of the variables.

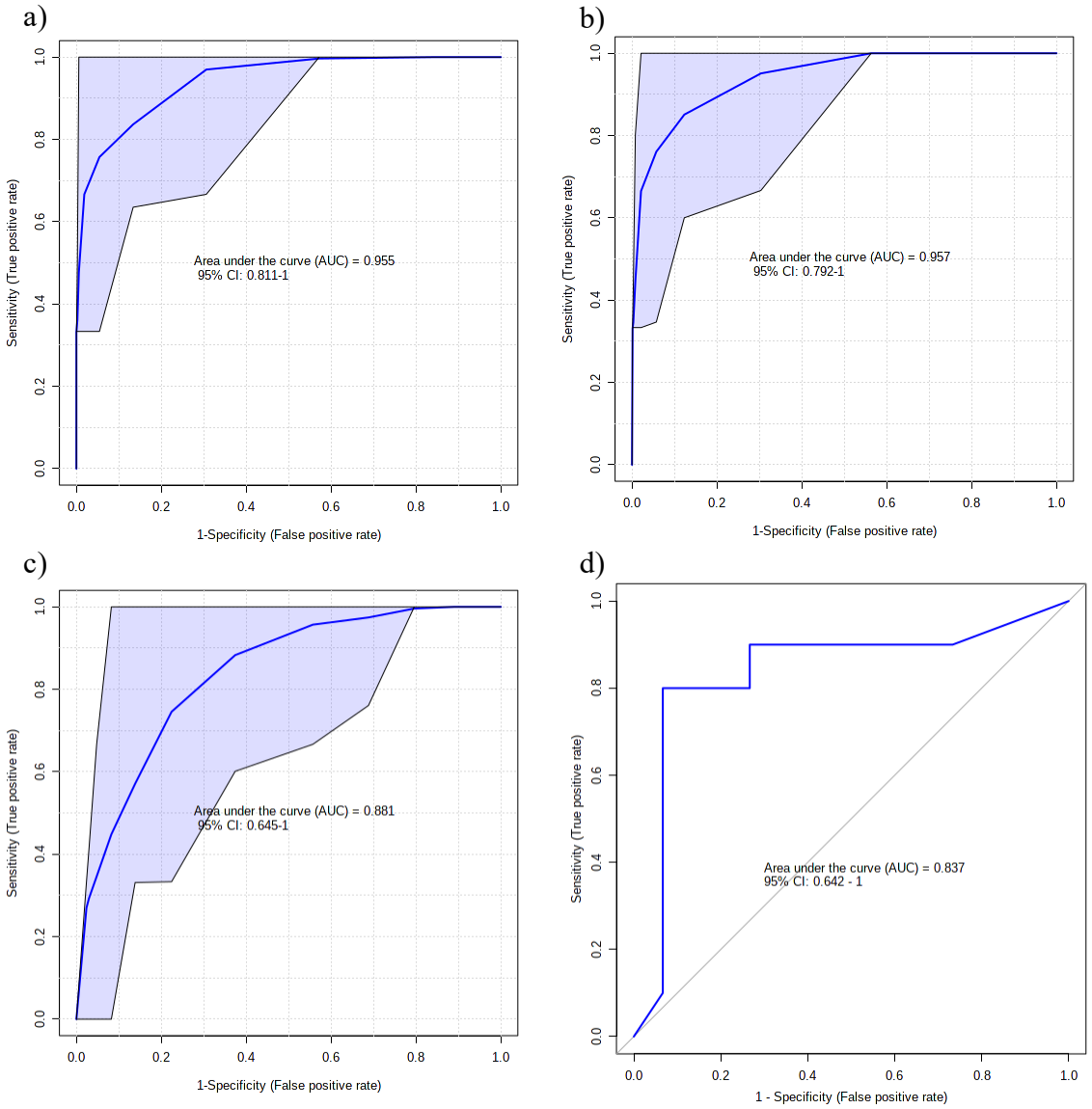
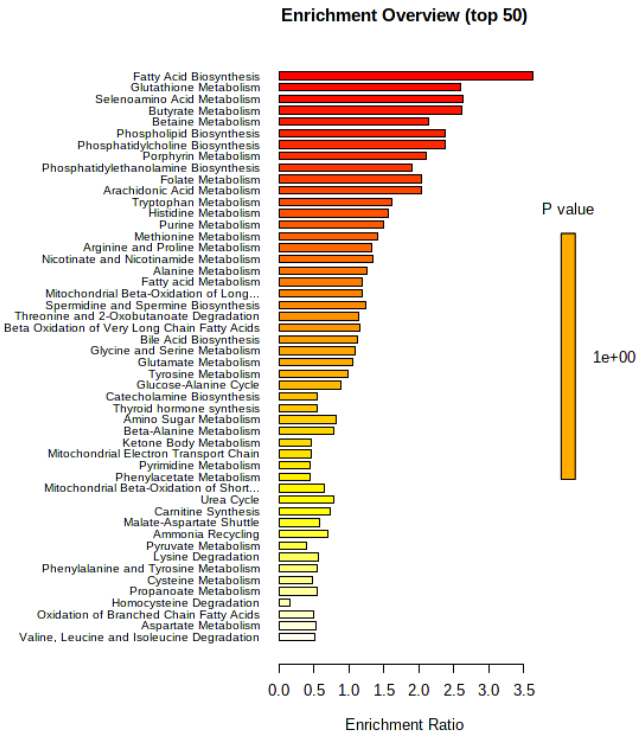


Figure 3.4. a) AUC of the top 5 VIP PLS-DA metabolites; **b)** AUC of the best biomarker model's classification; **c)** default linear SVM AUC model (AUC = 0.88, $P = 0.03$), and **d)** AUC of logistic regression model (AUC = 0.83, $P = 0.05$).

a)



b)

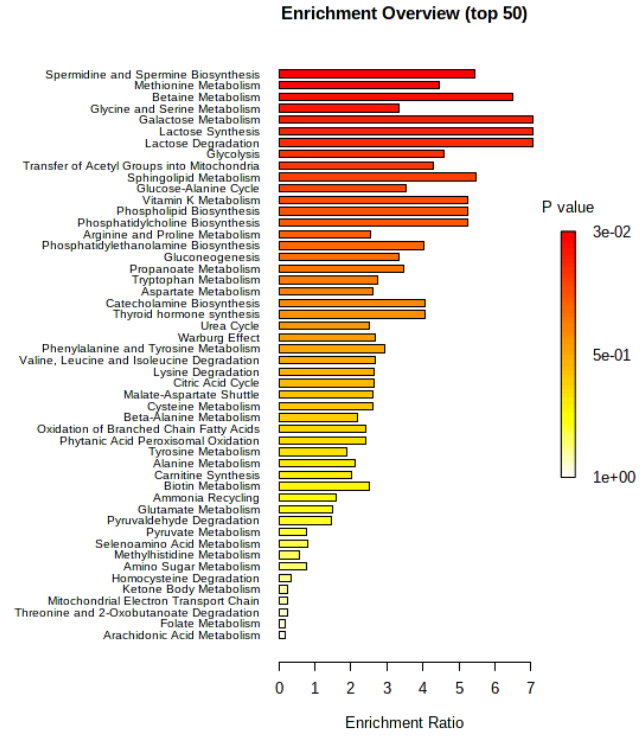


Figure 3.5. Summary plots for quantitative enrichment analysis (QEA) from the MSEA at **a)** –8 wks, and **b)** –4 wks**.**

Chapter 4 – Overall Discussion and Conclusions

4.1 Alterations of blood and urinary metabolites precede the occurrence of subclinical mastitis in dairy cows

A metabolomics approach was used to investigate serum and urinary metabolic signatures of pre-SCM cows at –8 and –4 weeks prior to parturition and after been diagnosed with SCM after calving. Our primary hypotheses were that: 1) Cows affected postpartum by SCM might show blood metabolic changes at the beginning of dry off (–8 wks prepartum) as well as at –4 wks prior to parturition that can be detected by MS-based metabolomics; 2) Cows affected postpartum by SCM might show urinary metabolic changes at –8 and –4 wks prior to parturition that can be detected by MS-based metabolomics; 3) Both blood and urinary metabotypes show specific biomarkers for dairy cows at risk of mastitis during the dry-off period.

This study contributes new data on the metabolic response of dairy cows to subclinical mastitis as related to various metabolite classes and species during the dry-off period when cows look healthy from the clinical point of view. Additionally, these data revealed a number of metabolic pathways involved in the host response to subclinical mastitis. These results throw some light on how the various metabolites might support or oppose the host response to the subclinical condition.

Indeed, our results demonstrated that by using an MS-based metabolomics approach we could identify metabolic fingerprints in the serum of pre-SCM cows that differentiated them from the healthy controls, at both –8 wks and –4 wks prior to the expected date of parturition. Specifically, around 36.5% of the metabolites measured in the

serum were altered in the pre-SCM cows compared to healthy ones from the beginning of the dry off period. The reason for these changes is not clear yet; however, it is speculated that they might be related to the subclinical infection of the udder during the dry off period (Eckel and Ametaj, 2016; Zwierzchowski et al., 2020). The metabolites identified as being significantly altered belong to five chemical groups including lipids, AAs, methyl donors as well as organic acids and carbohydrates. We were also able to identify and build several serum panels of biomarkers that could be used to monitor dairy cows for the risk of being affected by SCM, well in advance of occurrence of the disease. The top 4 metabolites were selected and fitted with logistic regression model for each time point (–8 and –4 wks prepartum). Our data also showed that urinary metabotyping of pre-SCM cows, at both –8 and –4 wks prepartum, can be used to distinguish pre-SCM cows from healthy controls through a number of metabolite species that were identified as being significantly altered. Two urinary panels of potential biomarkers were also identified that could be used to predict the risk of SCM starting from –8 and –4 wks prepartum. The details of these findings will be discussed below.

4.2 Identification of monitoring biomarkers in the serum of pre-subclinical mastitis dairy cows

In chapter 2 we identified that 36.5% of all metabolites measured in the serum could be used to differentiate pre-SCM cows from the healthy controls at –8 and –4 wks prepartum. Most of the metabolites identified as significantly changed were related to lipid metabolism, AA metabolism, methyl-donor metabolism as well as organic acids and glucose. Four metabolites, from each period, were selected to construct a panel of

biomarkers with predictive potential for cows predisposed to SCM at the beginning and the middle of the dry off period. More specifically, at the beginning of the dry off period (–8 wks from the expected date of parturition) 4 metabolites were used to construct a panel of biomarkers with excellent predictive accuracy. These metabolites included alanine, leucine, betaine, and ornithine (AUC = 0.92). Another panel of biomarkers was constructed for the other time point studied (–4 wks prior to parturition) that can also be used to screen cows for the risk of developing subclinical or clinical mastitis. Alanine appeared again as an important metabolite that can differentiate pre-SCM cows from the healthy controls, together with 3 organic acid species including lactate, pyruvate, and methylmalonate (AUC = 0.92). These two panels of biomarkers were validated using a permutation test of 1000 random models to assess this support vector machine (SVM) accuracy. The predictive accuracy of the logistic regression model was assessed with a 10-fold cross validation. The area under the curve (AUC) for both biomarker models and both types of analysis was significant ($P \leq 0.05$). The logistic regression model revealed an AUC = 0.81 and P = 0.05. Whereas for the –4 wks prepartum time point, the logistic regression analysis showed an AUC = 0.81, P = 0.04. These results show that the panels of biomarkers identified can provide a "good" accuracy test. The guide for assessing the utility of a biomarker panel is based on its AUC as follows: 0.9~1.0 = excellent; 0.8~0.9 = good; 0.7~0.8 = fair; 0.6~0.7 = poor; 0.5~0.6 = fail.

4.3 Identification of predictive biomarkers in the urine of pre-subclinical mastitis cows

As already explained throughout this thesis, the current diagnostic approach for subclinical or clinical mastitis is counting of somatic cells in the milk, which is performed

during the lactation period, starting after parturition. There are no predictive or monitoring tests available to foresee the risk of dairy cows developing SCM or CM during the dry off period. In chapter 3, data showed that it is possible to use the two panels of urinary biomarkers identified in this study to screen cows during the dry-off period for the risk of being affected by SCM. More specifically, at –8 wks a logistic regression model that used a combination of asymmetric dimethylarginine (ADMA), leucine, proline, and homovanillic acid was constructed to forecast SCM (AUC = 0.71, $P = 0.04$). Another 4-metabolite biomarker profile was constructed at –4 wks prior to calving that included ADMA, methylmalonate, spermidine, and citrate. The predictive accuracy of this logistic regression model was AUC = 0.83, $P = 0.05$. It should be noted that these panels of metabolites need to be further validated in another larger study. Our urine metabolomics study found that these 4 metabolites as well as another 23 metabolites (not part of the panel) were significantly different at –8 and –4 wks, respectively. These compounds belonged to the classes of ACs, organic acids as well as AAs and their derivatives.

4.4 Identification of perturbed pathways involved in pre-subclinical mastitis cows

Metabolite set enrichment analyses (MSEA) indicated several perturbed metabolic pathways at –8 wks prior to the diagnosis of subclinical mastitis. Most significant pathways were discovered in the serum samples. At the beginning of the dry off period, glycine and serine, methionine, and betaine metabolisms played key roles in the separation of pre-SCM and CON cows. These are all important pathways related to the one-carbon metabolism. At –4 wks before calving, the glucose-alanine cycle and seleno-amino acid metabolism were identified as important pathways, due to their role as energy providers. With regard

to the results from our urine studies, spermidine and spermine biosynthesis was the only perturbed pathway detected at –4 wks prior to calving.

4.5 Host metabolite responses suggest that some of the serum and urinary metabolites support host proinflammatory responses and others contribute to keeping inflammatory responses under control

Based on our previous research and research conducted by other labs along with the detailed discussion of the data in chapters 2 and 3, it can be concluded that most of the metabolites that differentiated the pre-SCM cows from healthy controls in both serum and urine might be grouped into: 1) those that support the proinflammatory response of the host to the potential bacterial by-products or cytokines generated in the mammary gland in cows affected by subclinical mastitic, and 2) those that contribute to keep the host inflammatory response under control. Overall, these metabolite responses in both serum and urine seem to be related to the presence of chronic low-grade inflammatory state in pre-SCM cows at –8 and –4 wks prepartum, as identified and reported in a similar study conducted by our lab (Dervishi et al., 2015).

In support of this hypothesis are the identified alterations in the lipid classes and lipid species in the serum of pre-SCM cows. For example, lowered concentrations of PCs in the blood in pre-SCM cows might support a chronic low-grade inflammatory state in those cows and unfortunately predispose them to developing fatty liver (Dervishi et al., 2015). Considering that multiple species of SMs also were lowered in pre-SCM cows and given their reported positive effects on neutrophil migration and their killing capacity

(Nakamura et al., 1994; Feldhaus et al., 2002; Sitrin et al., 2010) the lack of SMs in the circulation might increase the host susceptibility to infections. Beside the lowered concentration of multiple metabolite species of PCs and SMs, our data also showed lowered concentration of several serum LPCs. The latter have been shown to support the mounting of an inflammatory response. Again, lowered LPC metabolites during the dry off period are supportive of the idea that the host is trying to keep the inflammatory response under control.

As already discussed in chapter 2, serum BCAA (valine, leucine, and isoleucine) were increased in pre-SCM cows. These metabolites have been reported to support mounting of an efficient proinflammatory response (Zhenyukh et al., 2017). These three AAs are essential for protein synthesis, stimulation of secretion of proinflammatory cytokines, and migration of neutrophils to the site of infection (Calder, 2006; Zhenyukh et al., 2017). Besides BCAA, several other serum metabolites with proinflammatory activities were increased in the serum including arginine and ornithine. Additionally, two other proinflammatory AAs including glycine and serine were lowered potentially to keep the inflammatory response within acceptable limits.

In order for the immune cells to grow, proliferate, and respond properly to the inflammatory stimuli, they require energy. A potential source of energy for immune cells is alanine, which was lowered in the circulation of pre-SCM. This could reflect the extensive utilization of alanine by the immune cells but also by the liver for mounting of an acute phase response (Druml et al., 2001; Li et al., 2007; Garcia et al., 2016). Two other metabolite species that were elevated in the serum of pre-SCM cows were choline and

betaine. Both these metabolites have been shown to lower the proinflammatory response (Go et al., 2005, 2007; Abdelmegeid et al., 2017; Garcia et al., 2018).

Overall, our results for the serum findings suggest that it is possible that cows under a subclinical state of mastitis mobilize or lower their metabolic responses based on whether those metabolites support or oppose the inflammatory response of the immune cells to the inflammatory stimuli. Most of the identified metabolites support the mounting of an immune response; however, several others either suppress the immune responses or are lowered to not provide more fuel to the immune cells. This is an interesting finding that certainly needs to be pursued in the future.

Regarding the urinary metabolite species identified as altered in the pre-SCM cows, it should be noted that ACs were excreted in much higher quantities in the urine of pre-SCM cows than in the CON ones. Urinary data align with the serum findings that showed increased concentrations of multiple species of ACs. It is obvious that high concentration of ACs in the serum are associated with increased excretion of these compounds in the urine. There is mounting evidence that during inflammatory states there is some malfunctioning of β-oxidation of fatty acids in the mitochondria, leaving large amounts of unoxidized AC species in the cytoplasm and then moving those into the systemic circulation (Minuti et al., 2015).

Results showed that there was higher excretion of histidine and methyl-histidine in the urine of pre-SCM cows. Moreover, BCAA and arginine also were excreted in higher amounts in the pre-SCM cows versus healthy cows. It is possible that these metabolic responses might allow the host to elicit a proportional immune response (Wu et al., 2016). Disproportional immune responses might sicken the host instead of contributing to

resolving the inflammatory status. Data also showed that the host responded with higher excretion of anti-inflammatory compounds such as choline, betaine, ADMA, and TDMA, which can be considered as a metabolic host response to suppress the proinflammatory reaction against the presence of a potential subclinical infection in the udder (Go et al., 2005; Parrish et al., 2008; Tsikas, 2017; Tsikas et al., 2018). Beside the host urinary responses to lower the metabolomic proinflammatory load in the systemic circulation and body fluids, cows responded to subclinical mastitis through urinary excretion of several other metabolites that could impair the immune response itself. For example, higher glucose in the blood has been reported to impair functions of neutrophils and NK cells (Kim et al., 2019). Therefore, higher excretion of glucose in the urine might diminish its negative effects on immune cells and host immune responses. On the other hand, higher excretion of urinary citrate in pre-SCM cows is another example of a host response that lowers the metabolic anti-inflammatory load (Infatino et al., 2013).

Overall, the excretion of the anti-inflammatory compounds through the urine seems to be a response from the pre-SCM cows trying to support an inflammatory response. However, at the same time, some of those metabolites build up into the systemic circulation to keep the inflammatory response under control.

4.6 Shared and specific metabolite species in cows affected by pre-SCM and those affected by pre-SCM and another disease

We have thoroughly discussed serum and urinary metabolite differences and alterations between pre-SCM cows and the healthy controls in chapters 2 and 3.

Considering that 34 cows (out of a total of 44 affected by SCM) in our study were affected by SCM and another disease (SCM-P) this is an important issue with respect to our final goal of developing pen-side tests for predicting SCM in dairy cows. Given the presence of concurrent diseases in cows affected by SCM, this might complicate the development of pen-side tests specific for SCM. We will discuss these data briefly in order to identify shared and specific metabolites for cows affected by SCM only (SCM-O) and those that were affected by SCM and another disease (SCM-P).

It should be noted that there was a total of 44 cows that were diagnosed postpartum with SCM (SCM-T); however, only 10 cows were affected by SCM-O. There were also 20 cows affected by SCM and leukosis, 9 cows affected by SCM and ketosis, 2 cows affected by SCM and milk fever, and 2 cows affected by SCM, ketosis, and retained placenta, and 1 cow affected by SCM and lameness. All the cows affected by SCM and one or more diseases are identified as SCM plus (SCM-P). It should be noted that detailed discussion of these data is beyond the objective of this thesis; however, we will discuss them briefly as a particularly important finding of this study.

Results obtained from the serum analyses at –8 wks prepartum demonstrated that there was a total of 52 metabolites ($P \leq 0.05$) that differentiated SCM-O and SCM-P from the CON (healthy) cows (Table 4.1). Out of those, only 8 metabolite species were shared by SCM-O and SCM-P including Gly, Val, carnosine, alpha-aminoadipate, LPC-C18:1, LPC-C18:2, C5DC, and C14:1OH. The 8 shared metabolites were not different between SCM-O and SCM-P; however, those metabolites differentiated both groups from the healthy controls. Five of the metabolites were lowered in all cows affected by SCM-T (T for total) vs healthy controls and 3 others were higher in SCM-T, at –8 wks prepartum.

Given that these metabolites differentiated all cows affected by SCM (SCM-T), independently whether they were affected by SCM-O or by SCM-P, then, these metabolites might serve as potential screening biomarkers for SCM.

Another 27 serum metabolites (at –8 wks) were specific only to SCM-O cows. There were only 2 metabolites (hippurate and LPC-C20:4) that resulted to be specific for SCM-P cows. Another 15 metabolites were not specific to any of the SCM conditions. They included lipid species such as 3 LPC- (C26:1, C28:0; and C28:1), 3 PCs (PC ae C36:0, PC aa C36:6, and PC aa C38:0), 4 SMs (14:1-SMOH, 16:1SMOH, 18OSM, and 22:1SMOH), and 5 ACs (C0, C5:1DC, C6:1, C8, and C12:1).

At –4 wks prepartum there was a total of 40 metabolite species ($P \leq 0.05$) that differentiated SCM-O and SCM-P from the healthy control cows (Table 4.2). Only 3 metabolites were common to both SCM-O and SCM-P (alanine, proline, and methionine-sulfoxide). Another 15 metabolites were specific only for the SCM-O cows (ornithine, choline, methylmalonate, lactate, LPC-C28:1, PC aa C36:6, PC aa C40:1, PC aa C40:6, 18:OSM, 22:2SMOH, C5:1, C5DC, C9, C10:2, and C14:1). The group of cows affected by SCM-P had 4 metabolite species that were specific for this group only and these included betaine, LPC-C18:0, LPC-C18:2, and C3OH. The other 17 metabolites including 1 AA (methionine), 2 LPCs, 7 PCs, 2 SMs, and 5 ACs were not shared between the two SCM groups and were not specific for either SCM-O or SCM-P.

Regarding urinary data it is interesting to note that there were no significant differences between the SCM-P and healthy cows. All 21 metabolites ($P \leq 0.05$) identified as altered at –8 wks (Table 4.3) belonged to SCM-O cows; they differentiated this group

from the healthy controls and were higher than both SCM-P and CON cows. These metabolites can be considered as specific for SCM-O cows.

There was a total of 14 urinary metabolites at –4 wks prepartum for both SCM-O and SCM-P cows (Table 4.4). Our data showed that there were no shared or common metabolites between SCM-O and SCM-P cows at this sampling date. However, there were 12 metabolites (isoleucine, glutamine, threonine, carnosine, methylhistidine, methylmalonate, pyruvate, citrate, lactate, urate, C5DC, and C12:1) that were specific for SCM-O cows. Two metabolites (homovanillic acid and C5:1DC) were found to differentiate the two SCM groups but there were no differences between the two SCM groups and healthy control cows.

Overall, it can be concluded that there is a potential to identify cows that are affected by SCM independently of whether they are free of other diseases (SCM-O) or whether they are affected by SCM and one or more other diseases (SCM-P) based on 8 identified shared serum metabolites. Additionally, the same can be said for the 3 shared serum metabolites between SCM-O and SCM-P, at –4 wks prepartum.

Regarding our urinary results it appears that there are no shared metabolites identified between SCM-O and SCM-P. This means that, at this time, no SCM-T test can be developed for urine. The only metabolites identified as important in the urine for both –8 and –4 wks prepartum are those that are specific for SCM-O and not for the SCM-P group.

4.7 Panels of common selected biomarkers between SCM-O and SCM-P at –8 and –4 weeks compared with healthy controls

The last question for this study was whether we could identify panels of biomarkers that could be used under a central lab conditions or as a pen-side test to monitor cows for the risk of being affected by SCM. Considering that there were only 8 serum metabolites commonly shared between SCM-O and SCM-P cows at –8 wks prepartum, we evaluated various combinations of metabolites to generate the highest accuracy test possible. Indeed, the combination of alpha-aminoadipate, C14:1OH, and C5DC yielded the best logistic regression curve with the highest accuracy for a potential lab test (Figures 4.1.a; 4.1.b). Cows affected by SCM-O demonstrated an AUC of 0.81 for a potential test (with those 3 metabolites (P = 0.05), whereas cows affected by SCM-P presented an AUC of 0.86 for a test with the same 3 metabolites (P = 0.001). On the other hand, glycine and carnosine showed considerable utility for potential pen-side tests to detect SCM-T. Logistic regressions of glycine and carnosine for SCM-O cows showed an AUC of 0.79 for the test (P = 0.05) (Figure 4.2.a), whereas the AUC for SCM-P was 0.71 (P = 0.05) (Figure 4.2.b). These data show that it should be possible to develop both a central lab test and a pen-side test for all cows at risk of SCM even though they might be affected by only SCM or have one or more concurrent periparturient diseases, at –8 wks prepartum. On the other hand, cows at –4 wks prior to parturition (both SCM-O and SCM-P cows) had only 3 shared serum metabolites. Biomarker analysis showed that alanine and methionine-sulfoxide displayed an AUC of 0.88, for a potential test for identifying dairy cows at risk of SCM-O (P=0.03) (Figure 4.3.a). The same model for SCM-P had an AUC of 0.72, for a potential test, although the test was only marginally significant (P = 0.06) (Figure 4.3.b).

Finally, since no shared metabolites were found in the urine of both SCM-O and SCM-P cows, it appears that it is not yet possible to develop a test to detect cows at risk of SCM at –8 or –4 wks prepartum by using the metabolites from the current data.

4.8 Future Implications

Results of this study have shown that there is a possibility to develop both a central lab test and a pen-side test for identifying dairy cows at risk of being affected by SCM postpartum. In a cohort of 145 dairy cows included in the study, at the beginning and the middle of the dry-off period (–8 and –4 wks prepartum), 44 cows were diagnosed with SCM postpartum. Analysis of the MS-based metabolomics data, for both serum and urine, were complicated by the fact that just 10 dairy cows were affected by SCM only. Meanwhile, there was a total of 34 cows that were diagnosed with SCM and one or more other disease. This finding challenges the aim of developing a pen-side or a lab test to identify cows at risk of being affected by SCM because another concurrent disease might affect the metabolic responses and metabolite species released into systemic circulation and excreted in the urine from the host. However, a thorough analysis of all the data generated showed that a potential test for identifying dairy cows at risk of SCM, both at –8 and –4 wks prepartum, independently whether they are affected only by SCM or SCM and another disease(s), could be developed.

Interestingly the data from this study showed that the serum and urinary metabolites identified in a previous similar study, conducted at DRTC dairy farm, were different from the panels of metabolites identified in this study (Dervishi et al., 2017, Zwierzchowski et al., 2020). In our previous study the most important metabolites that were selected for a

potential monitoring test for the risk of SCM at –8 wks prior to parturition were trans-aconitate, hypoxanthine, leucine, and xylose. The AUC was 0.95, or 95% sensitivity and specificity of the test for identifying cows at risk of SCM only. The precise reason for this discrepancy it is not known. However, some of the reasons for the differences in metabolite species might be that the cohorts of dairy cows were selected from two different farms. Currently, we do not know whether feeding or the ration composition might affect each cows' metabolite responses to the development of SCM. Another more important reason for the discrepancy might be the different instruments used for identifying and quantifying the potential serum biomarkers. In our previous study NMR (Nuclear Magnetic Resonance) was used to analyze the serum samples. In the current study we used a LC-MS to identify and measure serum metabolites. In particular, the LC-MS platform we used could not measure three of the four NMR-detected metabolites: xylose, trans-aconitate or hypoxanthine.

Another question to consider is whether we will use specific metabolites to screen cows for each disease separately or construct panels of metabolites that can identify cows that will be affected by a serial or concurrent periparturient diseases that might be considered as part of a general sickness syndrome. Given that the identified metabolite panels in Chapters 2 and 3 show high accuracy for the detection of SCM only, such a test would leave out cows that were affected by SCM and another disease. Additionally, given that only 10 cows were diagnosed with SCM only, out of a total of 44 affected by SCM, there were 34 cows affected by SCM and another disease(s). Searching for high sensitivity and specificity biomarkers for only one disease (e.g., SCM), we can identify only 7% of cows affected by SCM-O in a herd where the prevalence of SCM is 33% of all the dairy

cows in a farm. The question is whether we should move to reductionist approach to identify one disease at a time with one or two specific metabolites or whether we should expand our approach and apply systems biology to better understand periparturient diseases and identify them at the very earliest stage in disease development. This suggests that to move forward we will need to develop very sophisticated pen-side tests with a multitude of metabolites in order to increase the accuracy, the sensitivity and the specificity of the tests for predicting the risk of all cows affected by SCM, independently of concurrent diseases.

4.9 References

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Table 4.1. Serum metabolites at –8 wks before calving between the three groups of cows and their respective *P* values for multiple comparison groups.

Metabolites (μM)	MEAN ± SEM[1]			*P* value	*P* value[2]		
	SCM-O[3]	SCM-P[4]	CON		SCM-O vs CON	SCM-P vs CON	SCM-O vs SCM-P
Glycine	319 ± 23.8	335 ± 12.9	389 ± 19.5	0.03	0.03	0.03	0.62
Isoleucine	142 ± 7.85	120 ± 4.26	117 ± 6.41	0.03	0.04	0.9	0.03
Leucine	255 ± 14.8	182 ± 8.03	181 ± 12.08	<0.001	<0.001	0.9	<0.001
Lysine	95.4 ± 7.35	75.6 ± 3.98	73.7 ± 6	<0.001	0.03	0.8	0.02
Valine	264 ± 16.42	217 ± 8.91	205 ± 13.41	0.01	0.01	0.75	0.03
Carnosine	12.9 ± 2.18	16.4 ± 1.18	21.5 ± 1.78	<0.01	0.01	0.05	0.3
Ornithine	65.2 ± 3.58	48.2 ± 1.94	47.9 ± 2.92	<0.001	<0.001	0.9	<0.001
Betaine	152.8 ± 14.31	93.2 ± 7.76	82.5 ± 11.68	<0.001	<0.001	0.7	<0.001
Choline	15.3 ± 1.148	11 ± 0.623	10.4 ± 0.938	<0.01	<0.01	0.8	<0.01
alpha-Aminoadipic acid	2.89 ± 0.318	2.69 ± 0.173	1.95 ± 0.26	0.03	0.02	0.02	0.7
Asymmetric dimethylarginine	0.883 ± 0.0542	0.737 ± 0.0294	0.653 ± 0.0442	<0.01	<0.01	0.2	0.05
Citric acid	220 ± 20.3	286 ± 11	282 ± 16.6	0.01	0.05	0.9	0.01
Fumaric acid	1.15 ± 0.235	1.71 ± 0.128	1.92 ± 0.192	0.04	0.03	0.6	0.1
Hippuric acid	55.6 ± 4.08	67.9 ± 2.21	65.3 ± 3.33	0.03	0.1	0.7	0.02
LYSOC14:0	0.942 ± 0.0874	1.234 ± 0.0474	1.432 ± 0.0713	<0.001	<0.001	0.06	0.01
LYSOC16:1	26.3 ± 2.05	27.4 ± 1.11	32.1 ± 1.67	0.03	0.02	0.2	0.2
LYSOC18:0	16.9 ± 1.612	20.7 ± 0.874	22.1 ± 1.316	0.04	0.04	0.6	0.1
LYSOC18:1	13.5 ± 1.33	16.7 ± 0.721	20 ± 1.086	<0.001	<0.001	0.03	0.1
LYSOC18:2	29.8 ± 2.86	35.7 ± 1.55	45.2 ± 2.34	<0.001	<0.001	<0.01	0.1
LYSOC20:4	2.32 ± 0.1793	2.23 ± 0.0973	2.66 ± 0.1464	0.05	0.3	0.04	0.8
LYSOC26:0	0.118 ± 0.0232	0.151 ± 0.0126	0.196 ± 0.0189	0.03	0.03	0.1	0.4
LYSOC26:1	0.0402 ± 0.0057	0.0568 ± 0.00309	0.0661 ± 0.00466	<0.01	<0.01	0.2	0.03
LYSOC28:0	0.228 ± 0.0263	0.322 ± 0.0143	0.408 ± 0.0215	<0.001	<0.001	<0.01	<0.01
LYSOC28:1	0.278 ± 0.0383	0.466 ± 0.0208	0.559 ± 0.0313	<0.001	<0.001	0.04	<0.001
PC32:2AA	8.43 ± 0.973	13.38 ± 0.528	15.55 ± 0.795	<0.001	<0.001	0.06	<0.001
PC36:0AA	12.3 ± 2.01	22.2 ± 1.09	26.6 ± 1.64	<0.001	<0.001	0.07	<0.001
PC36:0AE	2.3 ± 0.27	3.68 ± 0.146	4.29 ± 0.22	<0.001	<0.001	0.06	<0.01

PC36:6AA	2.77 ± 0.284	3.79 ± 0.154	4.09 ± 0.232	<0.001	<0.001	0.5	<0.001
PC38:0AA	1.83 ± 0.282	3.3 ± 0.153	4.08 ± 0.23	<0.001	<0.001	0.01	<0.001
PC38:6AA	2.93 ± 0.368	4.46 ± 0.2	4.96 ± 0.3	<0.001	<0.001	0.3	<0.001
PC40:1AA	0.299 ± 0.0277	0.45 ± 0.015	0.505 ± 0.0226	<0.001	<0.001	0.1	<0.001
PC40:2AA	0.912 ± 0.153	1.705 ± 0.083	2.053 ± 0.125	<0.001	<0.001	0.06	<0.001
PC40:6AA	1.93 ± 0.287	2.76 ± 0.155	2.83 ± 0.234	0.03	0.04	0.9	0.03
PC40:6AE	1.02 ± 0.1041	1.43 ± 0.0565	1.57 ± 0.085	<0.001	<0.001	0.3	<0.01
14:1SMOH	2 ± 1.03	4.08 ± 1.169	2.08 ± 0.888	<0.01	<0.01	0.05	0.1
16:0SM	123 ± 10.51	151 ± 5.7	170 ± 8.58	<0.01	<0.01	0.1	0.05
16:1SM	13.9 ± 1.135	16.6 ± 0.616	18.9 ± 0.927	<0.01	<0.01	0.09	0.1
16:1SMOH	12.6 ± 1.067	15.7 ± 0.579	18.1 ± 0.871	<0.001	<0.001	0.05	0.04
18:0SM	19.3 ± 1.478	24.3 ± 0.801	28.4 ± 1.206	<0.001	<0.001	0.01	0.01
18:1SM	20.2 ± 1.805	26.1 ± 0.979	30 ± 1.474	<0.001	<0.001	0.07	0.01
20:2SM	2.26 ± 0.231	3.18 ± 0.125	3.62 ± 0.189	<0.001	<0.001	0.1	<0.01
22:1SMOH	20.4 ± 1.822	27.1 ± 0.988	31.5 ± 1.488	<0.001	<0.001	0.04	<0.01
22:2SMOH	10.6 ± 0.784	13.7 ± 0.425	15.1 ± 0.64	<0.001	<0.001	0.1	<0.01
24:1SMOH	2.39 ± 0.173	3.06 ± 0.094	3.38 ± 0.141	<0.001	<0.001	0.1	<0.01
C0	4.11 ± 0.327	3.12 ± 0.177	3.43 ± 0.267	0.03	0.2	0.5	0.02
C4OH	0.0234 ± 0.00192	0.0313 ± 0.00104	0.0348 ± 0.00157	<0.001	<0.001	0.1	<0.001
C5:1DC	0.0138 ± 0.001213	0.0166 ± 0.000658	0.0184 ± 0.00099	<0.01	0.01	0.2	0.1
C5DC	0.01021 ± 0.000723	0.00974 ± 0.000392	0.01242 ± 0.00059	<0.001	0.05	<0.001	0.8
C6:1	0.0238 ± 0.001611	0.028 ± 0.000874	0.0305 ± 0.001315	<0.001	<0.01	0.2	0.05
C8	0.0174 ± 0.001359	0.0123 ± 0.000737	0.0119 ± 0.00111	<0.01	<0.01	0.9	<0.01
C12:1	0.0444 ± 0.00309	0.0401 ± 0.00168	0.0473 ± 0.00252	0.05	0.7	0.05	0.4
C14:1OH	0.00789 ± 0.000553	0.00872 ± 0.0003	0.01005 ± 0.000452	<0.01	0.01	0.04	0.3

[1]ANOVA three group comparison

[2]Tukey test for multiple group comparison

[3]SCM-O = subclinical mastitis only (cows affected by SCM only)

[4]SCM-P = subclinical mastitis plus (all cows affected by SCM and another disease)

Table 4.2. Serum metabolites at –4 wks before calving between the three groups of cows and their respective *P* values for multiple comparison groups.

Metabolites (μM)	MEAN ± SEM[1]			*P* value	*P* value[2]		
	SCM-O[3]	SCM-P4	CON		SCM-O vs CON	SCM-P vs CON	SCM-O vs SCM-P
Alanine	188 ± 11.94	213 ± 6.48	240 ± 9.75	<0.01	<0.01	0.05	0.1
Proline	77.9 ± 5.5	85.1 ±2.98	97.8 ± 4.49	0.01	<0.01	0.05	0.4
Valine	244 ± 15.7	250 ± 8.5	294 ± 12.8	0.01	0.04	0.01	0.9
Methionine	23.6 ± 1.617	27 ± 0.877	29.5 ± 1.32	0.02	0.01	0.2	0.1
Methionine-sulfoxide	1.91 ± 0.238	2.16 ± 0.129	2.82 ± 0.194	<0.01	0.01	0.01	0.6
Ornithine	159.5 ± 5.1	57.2 ± 2.77	63.8 ± 4.17	<0.001	<0.001	0.3	<0.001
Betaine	156 ± 12.57	136 ± 6.81	165 ± 10.26	0.05	0.8	0.05	0.3
Choline	19 ± 1.071	12 ± 0.581	11.7 ± 0.874	<0.001	<0.001	0.9	<0.001
Methylmalonic acid	0.562 ± 0.0525	0.387 ± 0.0285	0.349 ± 0.0429	<0.001	<0.01	0.7	0.01
Lactic acid	2242 ± 270	1330 ± 147	1421 ± 221	0.01	0.05	0.9	0.01
LYSOC18:0	14.2 ± 1.083	12.5 ± 0.587	15.9 ± 0.884	<0.01	0.4	<0.01	0.3
LYSOC18:1	9.3 ± 1.098	8.56 ± 0.595	11.18 ± 0.896	0.05	0.3	0.04	0.8
LYSOC18:2	17.4 ± 2.02	15 ± 1.1	20 ± 1.65	0.04	0.5	0.03	0.5
LYSOC20:3	2.68 ± 0.328	2.45 ± 0.178	3.32 ± 0.267	0.03	0.2	0.02	0.8
LYSOC28:1	0.196 ± 0.0327	0.256 ± 0.0177	0.313 ± 0.0267	0.02	0.01	0.1	0.2
PC32:2AA	6.3 ± 0.85	9.46 ± 0.461	11.85 ± 0.694	<0.001	<0.001	0.01	<0.01
PC36:0AA	7.5 ± 0.897	12 ± 0.486	14.5 ± 0.732	<0.001	<0.001	0.01	<0.001
PC36:0AE	1.95 ± 0.209	2.8 ± 0.113	3.38 ± 0.171	<0.001	<0.001	0.01	<0.01
PC36:6AA	1.99 ± 0.298	2.97 ± 0.161	3.59 ± 0.243	<0.001	<0.001	0.09	0.01
PC38:0AA	0.899 ± 0.1471	1.574 ± 0.0798	1.925 ± 0.1201	<0.001	<0.001	0.04	<0.001
PC38:6AA	1.89 ± 0.223	2.89 ± 0.121	3.45 ± 0.182	<0.001	<0.001	0.03	<0.001
PC40:1AA	0.399 ± 0.0204	0.347 ± 0.0136	0.225 ± 0.025	<0.001	<0.001	0.09	<0.001
PC40:2AA	0.479 ± 0.0671	0.789 ± 0.0364	0.988 ± 0.0548	<0.001	<0.001	0.01	<0.001
PC40:6AA	1.48 ± 0.2	2.3 ± 0.109	2.54 ± 0.164	<0.001	<0.001	0.4	<0.001
PC40:6AE	0.687 ± 0.0712	0.968 ± 0.0386	1.152 ± 0.0581	<0.001	<0.001	0.02	<0.01
18:0SM	13.3 ± 1.191	16.6 ± 0.646	17.2 ± 0.973	0.02	0.03	0.8	0.04
22:1SMOH	12.8 ± 1.414	17.7 ± 0.767	18.1 ± 1.155	<0.001	0.01	0.9	0.01

22:2SMOH	7.32 ± 0.661	9.62 ± 0.358	10.01 ± 0.539	<0.001	<0.01	0.8	<0.01
24:1SMOH	1.76 ± 0.1783	2.38 ± 0.0967	2.27 ± 0.1456	0.01	0.07	0.8	<0.01
C3OH	0.0251 ± 0.00192	0.0193 ± 0.00104	0.0239 ± 0.00157	<0.01	0.8	0.04	0.02
C4.1	0.021 ± 0.00145	0.0152 ± 0.000786	0.0154 ± 0.001184	<0.01	0.01	0.9	<0.01
C5:1	0.0204 ± 0.001469	0.0137 ± 0.000797	0.0145 ± 0.0012	<0.001	<0.01	0.8	<0.001
C5:1DC	0.0167 ± 0.001261	0.0113 ± 0.000684	0.0129 ± 0.00103	<0.001	0.05	0.4	<0.001
C5DC	0.0169 ± 0.001792	0.00884 ± 0.000972	0.00921 ± 0.001463	<0.001	<0.01	0.9	<0.001
C9	0.02195 ± 0.00272	0.00789 ± 0.00147	0.00798 ± 0.00222	<0.001	<0.001	0.9	<0.001
C10	0.0433 ± 0.00377	0.0331 ± 0.00205	0.0375 ± 0.00308	0.05	0.4	0.4	0.05
C10:2	0.0243 ± 0.00185	0.0182 ± 0.00101	0.018 ± 0.00151	0.01	0.02	0.9	0.01
C14:1	0.0337 ± 0.00529	0.053 ± 0.00287	0.0532 ± 0.00432	<0.01	0.01	0.9	<0.01
C14:2OH	0.00798 ± 0.000509	0.00645 ± 0.000276	0.00676 ± 0.000416	0.03	0.1	0.8	0.02
C18:1	0.0126 ± 0.001631	0.0167 ± 0.000884	0.0188 ± 0.001332	0.01	0.1	0.3	0.07

[1]ANOVA three group comparison

[2]Tukey test for multiple group comparison

[3]SCM-O = subclinical mastitis only (cows affected by SCM only)

[4]SCM-P = subclinical mastitis plus (all cows affected by SCM and another disease)

Table 4.3. Urinary metabolites at –8 wks before calving between the three groups of cows and their respective *P* values for multiple comparison groups.

Metabolites (μM)	MEAN ± SEM[1]			*P* value	*P* value[2]		
	SCM-O[3]	SCM-P[4]	CON		SCM-O vs CON	SCM-P vs CON	SCM-O vs SCM-P
Putrescine	0.813 ± 0.105	0.354 ± 0.0574	0.42 ± 0.0864	<0.001	0.01	0.8	<0.001
Leucine	12.11 ± 1.534	7.4 ± 0.832	6.79 ± 1.252	0.01	0.02	0.9	0.02
Valine	18.6 ± 2.38	10.8 ± 1.29	10.5 ± 1.95	0.01	0.02	0.9	0.01
Tyrosine	29.5 ± 3.12	20.4 ± 1.69	21.4 ± 2.55	0.04	0.01	0.9	0.03
Threonine	89.9 ± 10.93	57.8 ± 5.93	67.1 ± 8.92	0.04	0.2	0.6	0.03
Methionine-sulfoxide	3.94 ± 0.556	2.15 ± 0.306	2.65 ± 0.454	0.02	0.1	0.6	0.01
Acetyl-ornithine	74 ± 9.22	48.4 ± 5	51.9 ± 7.52	0.05	0.1	0.9	0.04
Choline	90.2 ± 17.58	33 ± 9.53	26.8 ± 14.35	0.01	0.01	0.9	0.01
Betaine	275 ± 44.1	132 ± 23.9	113 ± 36	0.01	0.01	0.9	0.01
Creatine	5643 ± 576	3380 ± 313	3141 ± 471	<0.01	<0.01	0.9	<0.01
Total dimethylarginine	32.2 ± 3.78	19.8 ± 2.05	20.4 ± 3.09	0.01	0.04	0.9	0.01
Asymmetric dimethylarginine	8.89 ± 1.173	3.79 ± 0.636	3.07 ± 0.95	<0.001	<0.001	0.8	<0.001
Homovanillic acid	12.85 ± 1.593	7.34 ± 0.864	6.59 ± 1.301	<0.01	<0.01	0.8	0.01
C0	2.17 ± 0.261	1.14 ± 0.141	1.12 ± 0.213	<0.01	<0.01	0.9	<0.01
C2	0.662 ± 0.0613	0.388 ± 0.0332	0.338 ± 0.05	<0.001	<0.001	0.6	<0.001
C3:1	0.0305 ± 0.00304	0.022 ± 0.00165	0.0205 ± 0.00248	0.03	0.03	0.8	0.04
C4	0.572 ± 0.0679	0.24 ± 0.0368	0.191 ± 0.0554	<0.001	<0.001	0.7	<0.001
C4OH	0.0895 ± 0.00696	0.0679 ± 0.00378	0.0696 ± 0.00569	0.02	0.7	0.9	0.02
C5OH	0.1266 ± 0.01083	0.0814 ± 0.00587	0.0828 ± 0.00884	<0.001	<0.01	0.9	<0.001
C5:1	0.226 ± 0.0229	0.138 ± 0.0124	0.141 ± 0.0187	<0.01	0.01	0.9	<0.01
C8	0.0503 ± 0.00553	0.0329 ± 0.003	0.032 ± 0.00452	0.01	0.03	0.9	0.02

[1]ANOVA three group comparison

[2]Tukey test for multiple group comparison

[3]SCM-O = subclinical mastitis only (cows affected by SCM only)

[4]SCM-P = subclinical mastitis plus (all cows affected by SCM and another disease)

Table 4.4. Urinary metabolites at –4 wks before calving between the three groups of cows and their respective *P* values for multiple comparison groups.

Metabolites (μM)	MEAN ± SEM[1]			*P* value	*P* value[2]		
	SCM-O[3]	SCM-P[4]	CON		SCM-O vs CON	SCM-P vs CON	SCM-O vs SCM-P
Isoleucine	8.3 ± 0.653	6.86 ± 0.354	6.07 ± 0.533	0.03	0.02	0.4	0.1
Glutamine	318 ± 32.9	277 ± 17.8	216 ± 26.9	0.05	0.05	0.1	0.5
Threonine	77.3 ± 6.57	57.9 ± 3.56	51.4 ± 5.36	0.01	<0.01	0.5	0.03
Carnosine	14.68 ± 1.177	9.46 ± 0.639	11 ± 0.961	<0.001	0.04	0.3	<0.001
Methylhistidine	378 ± 28.2	307 ± 15.3	249 ± 23	<0.01	<0.01	0.09	0.07
Homovanillic acid	13.11 ± 1.479	8.85 ± 0.802	8.93 ± 1.208	0.04	0.08	0.9	0.03
Methylmalonic acid	26.2 ± 2.6	14.1 ± 1.41	10.8 ± 2.13	<0.001	<0.001	0.4	<0.001
Pyruvic acid	18.7 ± 3.62	10.29 ± 1.96	6.27 ± 2.95	0.03	0.02	0.4	0.1
Citric acid	1875 ± 414	666 ± 225	321 ± 338	0.01	0.01	0.6	0.03
Lactic acid	214.6 ± 42.7	103.2 ± 23.1	85.6 ± 34.8	0.04	0.05	0.9	0.06
Uric acid	4682 ± 482	4011 ± 261	3133 ± 393	0.04	0.04	0.1	0.4
C5DC	0.0495 ± 0.00447	0.0392 ± 0.00243	0.0311 ± 0.00365	<0.01	<0.01	0.1	0.1
C5:1DC	0.0437 ± 0.00307	0.0342 ± 0.00166	0.036 ± 0.0025	0.03	0.1	0.8	0.02
C12:1	0.103 ± 0.0333	0.163 ± 0.018	0.214 ± 0.0272	0.04	0.03	0.2	0.2

[1]ANOVA three group comparison

[2]Tukey test for multiple group comparison

[3]SCM-O = subclinical mastitis only (cows affected by SCM only)

[4]SCM-P = subclinical mastitis plus (all cows affected by SCM and another disease)

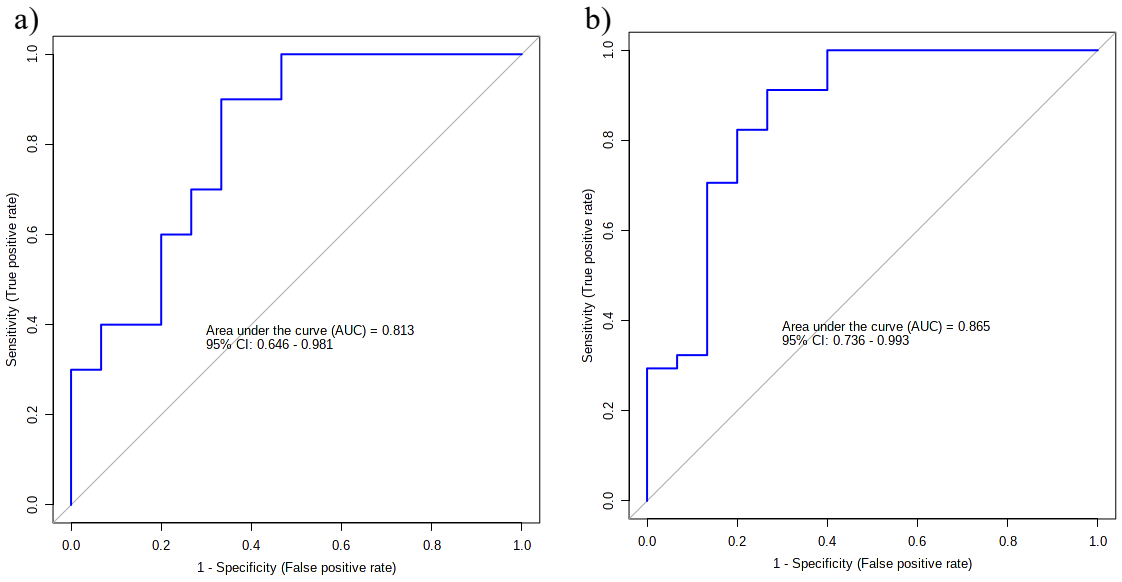

Figure 4.1. AUC of the logistic regression for α-aminoadipic acid, C5DC and C14:1OH at –8 wks in serum of **a)** SCM-O vs CON, and **b)** SCM-P vs CON.

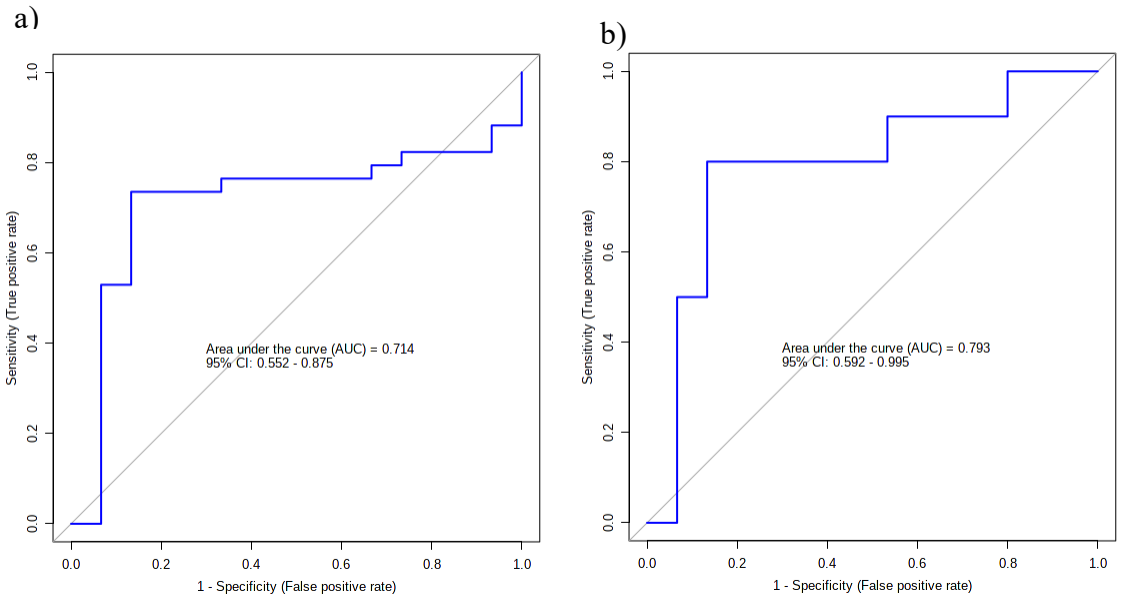

Figure 4.2. AUC of the logistic regression for glycine and carnosine at –8 wks in the serum of **a)** SCM-O vs CON, and **b)** SCM-P vs CON.

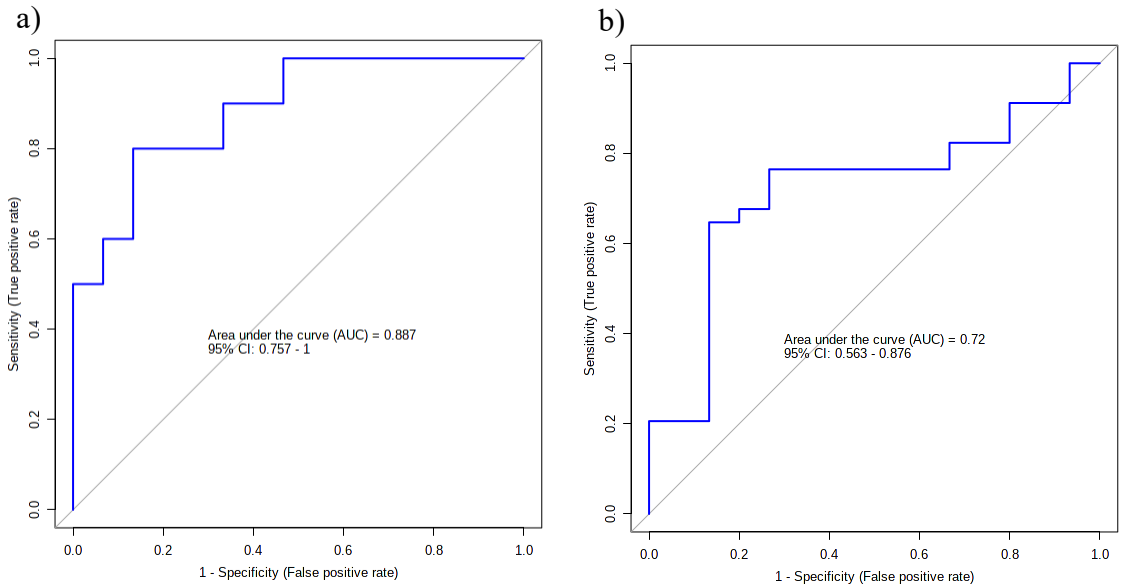

Figure 4.3. Area under the curve of the logistic regression for alanine and methionine-sulfoxide at –4 wks in the serum of **a)** SCM-O vs CON, and **b)** SCM-P vs CON.

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