Acute Phase Proteins, Proteomics and Metabolomics in the Diagnosis of Bovine Mastitis

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Abstract

Bovine mastitis continues to pose a major economic challenge to the dairy industry worldwide. Critical to the management and control of this condition, is the need for prompt and accurate diagnosis in field conditions, therefore a search for more sensitive and reliable biomarkers is required.

In this thesis, studies focused on assessing milk samples from cows with various forms of mastitis were undertaken with a view to identifying new biomarkers for bovine mastitis.

Three acute phase proteins (APP); haptoglobin (Hp), mammary associated serum amyloid A3 (M-SAA3) and C-reactive protein (CRP) were measured in milk samples from composite milk samples of all lactating cows in a commercial dairy herd, mastitis cases, submitted to a diagnostic laboratory and following an experimental mastitis challenge of cows with *Streptococcus uberis*. A new enzyme linked immunosorbent assay (ELISA) was developed for measuring Hp, while commercial ELISA assay kits were used to assay M-SAA3 and CRP. Other mastitis related parameters evaluated in the samples included the somatic cell counts (SCC) and the presence of pathogens.

A reliable and sensitive ELISA was developed and optimized for measuring milk Hp. A cut off value for Hp of 7.9 µg/ml was established for milk with SCC less than 200,000 cells/ml. Pathogen-specific variations were observed in the concentration of each APP in mastitic milk. It was observed that the environmental pathogens showed higher concentrations of APP compared to other pathogens, from the study of mastitis milk samples submitted to the diagnostic laboratory. Also, it was possible to distinguish between samples from subclinical and clinical mastitis and between samples from subclinical and healthy udders using each of the APP ($P<0.05$). Haptoglobin, M-SAA3 and CRP showed corresponding variation with stage of infection during the course of experimental mastitis, and specifically CRP was observed to rise earlier than other two APP.

Furthermore, characterization of the profile of these APP in the immediate post-calving milk samples was carried out to determine how valuable they would be in recognizing new mastitis infections arising at the post-partum period. It was

observed that there is a general moderately-high level of APP in milk immediately following parturition which drops a few days later in healthy milk. The immunohistochemical localization of Hp in the bovine mammary gland was also assessed. It could be concluded from that study that neutrophils and the mammary epithelial cells secrete Hp into milk during mastitis.

Gel and non-gel based proteomics approaches were employed to study the protein profiles and variation in mastitic milk from normal samples. Several proteins were identified that confirmed previous findings and project new mastitis markers, for example, serotransferrin, serpins, alpha-macroglobulin and neutrophil gelatinase associated lipocalins. A capillary electrophoresis mass spectrometry system (CE-MS) was also employed to elucidate the changing peptidome in milk during the course of an experimental mastitis, which lead to the generation of a panel of 77 polypeptides, which were able to significantly differentiate critical stages of mastitis. Three of these polypeptides were found in mastitic milk samples from previous peptidomic analyses thereby indicating strong biomarker value.

Finally, a liquid chromatography mass spectrometry based metabolomics approach was used to study the changing profile of small metabolites in milk during the course of an experimental infection. Several pathway-based changes that highlighted metabolites of potential significance in mastitis diagnosis were recognized including lactose synthesis, nitrogen containing compounds such as betaine, L-carnitine and lipid metabolites pathways namely sn-glycerophosphocholine and choline among others.

Overall, this study has shown the value of APP, milk proteomics and metabolomics in bovine mastitis diagnosis; the changing proteins and metabolites or their patterns need to be further experimentally and clinically validated as specific and sensitive markers of mastitis. Ultimately, the applicability of APP, proteins, peptides and metabolites and/or their changing patterns as mastitis biomarkers would require their adaptation to rapid (on farm) and robust measurement formats.

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- Excel spread sheet of peptidomics analysis LC-MS/MS polypeptides sequences

Dedication

This PhD thesis is dedicated to God Almighty, for His never failing love for me……and to the loving memory of Daniel Oluwafemi Thomas.

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To the only wise God, the everlasting and Almighty be Glory, honour, power and majesty for giving me the grace, strength and inspiration to start and complete this thesis and indeed the PhD. He it was, who brought the opportunity, positioned all necessary, and sustained me though it all and sure enough is leading me to greater heights. Lord Jesus, Thank you so much.

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To my Dad; Pa J.A Amode, late mum; Mrs G.A Amode, mother in-law, brothers; Segun and Olaolu, sisters; Gloria and Tayo; Uncle; Pastor Muyiwa, Uncle Alfred, aunties; Funke, Tope, Aina, Bimbo, and so many more too numerous to mention I want to say God bless you real God for being there and giving me all the moral backing you did through my programme.

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Author's Declaration

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

Funmilola Clara Thomas,

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Definitions/Abbreviations

%	Percentage
~	Approximately
£	pound
+	plus
-	minus
<	less than
>	greater than
°C	degree Celsius
=	equals to
ε	extinction coefficient
1D	one dimensional
1DE	one dimensional electrophoresis
2D	two dimensional
2DE	two dimensional electrophoresis
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
(optic)-4CN	4-choro-1-naphthol
4PL	4 parameter logistic
A	absorbance
A-SAA	acute phase serum amyloid A
AA	amyloid A
AAs	amino acid(s)
Ab	antibody
ACN	acetonitrile
AcP	acid phosphatase
ADP	adenosine diphosphate
Aer. viridans	*Aerococcus viridans*
AGP	acid glycoprotein
Ala	alanine
A_1TI	alpha$_1$-trypsin inhibitor
am	ante meridian
AMS	automatic milking systems
amu	atomic mass unit

AP	alkaline phosphate
APCI	atmospheric pressure chemical ionisation
APP	acute phase protein
APR	acute phase reaction/response
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
AUC	area under the curve
BCA	bicinchoninic acid
BH	Benjamini-Hochberg
BHBA	ß-hydroxybutyric acid
bHp	bovine haptoglobin
BSA	bovine serum albumin
BMDB	bovine metabolome database
BME	ß-mercaptoethanol
c	concentration (protein)
C	carbon (symbol)
C_1	initial concentration
C_2	final concentration
C/E	carbohydrate and energy (metabolites)
C-SAA	constitutive serum amyloid A
$C_3H_6BrNO_4$	2-bromo-2-nitro-1,3 propanediol
CA	California
CBB	Coomasie brilliant blue stain
CE	capillary electrophoresis
cells/ml	cells per millilitre
CE-MS	capillary electrophoresis-mass spectrometry
CFU	colony forming unit
CHAPS	3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate
CI	chemical ionisation
CID	collision induced dissociation
CI	confidence interval
Cl^-	chloride ion
cm	centimeters
CM	clinical mastitis

CMT	California mastitis test
CN	casein
CNS	Coagulase negative staphylococci
Co.	corporation
CPLL	combinatorial peptide ligand library
CPS	coagulase positive staphylococci
CRP	C-reactive protein
CS	confidence score
CSCC	composite milk somatic cell counts
CSF	cerebrospinal fluid
Cu	copper
CV	coefficient of variance
Cy	cyanine
Cy2	cyanine dye 2
Cy3	cyanine dye 3
Cy5	cyanine dye 4
CyDye	cyanine dye
Cys	cysteine
D-	dextrorotatory (isomer)
Da	Dalton
DB	database
DCC	DeLaval cell counter
DC&M	dehydrated, cleared and mounted
DHEA	dehydroepiandrosterone
DiGE	difference gel electrophoresis
DIM	days in milk
Dm	error difference between the theoretical mass value of the peptide and the experimental mass
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
e.g.	example
EB	equilibration buffer
EDTA	Ethylenediaminetetraacetic acid
EC	electrical conductivity
ECM	extracellular matrix

ETD	electron transfer dissociation
ELISA	enzyme linked immunosorbent assay
ESI	electrospray ionisation
ESI-MS	electrospray ionisation mass spectrometry
Etc	Et cetera (and so on)
EU	European Union
FA	fatty acid
FC	fold change
FDR	false discovery rate
Fe	iron
FFA	free fatty acid
FFPE	fixed in formalin and embedded in paraffin wax
FTICR	Fourier transform ion- cyclotron resonance
FTIS	Fourier transform infrared spectroscopy
g	gram(s)
g/l	gram per litre
GABA	gamma amino butyric acid
GCAT	glycine-C-acetyltransferase
GC-MS	gas chromatography mass spectrometry
GIT	gastrointestinal tract
Gly	Glycine
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
Glu	glutamic acid
GSH	glutathione
H	histidine
h	hour(s)
H&E	Hematoxylin and eosin staining
H_2O	water
H_2O_2	hydrogen peroxide
H_3PO_4	phosphoric acid
Hb	haemoglobin
Hb-Hp	haemoglobin-haptoglobin
HbCN	cyanomethaemoglobin
HCA	hierarchical cluster analysis
HCL	hydrochloric acid

HDL	high density lipoprotein
His	histidine
HMW	high molecular weight
Hp	haptoglobin
Hp-HbCN	haptoglobin-cyanomethaemoglobin
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IBM	International Business Machines
ICAT	isotope-coded affinity tag
ICC	immunocytochemistry/immunocytochemical
ID	identification
IDF	International Dairy Federation
i.e.	that is
IEF	isoelectric focusing
IEX	ion exchange chromatography
Ig	immunoglobulin
IHC	immunohistochemistry
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-19	interleukin-19
IL-1ß	interleukin-1ß
Ile	isoleucine
IM	ionisation mode
IMI	intramammary infection/inflammation
Inc.	incorporation
IPG	immobilized pH gradient
IRT	infrared thermography
ITIH	Inter-alpha (globulin) inhibitor H4
iTRAQ	isobaric tags for relative and absolute quantitation
K	lysine
K^+	potassium ion
$K_2Cr_2O_7$	potassium dichromate

KDa	kilo Dalton
KEGG	Kyoto Encyclopaedia of Genes and genomes
kV	kilo volts
ℓ	path length
L-	levorotatory (isomer)
lb	pound (weight)
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry
LC-MRM-MS/MS	Liquid chromatography multiple reaction monitoring tandem mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDH	lactate dehydrogenase
Leu	leucine
Lf	lactoferrin
LIFD	laser-induced fluorescence detection
LL	lipids and lipid related (metabolites)
LMW	low molecular weight
LN	lactation number
LOD	limit of detection
Log	logarithm
LogSCC	logarithmic somatic cell count
LOQ	intensity filter
LPB	lipopolysaccharide binding protein
LPS	lipopolysaccharide (endotoxin)
LTA	lipotechoic acid
LT	leukotriene
LTA_4	leukotiene A_4
LTB_4	Leukotriene B_4
LX	lipoxins
Lys	lysine
M	molar
MAA	milk amyloid A
MALDI	matrix assisted laser desorption ionisation
MALDI-TOF MS	matrix assisted laser desorption ionisation time of flight mass spectrometry

MALDI-TOF MS/MS	matrix assisted laser desorption ionisation time of flight tandem mass spectrometry
Max	maximum
MEC	mammary epithelial cells
Met	methionine
MFGM	milk fat globule membrane
milli Q	ultrapure water
Minim.	minimum
min	minute(s)
ml	millilitre
mM	millimolar
MOWSE	molecular weight search
MPO	myeloperoxidase
Mr	relative molecular mass
MRes	Master of research
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
M-SAA3	mammary associated serum amyloid A3
MudPIT	multidimensional protein identification
MUFA	mono unsaturated fatty acid
Mw	molecular weight
M/Yield	milk yield
m/z	mass to charge ratio
n/ N	total number
Na^+	sodium ion
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADA	N-gamma-Acetyldiaminobutyrate
NADH	reduced nicotinamide adenine dinucleotide
NAGase	N-acetyl-ß-D-glucosaminidase
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NASBA	nucleic acid sequence based amplification
NBT/BCIP	nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate

NCBI	National centre for biotechnology information
NCI	National Cancer Institute
NCM	nitrocellulose membrane
NEB	negative energy balance
NEFA	non esterified fatty acid
nESI-MS/MS	nanoelectrospray-tandem mass spectrometry
ng/ml	nanogram per millilitre
NH_4	ammonium
NH_4OH	ammonium hydroxide
NH_4 SO_4	ammonium sulphate
NHS	N-hydroxysuccinimidyl
NHS-PEO_4	N-Hydroxysuccinimide polyethylene oxide 4
NIH	National Institute of Health
NIRD	National Institute of Research in Dairying
nm	nanometer
NMC	National Mastitis Council
NMR	nuclear magnetic resonance
NMR co.	National Milk Recording Company
NSI	Nano spray ionisation
NT	nucleotides and related (metabolites)
OH^-	hydroxyl radical
OD	optical density
OH	hydroxyl ion
OSC	orthogonal signal correction
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	post challenge
PCA	principal components analysis
PCR	polymerase chain reaction
PCR-SSCP	polymerase chain reaction- single strand conformation polymorphism
PDB	protein data bank
Pers. comm.	personal communication
PGF	prostaglandins
PGI	prostacyclins
pH	power of hydrogen

pi	isoelectric point
PLS-DA	partial least squares discriminant analysis
pm	post meridiem
PMC	polymorphonuclear cells
PMF	peptide mass fingerprinting
PMN	polymorphonuclear
PMNL	polymorphonuclear leucocyte
PMSF	phenyl methylsulfonyl fluoride
PP	peptides
ppm	parts per million
PPV	positive predictive value
PRIDE	Proteomics identifications database
Pro	proline
PSD	post-source decay
psi	pounds per square inch
PTM	post translational modification
P-value	probability that null hypothesis is true
Q	quadrupole
QC	quality control
QSCC	quarter milk somatic cell counts
Q-TOF MS/MS	quadruopole-time of flight tandem mass spectrometry
r	correlation coefficient
RABHp	rabbit anti bovine Haptoglobin
RBP	retinol binding protein
RF	random forest
RHB	rehydration buffer
RIA	radioimmunoassay
ROC	receiver operating characteristics
RP	reversed phase
RP-HPLC	reversed phase high performance liquid chromatography
RT	room temperature
RT	retention time
RT-PCR	reverse transcription polymerase chain reaction
RSD	relative standard deviation
S. agalactiae	*Staphylococcus agalactiae*

S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. uberis	*Streptococcus uberis*
S. dysgalactiae	*Streptococcus dysgalactiae*
S/N	serial number
SAA	serum amyloid A
SCC	somatic cell counts
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of mean
Ser	serine
SELDI	surface enhanced laser desorption ionization
SELDI-TOF	surface enhanced laser desorption ionization time of flight
SFA	saturated fatty acid
SIB	Swiss Institute of Bioinformatics
SILAC	stable incorporation of labelled amino acids in culture
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
spp	species
SPE	solid phase extraction
SPME	solid phase microextraction
SPSS	statistical package for social sciences
SRID	single radial immunodiffusion
SVM	support vector machine
T	trace
TBS	Tris buffered saline
TCA	citric acid cycle
TLR	toll like receptor
TMAB	secondary antibodies
TMB	Tetra methyl benzidine
TNF-α	tumour necrosis factor alpha
TOF	time of flight
Tris-HCL	Tris hydrochloride
TTBS	Tris buffered saline and tween-20

TXA	thromboxane
UFA	unsaturated fatty acid
UK	United Kingdom
USA	United States of America
UPLC	ultrahigh pressure liquid chromatography
UPLC-TOF MS	ultrahigh pressure liquid chromatography time of flight mass spectrometry
USD	United States Dollar
UST	udder skin/surface temperature
UV	ultra violet
V	volts/voltage
V_1	initial volume
V_2	final volume
Val	valine
VC	vitamins and Co-factors (metabolites)
VDS	Veterinary Diagnostic Services
vs.	versus
v/v	volume per volume
W	watts
w/v	weight per volume
X	timesXCMS
x *g*	centrifugal force in gravity
Zn	zinc
z-value	standard score
α-1-AGP	alpha 1 acid glycoprotein
α-1-PI	alpha 1-acid proteinase
α-CN	alpha casein
α-LA	alpha lactalbumin
α-S1-CN	alpha S1 casein
α-S2-CN	alpha S2 casein
β-CN	beta casein
β-LG	beta lactoglobulin
γ	gamma
γ1-CN	gamma 1 casein
γ2-CN	gamma 2 casein

γ3-CN	gamma 3 casein
δ-CN	delta casein
κ-CN	kappa casein
µg	microgram
µg/ml	microgram per litre
µl	microlitre

Amino acids	**Symbols**	
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

1 General Introduction

1.1 Bovine mastitis

Mastitis refers to the inflammation of the udder or mammary gland. This usually follows invasion by micro-organisms, although other physical or chemical causes such as trauma or harmful toxins/chemicals can also lead to mastitis. There are two main forms of mastitis, clinical (CM) and subclinical mastitis (SM).

1.1.1 Epidemiology and economic Importance

For a long time, bovine mastitis has remained prominent as one of the most costly and prevalent diseases in the dairy industry (Hillerton and Berry, 2005; Halasa *et al.,* 2007; Akers and Nickerson, 2011; Hettinga *et al.*, 2008; Awale *et al.*, 2012). Losses accrued to mastitis are related to expenses due to cessation or reduction of milk production (accounting for up to two-thirds of total losses (Akers and Nickerson, 2011), costs of treatment, culling, extra labour, and wasted time and discarded milk as well as veterinary costs. It is often difficult to estimate the total costs of mastitis due to the myriad of factors that can contribute to it during mastitis episodes (Heikkilä *et al.*, 2012). Moreover, drug residues in milk, as a result of treatment, pose the danger of antibiotic resistance and other public health hazards in milk consumed by humans.

The cost of mastitis has been said to be highly variable depending on lactation stage and other factors (Heikkilä *et al.*, 2012), however, the annual cost of mastitis has been estimated to be about 1.8 billion United State dollars (USD) (Schroeder, 2012) in the USA and in the United Kingdom (UK) one hundred and sixty eight million pounds sterling (£168,000,000) by Bradley (2002). In the analysis of Heikkilä *et al*. (2012), using mastitis on Finnish dairy farms, 209 Euros (€209) and €112 were the lowest costs of a CM case in Ayrshire and Holstein-Friesian breeds respectively while €1006 and €946 was the highest cost for each breed.

Hillerton and Berry (2005) in their study arrived at a total of about £300 million, as cost implication of mastitis per annum in the UK. This total was arrived at by adding estimated costs of loss in production due to subclinical mastitis, to the

average national cost. In the analysis of Schroeder (2012), it was estimated that mastitis costs over $200 per annum, per cow.

Due to a rigorous control programme set up by the National Institute of Research in Dairying (NIRD) in the UK, which was adopted in the 1970s, the incidence of clinical mastitis in the UK declined. An estimate of the incidence of clinical mastitis in herds both within the UK and Wales was put at about 47-65 cases per 100 cows per annum (Bradley *et al.*, 2007), in another estimate, the average incidence of mastitis was put at 40 cases per 100 cows per year in the UK (Hillerton and Berry, 2005). This represents a drastic reduction from the former 150 cases per 100 cows per annum seen in the 1960s-80s (Hillerton and Berry, 2005). However, in recent decades, there has been a shift in pathogen causes and also the prevalence of subclinical mastitis (Bradley, 2002). It has been estimated that an average dairy farm has 20-50 % the herd suffering from one form of mastitis or the other, per annum.

Clinical mastitis is characterised by occurrence of the classical signs of inflammation on the affected quarter(s) and sometimes with systemic involvement. Subclinical mastitis, on the other hand, is characterized mainly by an increase in somatic cells in milk, without obvious clinical signs of an inflammatory disease.

1.1.2 Aetiology and risk factors

Micro-organisms including bacteria, Mycoplasma, algae and viruses are a variety of the pathogens capable of invading the mammary gland and leading to mastitis (Nicholas, 2011; Cheville *et al.*, 1984; Tomasinsig *et al.*, 2012; Zaini *et al.*, 2012; Wellenberg *et al.*, 2002). Up to 250 different pathogens have been reported to be able to cause mastitis in the bovine species (Sudhan and Sharma, 2010). Bacteria are however, the most prevalent cause of mastitis. Pathogens causing mastitis can be generally classified as environmental or contagious pathogens.

Coliform bacteria namely *Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), Klebsiella oxytoca (K. oxytoca)* and *Enterobacter aerogenes (E. aerogenes)* and other bacteria such as *Streptococcus uberis (S. uberis)* are considered environmental pathogens, because, being present in the

environment, they cause mastitis opportunistically and are not specifically adapted to the host for such a condition.

New intramammary infections (IMI) with coliforms occur more often in the dry period than during lactation (National mastitis council (NMC), 2004). Severity of mastitis caused by these groups of bacteria is based on host factors (Zadoks *et al.*, 2011). *E. coli* usually causes severe clinical mastitis that elicits massive increases in inflammatory and immune indices, usually resulting in disease which can either be rapidly eliminated or can become systemic and consequently fatal (Baeker *et al.*, 2002; Pyorälä *et al.*, 2011).

On the other hand, contagious pathogens are generally considered host adapted to cause mastitis and are transmitted from one cow, udder or quarter to the other in a herd, and include *Staphylococcus aureus (S. aureus), Streptococcus dysgalactiae (S. dysgalactiae)* and *Streptococcus agalactiae (S. agalactiae)* amongst others. Subclinical or chronic forms of mastitis are usually associated with contagious pathogens, because these organisms are adapted to survive for long periods in the mammary gland, thus, such infections are difficult to eliminate and often lead to a substantial rise in somatic cells in milk (Bradley, 2002).

Another group of bacteria, the coagulase negative staphylococci (CNS), which are opportunistic gram positive cocci, for example, *Staphylococcus chromogenes (S. chromogenes), Staphylococcus simulans (S. simulans) and Staphylococcus epidermidis (S. epidermidis)* which are just a few of up to twelve and possibly more different CNS species commonly isolated from mastitis cases in most dairy farms (Thorberg *et al.*, 2009; Pyorälä and Taponen, 2009; Simojoki *et al.*, 2009) and which characteristically cause more milder forms of subclinical infections that develop mostly around the dry period. Coagulase negative *Staphylococci* (CNS) are more commonly isolated from primiparous cows and cause high SCC in milk. CNS Infections also exhibit high spontaneous cure rates (Pyorälä *et al.*, 2011). *Corynebacterium pyogenes* (*C. pyogenes*) is a bacterium that causes a type of mastitis called summer mastitis which is usually characterised by purulent abscess formation.

In a retrospective study of mastitis cases in Swedish dairy farms by Unnerstad *et al.* (2009), *S. aureus*, followed by *E. coli* were the most common pathogens

isolated from cases of clinical mastitis between the years of 2002-2003. Other pathogens seen in order of prevalence included *S. dysgalactiae*, *S. uberis* and CNS, *Arcanobacterium pyogenes* (*A. pyogenes*) and *Klebsiella spp*. A similar trend was observed in an earlier study in the same region by Nilsson *et al.* (1998) between the years of 1994-1995. Identical pathogens were observed in epidemiological studies in other regions of the world including the UK (Bradley *et al.*, 2007); United States of America (USA) (Neuder *et al.*, 2003); Canada (Olde ReiKerink *et al.,* 2008) and in Norway (Waage *et al.,* 1999).

In a UK and Welsh survey of dairy herds, *S. uberis* and *E. coli* were the most prevalent causes of clinical mastitis, while *S. uberis,* coagulase positive staphylococci (CPS) and *S. aureus* were more frequently isolated from samples with high SCC (SM) in order of prevalence (Bradley *et al.*, 2007). In a more recent study by Kalmus *et al.* (2013), *S. uberis* was the most prevalent pathogen isolated from clinical mastitis milk samples containing a single pathogen from dairy farms in Estonia, using a molecular diagnostic polymerase chain reaction (PCR) kit. These *S. uberis* CM were however associated with mild clinical signs.

Individual cow factors such as breed and levels of PMN cells in the udder increase the risk of a cow developing mastitis, for example there is an increased risk in breeds of cows with pendulous udders developing mastitis than cows with non-pendulous udders. In a study comparing the innate immune response of Jersey and Holstein cows to experimental *E. coli* mastitis induction, Bannerman *et al.* (2008), however found only slight non-significant differences in temporal onset, duration and cessation of some immune variables between the two breeds. Findings from this study also agreed with reports of Youngerman *et al.* (2004); Biffa *et al.* (2005) and Berry *et al.* (2007). Breed susceptibility to mastitis may be related to the selection of features in cows that enhance milk production but which at the same time are characteristics that predispose the udder to easy invasion and colonization by pathogens.

The risks of development of new infections as well as the severity of these infections are also related to other factors including management systems, type of milking system, environmental factors, previous SCC, milk yield, and type of treatment(s) and antibiotics used during the dry period treatment programme;

this being a common measure of control employed in herds to prevent the occurrence of mastitis (O'Reilly *et al.*, 2006; Plozza *et al.*, 2011).

Occurrence of mastitis also depends on the season and herd location (Awale *et al.*, 2012). Lactation stage as well as lactation number (parity) have also been shown to be predisposing factors to IMI by studies demonstrating the higher susceptibility of primiparous cows to specific pathogens, for example *S. chromogenes,* compared to multiparous cows (Vangroenweghe *et al.,* 2005; Thorberg *et al.,* 2009; Heikkilä *et al.*, 2012). Dry period length has also been shown to influence the risk of subclinical mastitis and incidence of new cases of CM are observed to be highest during the dry and periparturient periods (Hillerton and Berry, 2005; Vangroenweghe *et al.,* 2005; Pinedo *et al.,* 2012).

Cow hygiene is one of the most important risk factors for poor udder health and IMI. The use of automatic milking systems (AMS), portends an additional risk factor over the conventional milking system as pre-milking cleaning of udders that is automated into the system does not discriminatingly clean cows with dirtier udders as compared with those with clean ones, and thus, compromises udder hygiene during milking. This has been shown to increase the occurrence of poor udder health (Dohmen *et al.*, 2010; Santman-Berends *et al.*, 2012). Paduch *et al.* (2013) in their study, found significant associations between the treatment of bedding material with an alkaline conditioner and Coliform bacteria counts of teat skin and canal, suggesting that pathogen levels in the bedding materials is a predisposing factor to the incidence of environmental IMI.

Position of the gland (whether front or hind quarters), contributes to the incidence of mastitis according to the findings of Awale *et al.* (2012).

Virulence factors of Major Mastitis organisms

Coliform mastitis is characterised by a rapid rise in milk SCC and its pathogenesis depends largely on the bacterial cell wall lipopolysaccharide (LPS). *Staphylococus aureus* mastitis on the other hand, presents with a milder inflammatory signs and lower milk SCC, which leads frequently to subclinical and chronic infection of the gland. *Staphylococcus aureus* mastitis virulence is mostly facilitated by peptidoglycans, lipoproteins and lipoteichoic acid (LTA). *Streptococcus uberis* induces a variable type of mastitis by inducing Il-1 and Il-8

secretion, through the LTA pathogenic factor (being a gram positive bacteria) (Wellnitz and Bruckmaier, 2012; Rambead *et al.,* 2003).

1.1.3 Transmission and pathogenesis

Transmission of bacteria that cause mastitis usually occurs through contact of teats or udder or cows with contaminated housing or pasture areas, milking machines and/or the milker (Hillerton and Berry, 2005). Pathogens access the mammary gland via the teat orifice, overcoming the innate immune barriers (Wellnitz and Bruckmaier, 2012) of tight teat orifice sphincter muscle, keratin layer and antimicrobial long chain fatty acids found around the mammary teat, and ascend up into the mammary gland through the teat duct. Exploiting the rich nutrient environment provided by milk as well as the optimum temperature of the udder, pathogens multiply in the teat and gland cisterns, and subsequently rise to and adhere to secretory cells (mammary epithelial cells) surfaces within the milk producing tissue. The action of a milking machine during milking as well as movement of the cow can aid the spread and establishment of microorganisms within the udder. Different pathogens express different degrees of virulence which assist their establishment within the gland and determine the severity of disease they cause (Blum and Leitner, 2013).

1.1.4 Acute phase response

Following pathogen invasion, macrophages present within the gland release chemo-attractant compounds that cause the recruitment of polymorphonuclear cells (PMC), mainly neutrophils, from the blood into the mammary tissues. There is also the release of a number of interleukins (IL); such as IL-6 and IL-8, tumour necrosis factor-α (TNF-α) and other chemokines. These compounds synergistically achieve an inflammatory response that is also characterized by an acute phase response (APR) (Wenz *et al.*, 2010). The liver and mammary gland itself are the sites of synthesis (following cytokine stimulation) of the acute phase proteins (APP); these being a group of proteins that are known to be further involved the APR (Murata *et al.*, 2004). In cattle the mammary form of serum amyloid A (SAA) or mammary-associated serum amyloid A (M-SAA3) and Haptoglobin (Hp) are the major APP synthesized and secreted into milk during inflammation (Ceciliani *et al.*, 2012).

Alpha acid glycoprotein (AGP) is another APP secreted into milk during mastitis, although a minor one, and it has been suggested to have immunological roles (Ceciliani *et al.*, 2007).

1.1.5 Clinical signs and pathology

Following pathogen invasion and establishment in the gland, either of the two major forms of mastitis may then result; CM, showing swelling, redness, pain and heat (or generalized fever) of the udder or quarter, as well as physical and chemical changes in milk such as presence of flakes, clots and blood (physical), increased proteolysis of milk caseins, increase in sodium and chloride ions and also a decrease in lactose and many more chemical changes.

Subclinical mastitis occurs with no noticeable physical signs of inflammation, but is commonly indicated by an increase in somatic cell counts in milk produced from affected quarters due to the migration of leukocytes from blood into milk.

Any of these two forms of mastitis may occur as a peracute, acute or chronic infection. Clinical mastitis is usually peracute or acute in duration while SM is often chronic. When chronic mastitis occurs, it is usually characterized by high SCC and reduction in milk production. It is a form of the infection that persists in the gland for long periods and from lactation to lactation (Awale *et al.*, 2012). *S. aureus* is the predominant cause of chronic mastitis.

All forms of mastitis have a negative impact on the quality and quantity of milk produced from affected animals; however, it is believed that SM is more costly overall than CM (Zhao and Lacasse, 2008).

The increased migration of neutrophils into the mammary gland as well as the penetration and multiplication of pathogens and release of toxins causes varying levels of damage to the mammary epithelial cells and renders them less secretory, blocks alveolar ducts and can sometime lead to fibrosis in the gland (Akers and Nickerson, 2011). In addition, compromise of the udder parenchyma and extracellular matrix (ECM) structural conformation leads to increased permeability of the blood-milk barrier membranes resulting in leakage of ions and other molecules from serum into milk (Pyorälä, 2003).

Polymorphonuclear cells assist in destroying invading pathogens utilizing intracellular enzymes systems such as proteases, myeloperodixases and other pathogen-destroying reactive oxidants and defensins (Wellnitz and Bruckmaier, 2012). However, the activities of the PMCs also destroy some of the mammary epithelial cells leading to the release of cytosolic enzymes such as lactate dehydrogenase (LDH), and N-acetyl-ß-D-glucosaminidase (NAGase) from the PMCs. The PMCs are later destroyed by apoptosis and engulfed by macrophages.

Signs of mastitis become severe when PMCs are unable to fight off the pathogens leading to massive damage to the epithelial cells; this causes seepage of extracellular fluid and blood into milk, which is reflected as an increase in electrical conductivity of milk, and change in colour. Presence of flakes, milk clots and blood can also occur in cases of severe pathogenicity.

Mastitis (clinical, subclinical or chronic) results in loss of vital milk composition and protein quality and invariably compromises the technological or processing properties of milk (Åkerstedt *et al.*, 2008; Sunderkilde *et al.*, 2012).

1.1.6 Treatment and control

Antimicrobial agents, chiefly antibiotics, are the drugs of choice for the treatment of cows with mastitis. Penicillins, cephalosporins and tetracycline are some of the commonly used antibiotics. As part of control measures for mastitis in dairy herds, treatment with antibiotics during the dry period of cows is routinely carried out. An additional therapeutic practice is the milking out of affected quarters/cows and/or even the use of oxytocin to stimulate flow of milk in order to get rid of the infected milk and decrease levels of medium (milk) of bacterial growth.

In the early 1970s, a five point mastitis control programme was initiated by the NMC which included prompt treatment of all clinical mastitis cases, treatment of cows in dry periods to eliminate existing infection and to prevent acquisition of new ones, culling of persistently infected cows, use of disinfectant dipping of teat after milking and annual testing of milking machinery (Hillerton and Berry, 2005). This programme was implemented by dairy farms in many countries and was successful in greatly reducing the incidence of clinical mastitis in the farms. In one study, similar control measures were utilized by Nagahata *et al*. (2007) to

successfully control even a persistent *S. aureus* mastitis on a farm. Since its introduction and adoption, the five point mastitis control plan, has become an integral practice in most commercial dairy farms in the western world.

Adopting hygienic practices in the general dairy farm management and especially during milking, represents a very important step in the control of mastitis. Pre and post milking dipping of teats with disinfectants; proper sanitation of stalls, litter and bedding materials as wells as milking machines, are some of these measures. Precalving antibiotic use has also been considered, as mastitis incidence tend to increase around the immediate post calving period (Bastan *et al.*, 2010).

Vaccination has also been employed in the control and prevention of mastitis, vaccines against *E. coli* (J5 vaccine), (Sudhan and Sharma, 2010) and CNS (Startvac® by HIPRA) have been introduced. The CNS vaccine is administered 45 days and 10 days before calving, and then at 52 days post calving to reduce the risk of development of CNS mastitis.

The use of teat sealers, which could be externally or internally applied, to occlude the udder from contact with environment during the dry period have also been evaluated as a means of mastitis control (Huxley, *et al.*, 2002; Dingwell *et al.*, 2003a). Recently, Leitner *et al.* (2013) have explored the use of immunotherapeutic agent, 'Y-complex' in the treatment of mastitis. Y-complex is made up of a microbead containing immunoglobulins against mastitis-causing-bacteria and a phagocytosis enhancer. In their study, they found the complex effective to clear infections, with no side effect or problem of residues as encountered in antibiotic use, and recommend it as a new approach for mastitis treatment.

Despite the stringent control measures, it has been observed that contagious pathogens are relatively easier to control than environmental pathogens (Rambeaud *et al.*, 2003), and there is a lot of work going on to explore or develop measures for optimum control of environmental pathogens. Prompt recognition of mastitis cases especially the subclinical is central to the effective control of pathogen-specific mastitis.

1.1.7 Diagnosis

Clinical signs of inflammation of the udder, namely painful swelling, heat, hyperaemia and in some cases generalized fever are indicative of CM. In milk, the presence of blood clots, flakes and change in colour towards a bloody or serum-like appearance, also points to the presence of mastitis. A definite diagnosis is usually made by bacteriological culture and isolation of causative organisms from milk in combination with somatic cell counts as recommended by the International Dairy Federation (IDF). A number of other diagnostic procedures are also used, as discussed in the next Sections.

1.1.7.1 Somatic cell counts

Rapid migration of leukocytes from blood into the udder in order to help combat invading pathogens occurs in mastitis (Leitner *et al.*, 2000). Damage to mammary epithelial cells also occurs. Somatic cells are composed of these leukocytes and damaged mammary epithelial cells (Wagner *et al.*, 2009) that are found in milk during IMI.

Neutrophils are the predominant type of leukocytes found in milk in acute mastitis and macrophages to a lesser extent. The marked increase of these cells in milk during the course of mastitis has been shown to affect milk quality and composition. Somatic cell counts (SCC) is the estimated number of somatic cells in milk and has been used as a gold standard for confirming mastitis in cases of SM (NMC, 2001; Pyorälä, 2003) and also used to determining milk quality (Ruegg and Pantoja, 2013). Somatic cells, predominantly macrophages, are known to be present to a limited extent in healthy udders, forming part of the host innate defence mechanisms. Cytokines and other chemoattractants released by these macrophages are thought to play a crucial role in the massive recruitment of neutrophils from blood following pathogen invasion (Pyorälä, 2003). The extent and magnitude of migration of leukocytes into the mammary gland in mastitis varies with host and pathogen virulence factors.

In a study to characterize the levels of SCC according to the presence or absence of mammary infection, an average cell count of 68,000 cells/ml was found in milk samples negative for bacteriological infection (by culture), 110,000-150,000 cells/ml for infections with minor pathogens and 350,000 cells/ml and above for

infections with major pathogens (Djabri *et al.*, 2002). It has now come to be generally accepted that SCC ≤100,000 cells/ml are consistent for healthy udders while ≥200,000 cells/ml could indicate presence of infection (Dufour and Dohoo, 2012).

Measurement of SCC can be carried out on herd or cow level, consisting of bulk milk SCC (BMSCC), composite or quarter milk SCC. Many dairy farms routinely carry out monthly cow composite milk SCC to determine the mastitis status of cows. BMSCC is carried out to determine overall milk quality of the herd as it relates to the presence of mastitis (Barkema *et al.*, 1998).

Since it is possible for an individual quarter of a cow's udder to be infected without contra-lateral or adjacent quarters of same udder being infected, due to the presence of intramammary septa separating each quarter from the other, quarter milk SCC (QSCC) is important for isolating the particular infected quarter(s) of the udder, as composite milk SCC (CSCC) can be diluted from quarters with low SCC (Forsback *et al.*, 2009).

Dufour and Dohoo's study (2012) confirmed the usefulness of quarter SCC for the determination of incidence and elimination rates of new dry period IMIs and their findings agreed with earlier reports of Mollenhorst *et al.* (2010) of the greater sensitivity of quarter milk than cow (composite) milk for monitoring udder health, using an online system.

Frequently used methods for the determination of SCC are direct microscopy (also called Breed method) and Fossomatic counter methods which are based on fluoro-optical properties. Another method used is the Coulter milk counter by counting cells as they flow through an electric field. Recently, a new milk somatic cell counter, DeLaval cell counter has been introduced (DCC; DeLaval International AB, Tumba, Sweden) (Leslie *et al.*, 2006; Kawai *et al.*, 2013). This counter uses a similar principle as the Fossomatic counter, and counts cell nuclei stained with a DNA specific dye using an optical digital camera. It is accurate when counting SCC of ≤4 x 10^6 cells/ml, but it has also been shown that samples can be diluted to give reliable counts (Kawai *et al.*, 2013). It has the added advantage of low initial cost and portability.

Given that direct SCC measurement is difficult to adapt to rapid field (on line; production milking) detection methods, indirect on line methods exists such as the California Mastitis Test (CMT). Another test was developed for the determination of SCC based on viscosity properties, and this is frequently used in AMS (Kamphuis *et al.*, 2008; Fosgate *et al.*, 2013). More advancement in measuring SCC came by way of a developed chip (Choi *et al.*, 2006) that was able to detect and measure SCC, pH, antibiotic residues and some bacteria pathogens in milk at the same time. Another means of measuring SCC indirectly is by analysis of adenosine triphosphate (ATP) (section 1.1.7.6).

Differential cell counts of milk have also been advocated as an alternative to total SCC, and when utilized in the study by Pilla *et al.* (2013), they showed a very high sensitivity and specificity to detect IMI in quarters that otherwise had low SCC, using a specified cut off value for neutrophil and lymphocyte ratio. Apart from the dilution effect on composite milk samples that compromises its reliability for diagnosis of mastitis using SCC, other factors other than IMI can affect the levels of SCC in milk such as lactation stage, season, lactation number, milking frequency, milking interval and type of milk sample collected (foremilk or stripping milk). SCC levels have been shown to remain high in milk long after the resolution of a mammary infection/inflammation (Pyörälä and Syvajarvi, 1987) thus further compromising its specificity in mastitis diagnosis. In addition, SCC has also been questioned and has not been found very dependable in its correlation to milk protein quality (Åkerstedt *et al.*, 2008).

1.1.7.2 California mastitis test (CMT)

The California mastitis test (CMT) is an indirect estimation of SCC designed by Schalm and Noorlander in 1957 (Sargeant *et al.*, 2001), and based on the formation of a gelatinous precipitate in milk mixed with a detergent reagent (3 % w/v sodium lauryl sulphate and bromocresol), as a result of the interaction of DNA released from cells with the detergent. Thus, the degree of gel precipitate formed can be scored to correlate with different SCC groups. CMT is inexpensive, fast and can easily be adaptable as a cow side test. However, some variability in detecting abnormal milk using CMT has been reported (Kawai *et al.*, 2013). Several studies have been carried out to ascertain the usefulness of CMT as a cow side test, in readily recognising samples with IMI for selection for

further bacteriological confirmation. In the study of Sargeant *et al*. (2001), CMT was found to be sufficiently sensitive and specific in the mid to late lactation stage and early (first week of) lactation respectively. Dingwell *et al*. (2003b) also confirmed the sufficient sensitivity of CMT in the first week of lactation. Good correlations have been found between CMT and other indicators of mammary inflammation such as SCC and electrical conductivity (EC) (Kaşikçi *et al*., 2012), and Seker *et al*. (2009) found a significant correlation between CMT scores and ultrasonographic teat measurements of different breeds of cows, indicating that teat features are possibly predisposing factors to mastitis occurrence. In another recent study, CMT was found to be more sensitive than EC in classifying infected samples with SCC above 200,000 cells/ml (Fosgate *et al*., 2013). California mastitis test results are usually scored on the basis of level of precipitate formed as described by the CMT kit manufacturers on scales of negative, weak positive, strongly positive and so forth (Fosgate *et al*., 2013).

1.1.7.3 Electrical conductivity

Measuring electrical conductivity (EC) of milk samples has been shown to indicate SM and CM (Milner *et al*., 1996). This assay relates to the increase in milk conductivity of electricity that is enhanced during IMI as a result of leakage of extracellular ions such as Na^+ and Cl^- into milk and the subsequent loss of predominantly lactose and K^+ (Pyorälä, 2003; Kaşikçi *et al*., 2012). Electrical conductivity was found to show similar sensitivity in detecting SM as did SCC and CMT in the study of Kaşikçi *et al*. (2012).

Although able to be adapted to an online system for mastitis diagnosis, many other factors can affect milk EC, and a lot of false positive rates have been associated with use of EC (Mottram *et al*., 2007) thereby limiting its potentials as a diagnostic tool for mastitis detection (Pyorälä, 2003). Hand held meters for measuring EC have been used in several countries (Fosgate *et al*., 2013). It is becoming increasingly common to combine EC determination with either quarter milk SCC (QSCC) or composite milk SCC (CSCC) for the monitoring of udder health (Kamphuis *et al*., 2008; Mollenhorst *et al*., 2010), particularly with the use of AMS (Mottram *et al*., 2007).

1.1.7.4 Infra-red thermography

Infra-red thermography (IRT) is a non-invasive method that utilizes heat absorbed following emission of infra-red radiation to generate images and can be used as an indicator of inflammation (Kotrba *et al.*, 2007). A large and growing body of literature has investigated the usefulness of IRT for the detection of SM in dairy animals (Polat *et al.*, 2010; Colak *et al.*, 2008; Samara *et al.*, 2013; Metzner *et al.*, 2014; Pezeshki *et al.*, 2011). Many of these studies have demonstrated its sensitivity in detecting changes in udder skin temperature that reflects the presence of mastitis. Kunc *et al.*, (2007) reported that mastitis infections cause udder surface temperatures to rise often before other clinical signs are observed. On the other hand, studies by Hovinen *et al.* (2008) and Pezeshki *et al.* (2011) found the use of IRT in SM and early mastitis not to be reliable. This study observed that changes in udder skin temperature (UST) occurred hours (h) after the appearance of local signs of inflammation, indicating that IRT might not be sufficiently reliable enough for the early detection of mastitis.

1.1.7.5 Milk Enzymes

Certain enzyme levels increase in milk during IMI. Enzymes originating from phagocytes, ruptured epithelial cells, and from serum contribute to the change in the physical and chemical properties of milk seen during mastitis.

N-acetyl-ß-D-glucosaminidase (NAGase), ß-glucuronidase (Nagahata *et al.*, 1987; Larsen and Aulrich, 2012) and catalase (Kitchen, 1976) are some lysosomal enzymes the activities of which increase in milk as they are released from neutrophils to facilitate the phagocytic process on pathogens. Assays of these enzymes are an important diagnostic test for mastitis (Polat *et al.*, 2010).

NAGase is also present in lysosomes of mammary epithelial cells and following cell lysis is released into the milk (Zhao and Lacasse, 2008).However much of this enzyme still remains within the cytoplasm of cells in milk. Kalmus *et al.* (2013) found a good association between the severity of CM, causative bacteria and the concentration of NAGase. A biosensor (electrochemical based) for measuring NAGase has been described by Pemberton *et al.* (2001).

Lactate dehydrogenase (LDH) is another enzyme the levels of which in milk, have been used as an indicator of inflammatory conditions of the udder. It is a cytoplasmic enzyme involved in carbohydrate metabolism that gets released into milk following damage to mammary epithelial cells.

Plasminogen concentration in milk is also increased due to leakage from blood as a result of the compromised membrane permeability. This causes proteolysis of milk proteins following its activation to plasmin in milk. Other enzymes in milk that have been suggested as markers of mastitis include alkaline phosphatase (AP) (Babaei *et al.*, 2007; Larsen *et al.*, 2010a; Guha *et al.*, 2012) and acid phosphatase (AcP) (Larsen *et al.*, 2010a) whose activity in milk tends to increase during IMI due to their release from leucocytes.

1.1.7.6 Adenosine triphosphate

Adenosine triphosphate (ATP) is often referred to as the energy currency of the body and it is present in all living cells. Its concentration in milk has shown a correlation with SCC (Olsson *et al.*, 1986) due to the fact that ATP is released by these SCC into milk; its measurement may therefore be useful to monitor IMI (Pyorälä, 2003). ATP level has been successfully used to group milk samples by health status, and was also found to correlate with acute phase proteins (APP) (Gronlund *et al.*, 2005).

Measurement of ATP is considered an indirect assay of the SCC and a bioluminescent assay of ATP released from SCC was proposed (Emanuelson *et al.*, 1988). Frundzhyan *et al.* (2008), went ahead to improve the bioluminescent assay for ATP to measure only non-bacteria ATP, however, measurement of ATP for mastitis diagnosis has not found widespread usage, probably because measuring ATP requires several sample preparation steps and technical machinery just as much as in the measurement of SCC.

1.1.7.7 Lactose

Lactose (β-D-galactopyranosyl-D-glucopyranoside) is the predominant form of milk sugar, and it is synthesized in the mammary gland secretory cells (Golgi apparatus). Since the synthetic ability of the mammary cells is massively affected in mastitis due to cell damage, lactose concentrations are known to fall (Pyorälä, 2003). Several studies have also demonstrated the correlation of a

reduction in lactose concentration with mastitis or SCC (Sharma and Misra, 1966; Malek dos Reis *et al.*, 2013). Berning and Shook (1992), however found that the change in lactose concentration does not correlate well with SCC and is not very indicative of IMI.

1.1.7.8 Bacteriology

This entails the culture of milk samples collected in suitable culture media. A standard of 0.01 ml of milk sample is plated onto suitable agar and usually incubated for 24-48 hours (h) at 37ºC, followed by identification of bacteria based on colony morphology, haemolysis and further tests such as catalase, coagulase tests.

Microscopic identification is carried out after gram staining. The NMC has stipulated guidelines for the culturing of milk for mastitis diagnosis (NMC, 2004). In these guidelines, some factors including presence of organisms of interest (mastitis pathogens), number of colonies of these organisms, whether it was isolated in pure culture and use of multiple sampling of individual glands is recommended for accurate diagnosis of IMIs. In a recent study, triplicate quarter samples were determined to be the most specific and sensitive in recognising IMIs (Dohoo *et al.*, 2011). However, it has been noted that in up to 30 % of clinical mastitis cases, bacteriological tests of milk yields no positive results (Hettinga *et al.*, 2008; Bradley *et al.*, 2007). Commonly, cultures where up to three different pathogens are isolated are regarded as contaminated and often not used in conclusively identifying mastitis aetiology (Randy *et al.*, 1986).

Bacteriological culture is generally accepted as the most reliable means of detecting intramammary infections (Dohoo *et al.*, 2011), however, major limitations of being time consuming, expensive and not practically adaptable to cow side or on line use, are associated with this method of diagnosis.

1.1.7.9 Molecular (PCR) diagnosis

Recently, polymerase chain reaction- single strand conformation polymorphism (PCR-SSCP) has been suggested as a useful alternative for the identification of pathogenic causes of mastitis, necessitated by increasing observations of culture-negative (about 30 %) milk samples from mastitis cows (Schwaiger *et al.*, 2012). Gurjar *et al.* (2012) presented several case reports of diagnosis of IMI

caused by *S. aureus*, *Mycoplasma bovis*, *S. uberis* and *Enterobacter spp* in farms using the DNA based molecular technique and cited advantages of the diagnostic measure in facilitating prompt control and prevention of further spread of infection.

A commercial PCR based mastitis diagnosis kit was introduced recently (PathoProof Mastitis PCR Assay; Thermo Fisher Scientific, Espoo, Finland) (Spittel and Hoedemaker, 2012) and has been reported to be more sensitive. However, even in cases of CM, there have been reports of no pathogens being detected in milk samples even with the PCR method (Kalmus *et al.*, 2013), moreover the molecular technique is labour intensive, expensive and not easily carried out on the complex milk matrix (Hettinga *et al.*, 2008).

1.1.7.10 Multisensors in automatic milking systems

Milking by the AMS presents new challenges for the detection of IMI as physical indicators of abnormal milk such as colour and presence of flakes, blood or clots cannot be seen as in conventional methods, by the milker. This has warranted the development and use of sensors for multiple attributes of milk such as yield, temperature, and electrical conductivity (Kamphuis *et al.*, 2008; Mottram *et al.*, 2007). A multisensor also called the 'electronic tongue' based on potentiometric chemical sensor has been advocated for the detection of clinical mastitis (Mottram *et al.*, 2007). A gas sensor array system (electronic nose) was used to differentiate mastitic from non-mastitic milk samples and has been described (Eriksson *et al.*, 2005) as offering promise for rapid mastitis detection.

1.1.7.11 Acute phase proteins

Following the release of cytokines and other proinflammatory mediators, predominantly interleukine -1 (IL-1), interleukine-6 (IL-6) and tumour necrosis factor-alpha (TNFα), by macrophages in the mammary gland upon pathogen invasion (Tassi *et al.*, 2013), several local and systemic responses are elicited in an acute phase response (APR), comprising of the release of acute phase proteins (APP) from the liver and the mammary glands into milk, and recruitment of other proinflammatory cells from the blood amongst many other systemic and local responses (Jensen and Whitehead, 1998). Figure 1-1 shows a schematic diagram of the pathway for secretion of APP.

Acute phase proteins are a group of proteins predominantly produced in the liver, that are changed (usually increased or decreased) by over 25 % during inflammation, infection or stressing conditions, and released into blood (Lomborg *et al.*, 2008; Ceron *et al.*, 2005; McDonald *et al.*, 2001). Elevated levels of APP in serum are generally used as non-specific indicators of inflammation and have been widely used in human clinical diagnosis for a long time (Eckersall and Bell, 2010). The use of APP as inflammation indicators has become important over the last few decades.

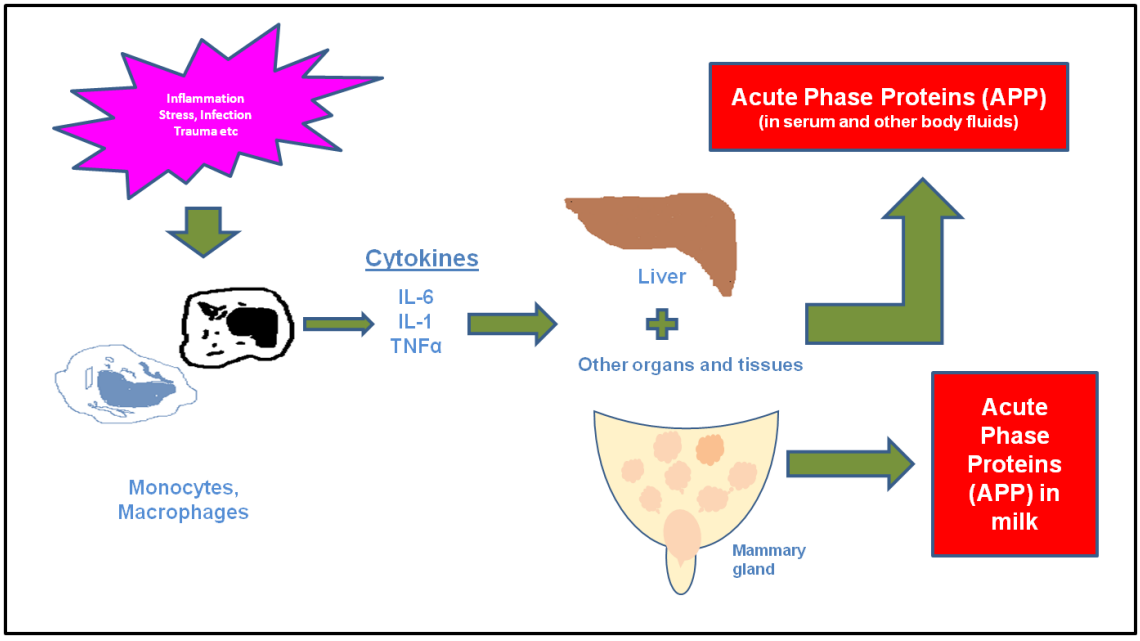

Figure 1-1: Schematic representation of the acute phase response leading to secretion of acute phase proteins.

There are three major classes of APP; including major, minor and negative APP. Major APP are proteins whose levels in serum increase by 1000 folds from baseline values during inflammation; haptoglobin (Hp) and serum amyloid A (SAA) are major APP of cattle. Moderate APP concentration increase less dramatically during inflammation (2-10 fold) and examples in cattle are alpha 1-glycoprotein and inter α-trypsin inhibitor H (ITIH), while minor APP increase in less than 2 fold proportion for example C-reactive protein (CRP) and negative APP whose synthesis are down regulated during APR for example albumin (Uhlar and Whitehead, 1999; Murata *et al.*, 2004).

Several studies have revealed the occurrence of APP not just in serum, but in other body fluids such as milk, colostrum, nasal secretion, abdominal fluid, synovial fluid (Eckersall *et al.*, 2001; McDonald *et al.*, 2001; Molenaar *et al.*, 2009). Bovine serum albumin and alpha$_1$-trypsin inhibitor (A_1TI) have been measured in milk as indicators of mastitis (Sandholm *et al.*, 1984 in Pyorälä *et al.*, 2011).

Of late, a large and growing body of literature has examined the relationship between the major bovine APP in milk with IMI and other indicators of inflammation (Viguier *et al.*, 2009). Eckersall *et al.* (2001) first reported the correlation of major APP; SAA and Hp in milk with presence mastitis. Many other workers have demonstrated the correlation of major bovine APP with other inflammatory indices (particularly SCC) during mastitis (Nielsen *et al.*, 2004; O'Mahony *et al.*, 2006; Åkerstedt *et al.*, 2008; Pyorälä *et al.*, 2011) and even with milk composition and protein quality (Åkerstedt *et al.*, 2008; Åkerstedt *et al.*, 2009) and with severity of the IMI (Pyorälä *et al.*, 2011). To further emphasize the advantage of these major APP in mastitis detection, only a small variation in their levels were observed in healthy cow's milk over 42 consecutive milkings, which shows that these APP are stable and able to discriminate between healthy and inflamed tissues reliably (Åkerstedt *et al.*, 2011).

Alpha$_1$-acid glycoprotein and alpha$_1$-trypsin inhibitor are moderate APP in cattle which increases in chronic conditions (Eckersall *et al.*, 2001; Pyörälä, 2003) therefore they are less effective in diagnosis or prognosis of mastitis in cows. Bovine serum albumin (BSA) is a negative APP in cattle and does not show significant changes with subclinical mastitis in the acute phase to be of diagnostic value.

Wenz *et al.* (2010) evaluated the usefulness of Lipopolysaccharide binding protein (LBP), another APP, for mastitis diagnosis. In studies by Suojala *et al.* (2008) LBP in serum and milk was found to increase in association with the course of an experimental *E. coli* mastitis infection, especially in milk.

It is now established that the major bovine APP (Hp and SAA) are synthesized in the mammary gland (Eckersall *et al.*, 2001; Eckersall *et al.*, 2006; Hiss *et al.*, 2004; Lai *et al.*, 2009; Theilen *et al.*, 2007). It has also been shown that Hp is

synthesized by the neutrophils that migrate into the gland during inflammation, but it is not clear if this is the only source of Hp in milk during mastitis.

Soyeurt *et al*. (2012) explored the use of mid-infrared spectrometry for the measurement of lactoferrin, an important immune glycoprotein in milk, developing an equation to quantify its level in milk and they also found that using Lactoferrin and SCC improved the predictability for mastitis over using SCC alone. Different pathogens have been shown to elicit differences in the level and time of onset of APP secretion (Suojala *et al*., 2008) and this was shown in a study by Pyörälä *et al*. (2011) and Kalmus *et al*. (2013) where coliforms had higher levels of APP (inflammatory response) than other pathogens assessed.

Major acute phase proteins of bovine milk

Serum amyloid A (Mammary associated serum Amyloid A3)

Serum amyloid A is an apolipoprotein made up of two major classes. The acute phase SAA (A-SAA), this is primarily produced in the liver in response to an acute phase stimulus, under the influence of inflammatory cytokines, mainly IL-1, IL-6 and TNFα. A-SAA is then released into the blood stream where it binds predominantly to the high density fraction of lipoproteins (HDL). Secondly, the constitutive SAA (C-SAA) is constitutively expressed in many tissues (Uhlar and Whitehead, 1999; Berg *et al*., 2011; Lecchi *et al*., 2012; Kovačević-Filipović *et al*., 2012), especially cells lining tissues that communicate with the external environment for example the gastrointestinal tract (GIT) and respiratory tract. A-SAA is reported to be able to bind to and transport cholesterol at its amino terminal region by a structural modification (diminished α-helical structure) that makes them different from the C-SAA, which does not bind cholesterol (Liang *et al*., 1996). This amino terminal of A-SAA molecules provides the HDL-binding domain comprising of the last 10 amino acids (Yamada, 1999).

It is a highly conserved protein in most vertebrates and invertebrates and has been shown to be a major and highly sensitive APP, the concentrations of which rise from basal to up to a thousand fold increases in serum shortly following inflammatory or stressor stimuli, in most mammalian species (Wilkins *et al*., 1994; Eckersall and Bell, 2010). It is a small protein, having a molecular weight between 11-14 kDa and about 104-112 amino acid residues (Rossevatin *et al*.,

1992; Yamada, 1999). The role of SAA in inflammation and immunity is not fully understood but it is thought to be involved in lipid transport and repair of damaged tissues (Takahashi *et al.*, 2009), and also thought to have antibacterial activity (Hari-Dass *et al.*, 2005; Molenaar *et al.*, 2009). Other speculated effects of SAA include functional modulation of immune response cells such as neutrophils (Gatt *et al.*,1998) and induction of secretion of mucin in the intestinal lining by the mammary associated isoform (SAA3), thereby preventing adhesion of pathogenic bacteria (Larson *et al.*, 2003). SAA has been speculated to have both pro and anti-inflammatory roles.

There are different isoforms of SAA and these are known to be heterogeneous in their amino acid sequences, and isoelectric points (Horadagoda *et al.*, 1993; Alsemgeest *et al.*, 1995; Kovačević-Filipović *et al.*, 2012). Major isoforms include SAA1, SAA2 and SAA3 (which are encoded for by the *SAA1, 2* and *3* genes to form the A-SAA) and SAA4 which is encoded for by the *SAA4* gene and comprise the C-SAA (Upragarin *et al.*, 2005). The SAA1, SAA2 and SAA4 are produced in the liver. SAA3 is the predominant isoform produced in extrahepatic sites and is the form found in colostrum and mastitis milk; it is also called mammary associated serum amyloid A (M-SAA3) or milk amyloid A (MAA)(Eckersall *et al.*, 2001; McDonald *et al.*, 2001; Nielsen *et al.*, 2004; Molenaar *et al.*, 2009). Serum amyloid A3 has also been shown in the equine synovial fluid (Jacobsen *et al.*, 2006) adipose tissue (Mukesh *et al.*, 2010) and gastric epithelial cells (Dilda *et al.*, 2012).

It was specifically demonstrated by Molenaar *et al.* (2009) using *in situ* hybridization, that the mammary secretory epithelial cells were responsible for the production of SAA3 during episodes of mammary infection. Several other isoforms (apart from SAA1 and SAA2) have been demonstrated in bovine serum by Takahashi *et al.* (2009) having pIs ranging from 5.2 to 8.6.

Moreover, multiple isoforms have also been demonstrated in milk including a three very alkaline forms (pI > 9.3) during mastitis which were not found in serum (Jacobsen *et al.*, 2005; Kovačević-Filipović *et al.*, 2012; Larson *et al.*, 2005; Weber *et al.*, 2006). The SAA isoform demonstrated in colostrum was also of very high alkaline pI and molecular weight (Mw) of 12.6 kDa (McDonald *et al.*, 2001). Serum AA exists mainly as complexes with high density lipoproteins (HDL) in serum and during periods of prolonged high concentration of SAA in the blood,

deposition of amyloid fibrils usually occurs causing a condition called amyloidosis in many species (Takahashi *et al.*, 2007; Murakami *et al.*, 2014).

Prolactin is a major physiological stimulus for the M-SAA3, and Larson *et al.* (2005) showed that, M-SAA3 is the main isoform synthesized by the mammary epithelial cells in response to prolactin and lipopolysaccharide (LPS) stimulation, suggesting a specific role of that isoform in the gland. Van Der Kolk *et al.* (1992) however demonstrated that adrenocorticotropic hormone failed to induce hepatic synthesis of SAA. Serum amyloid A has been described as a useful nonspecific marker of inflammation, which can also be used to monitor progression or prognostics of disease in Veterinary Medicine (Eckersall and Bell, 2010). According to Humblet *et al.* (2006), SAA (and Hp) measurement in serum within a 6 months study period was able to classify clinically diseased cows with higher specificity (though less sensitivity) than clinical examination.

The increase in serum of acute phase SAA during inflammation occurs rapidly and up to 1000 fold, hence it has been exploited as a useful biomarker of inflammation in man and animals and is designated a major APP in most species. Serum amyloid A is expressed in all animal species and their expression is highly conserved, suggesting its importance and functional integrity in all species. It has also been demonstrated to be synthesized in histologically normal extrahepatic tissues such as lungs, mammary gland, uterus and the gastrointestinal tract, supporting the hypothesis that this apolipoprotein plays a role in innate defence of the body against pathogen invasion (Berg *et al.*, 2011).

These M-SAA3 isoforms are made up of about 113 amino acids and a molecular weight of between 12-14 kDa (Takahashi *et al.*, 2009; Yamada, 1999 Rossevatn *et al.*, 1992) whereas the human A-SAA has 104 amino acids and a molecular weight of about 12 kDa (Uhlar or Whitehead, 1999). Moreover, the various serum A-SAA and the milk specific isoform of SAA, have been identified as having different isoelectric points (pI), with the M-SAA3 being highly alkaline (pI = 9.6) as compared with other isoforms (McDonald *et al.*, 2001; Jacobsen *et al.*, 2005).

Data from several sources have demonstrated the correlation of M-SAA3 to other established inflammatory indices of intramammary inflammation (IMI) (O'Mahony *et al.*, 2006; Jacobsen *et al.*, 2005; Gerardi *et al.*, 2009; Kovačević-Filipović *et al.*, 2012). M-SAA3 has been found to be more sensitive to changes in the

inflammatory condition of the mammary gland than its serum counterpart (Eckersall *et al.*, 2001; O'Mahony *et al.*, 2006; Jacobsen *et al.*, 2005).

Larson *et al.* (2006) and Molenaar *et al.* (2009) also demonstrated in their study that M-SAA3 is synthesized locally by the mammary epithelial cells, thereby improving the potentials of M-SAA3 as a biomarker for subclinical mastitis.

The presence of SAA in colostrum of healthy animals has suggested a role for these APP in conferment of some immunological characteristics on the new-born (McDonald *et al.*, 2001), or a role in the maintenance and remodelling of the mammary gland tissue during lactation. Moreover it may also be related to the stress induced APR on the cow during the process of parturition, as APP levels remain elevated only for a few days and then drop.

Assay of SAA has been predominantly by immunoassays such as ELISAs. Obtaining monoclonal as well as polyclonal antibodies for SAA has been known to be particularly difficult due to the fact that SAA is highly hydrophobic (thus binding to HDL in serum) and the high homology of individual species SAA isoforms (Yamada, 1999; Kho *et al.*, 2000). However a method for production of monoclonal antibodies against SAA has been described by McDonald *et al.* (1991) and has now been used to produce a commercial immunoassay format for the measurement of multispecies SAA (Tridelta Development Co., Ireland).

The usefulness of SAA (mammary associated serum amyloid A3) in diagnosis and prognosis of mastitis, particularly SM has also been investigated by various groups in the bovine species (Pyörälä *et al.*, 2011; Kovačević-Filipović *et al*, 2012; Szczubial *et al.*, 2012; Gerardi *et al.*, 2009; Suojala *et al.*, 2008) and in other species including ovine (Winter *et al.*, 2006; Miglio *et al.*, 2013). Using both experimental models (Eckersall *et al.*, 2001; Nielsen *et al.*, 2004; Jacobsen *et al.*, 2005; Gronlund *et al.*, 2005; Eckersall *et al.*, 2006) and field conditions (Pyörälä *et al.*, 2011; Kovačević-Filipović *et al.*, 2012; Kalmus *et al.*, 2013) the potentials of M-SAA3 in mastitis detection, assessment of severity of infection and prognostic evaluation of cases, has been identified.

The effect of sample storage temperature and conditions on SAA values was recently investigated by Tothova *et al.* (2012) who found that duration and storage temperature of milk and serum samples from dairy cows could affect SAA

(reducing) values significantly.

Haptoglobin

Haptoglobin (Hp) was discovered in the late 1930s by Polonovski and Jayle (Rowe, 1962). The name haptoglobin derives from two words 'hapto' (to bind) and 'globin', suggesting its role (Sadrzadeh and Bozorgmehr, 2004) in binding to haemoglobin (Hb). Haptoglobin is a major APP in cattle, a tetrameric glycoprotein having two α (alpha) and two ß (beta) chains linked by disulphide bonds. It is often found in polymeric (2-20 units) forms in bovine serum. The two α chains are different, with α 1 weighing approximately 8.9 kDa, and α 2 about 16 kDa; these correspond to the light chain of the human H2 haptoglobin. The two β-chains, however, are identical and have molecular weights (Mw) of about 40 kDa and corresponding to the heavy chain of human Hp (Morimatsu *et al.*, 1991a; Sadrzadeh and Bozorgmehr, 2004). Recently Andersen and co-workers elucidated the structure of the Hp-Hb (porcine) complex (Figure 1-2) after purification and crystallization by employing the use of ultra-visible and Raman spectroscopy and other advanced data processing techniques (Andersen *et al.*, 2012).

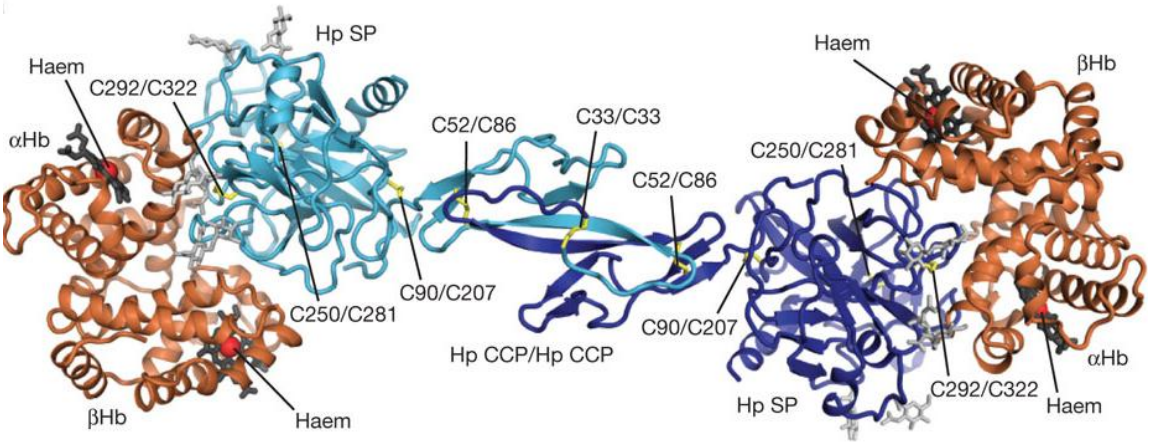

Figure 1-2: Crystal structure of porcine haptoglobin-haemoglobin complex.
Hp is coloured blue and cyan, αHb and βHb are orange. Haem groups are shown as dark grey sticks. Red spheres represent Fe ions. Glycosylations are shown as light grey sticks and disulphide bridges as yellow sticks.

Source; Andersen *et al*; Nature 489, 456–459 (20 September 2012) doi:10.1038/nature11369

Haptoglobin binds to free haemoglobin with very high affinity; the β-chains carry the haemoglobin binding portion and have sequences which are highly conserved

in most species. Haptoglobin also has broad anti-inflammatory activities and is involved in angiogenesis (Arredouani *et al.*, 2003; Tseng *et al.*, 2004; Sadrzadeh and Bozorgmehr, 2004). Potent antioxidant activities are associated with Hp, not only through its haemoglobin binding capabilities which serve to prevent the damaging oxidative effects of iron released from free haemoglobin to the body, but possibly through other structural conformation changes (Tseng *et al.*,2004). Roles in lipid metabolism have been suggested (Dobryszycka, 1997), while others have suggested immunomodulatory activities (Morimatsu *et al.*, 1991a; Arredouani *et al.*, 2003). The binding of Hp to Hb is particularly useful in preventing the growth and multiplication of pathogenic bacteria that require iron, thus Hp can be said to have antibacterial activity (Sadrzadeh and Bozorgmehr, 2004).

Haptoglobin is synthesized mainly by the hepatocytes in liver, following proinflammatory cytokine; IL-6 and TNFα stimulation during inflammation or infection. Extrahepatic synthesis has been demonstrated in several other sites including lungs (Abdullah *et al.*, 2012), skin (Li *et al.*, 2005) spleen, intestine (D'Armiento *et al.*, 1997), female reproductive organs in bovine (ovary and oviduct) (Lavery *et al.*, 2003), adipose tissue (Saremi *et al.*, 2012) and also in the mammary gland (Hiss *et al.*, 2004; Thielen *et al.*, 2005; Cooray *et al.*, 2007). Macrophages, epithelial cells, and granulocytes have also been reported to synthesize Hp (Mao *et al.*, 2001; Cooray *et al.*, 2007). Haptoglobin synthesis can also be stimulated by glucocorticoids as well as through cytokines.

Increased synthesis of Hp leads up to a thousand fold change in serum levels, and Hp has also been detected in several other body fluids including saliva (Gutierrez *et al.*, 2009), oviductal fluid (Lavery *et al.*, 2003), nasal secretion (Ghazali, personal communication (pers. comm.), synovial, cerebrospinal, ascitic and pleura fluids, urine (Sadrzadeh and Bozorgmehr, 2004) and in milk (Eckersall *et al.*, 2001; Gronlund *et al.*, 2003; Pedersen *et al.*, 2003; Eckersall *et al.*, 2006), with milk Hp level shown to rise dramatically in episodes of mastitis.

Increased milk Hp in IMI was initially thought to occur only as a result of increased permeability of blood milk barrier during udder infections (Eckersall *et al.*, 2001), however Hiss *et al.* (2004), Thielen *et al.* (2005) and Lai *et al.* (2009), have demonstrated the synthesis of Hp in mammary epithelial cells as well as in

milk somatic cells predominantly neutrophils. Measuring milk Hp has been shown to be a more sensitive indicator of mastitis than serum Hp, as Hp in milk increases several more folds during IMI than serum (Eckersall *et al.*, 2001; Gronlund *et al.*, 2005), and the increases appear earlier than in serum, for example a rise by just 3 h post challenge in milk as against 9 h in serum, following an intramammary LPS challenge in the study by Hiss *et al.* (2004). This was also demonstrated in the study by Eckersall *et al.* (2006).

Several isotypes of Hp were demonstrated in the study of Cooray *et al.* (2007) in the granules of granulocytes of healthy cattle indicating it is constitutively expressed with the different isotypes possibly being due to post translational modifications. Isoelectric points (pI) ranging from 8-9.5 for the 40 kDa (β) subunit and ranging from 6 to 8 for the 20 kDa (α) subunit of Hp, were also shown.

Unlike humans and rats, non-acute phase sera in cattle rarely contains detectable levels of Hp, but following an inflammatory stimulus, Hp is known to rise steadily from about 10 to 24 h after exposure and peaks by the 3rd to 4th day and then gradually drops by the 11th day (Conner *et al.*, 1988 in Jawor *et al.*, 2010; Eckersall *et al.*, 2006). This therefore enhances the potential of Hp in indicating inflammation in cattle.

Haptoglobin has also been described as a bovine adipokine, which is not confounded by varying degrees of adiposity when used as an inflammatory marker (Saremi *et al.*, 2012). It was also suggested that parturition may elicit a higher APR in primiparous cows than in multiparous. The potential usefulness and applications of serum Hp measurement in cattle continues to grow especially in the areas of clinical diagnosis, monitoring herd health and management, pre slaughter meat inspection, evaluating responses to stress and so on (Skinner *et al.*, 1991; Alsemgeest *et al.*, 1994; Nazifi *et al.*, 2009; Cooke *et al.*, 2012; Holland *et al.*, 2011; Blagojevic *et al.*, 2011; Eckersall and Bell, 2010).

Blagojevic *et al.* (2011) investigated the possibility of using serum Hp levels assayed during pre-slaughter ante-mortem meat inspection of cattle and pigs, to determine and establish values and cut off points that could be used for discriminating animals with abnormalities from those without abnormalities in order to reduce the needs for hands on meat inspection. This is in accordance

with previous studies (Saini *et al.*, 1998), which showed that there was a significant difference between Hp of group(s) of cattle having abnormalities compared with those without; however there were no difference observed at individual cow level. Crawford *et al*. (2005), also found serum Hp assessment useful for indicating inflammatory disease and other stressing factors in post calving dairy cows. It has been suggested that prolonged increases in serum Hp may lead to immunosuppression (Crawford *et al*., 2005; Murata and Miyamoto, 1993) and could thus be used to identify cows due for culling.

Serum Hp has been used to assess the severity of inflammation and also as a prognostic marker; in the study of Hisaeda *et al*. (2011), both serum and whey Hp showed prognostic value in predicting recovery from naturally occurring peracute Klebsiella *pneumoniae* mastitis in dairy cows.

Milk Hp has also been evaluated by several studies for its ability as a diagnostic marker for subclinical mastitis (Nilgun *et al*., 2012), although the authors did not find Hp analysis in both samples useful in diagnosing and evaluating treatment of SM and clinical mastitis (Wenz *et al*., 2010), as well as other conditions such as metabolic status during early lactation (Hiss *et al*., 2009). Milk Hp has been found to be elevated in the early pre-partum period (Crawford *et al*., 2005; Hiss *et al*., 2009). In the study of Kalmus *et al*. (2013), milk Hp performed better as an indicator of IMI than MAA, due to the fact that MAA was not able to correctly identify inflammation by *Arcanobacterium pyogenes*, the cause of a purulent acute form of mastitis.

Assays for Hp are based predominantly on two principles; the binding of Hp to antibodies as in immunoassays and on the high affinity of Hp for haemoglobin to form a Hb-Hp complex (Owen *et al*., 1960), which has innate peroxidase activity that can be measured using suitable substrates such as guaiacol. The level of Hb-Hp is proportional to the concentration of Hp. In addition a nephelometric assay for human Hp measurement was described by Vanlente *et al*. (1979). A high performance liquid chromatography method for determining serum Hp levels has also been described (Salonen *et al*., 1996).

A number of immunoassays and Hb binding assays for Hp have been developed and described in literature. Single radial immunodiffusion assay (SRID) was described by Morimatsu *et al*., (1992), ELISAs for Hp determinations which

maybe direct or indirect, sandwich or competitive ELISA have been developed and utilized (Sheffield *et al.*, 1994; McNair *et al.*, 1995; Jawor *et al.*, 2010). Immunoassays are more specific in determining Hp concentration in samples (McNair *et al.*, 1997). In bovine serum, sulphhydryl compounds have been employed to reduce polymerization of Hp before measurement (Morimatsu *et al.*, 1992; Eckersall and Conner, 1990). McNair *et al.* (1995) described a competitive time resolved fluorometric immunoassay that utilized a bovine specific mouse monoclonal antibody and had improved sensitivity as a result of use of lanthanide labelling.

The Hp-Hb binding assay described by Owen *et al.* (1960) was modified by Jones and Mould (1984), for use in a microtitre plate format. Eckersall *et al.* (1999) further modified the assay to take care of the confounding effects of serum albumin on the assay. Slocombe and Colditz (2012) were able to derive an equation to correct for the effect of haemolysis, which frequently occurs during blood sample collection especially from the coccygeal vein in cattle, on biochemical assays of serum Hp.

Haptoglobin-haemoglobin binding assays have the advantage of being cheap and suitable for measurement of Hp from all species, and unlike immunoassays, being less time consuming. However, measuring Hp using the Hb binding, presents the difficulty of interference by haemolysis and serum albumin and other factors that can affect peroxidase activities (McNair *et al.*, 1995; Slocombe and Colditz, 2012; Eckersall *et al.*, 1999) in blood samples. Indeed, Cooke and Arthington (2013) compared the Hb binding assay for measuring Hp in plasma to an ELISA and found significant correlations between the two different assay techniques. Although Slocombe and Colditz in their study were able to derive an equation to correct for the effect of haemolysis on blood Hp assay, in milk, it was observed that innate milk lactoperoxidase could compromise the assay of Hp using the biochemical method (peroxidase activity of Hb-Hp complex) (Eckersall *et al.*, 2001; Åkerstedt *et al.*, 2006).

Milk Hp is measured by immunoassays, especially ELISAs. An immunodiffusion assay was used by Eckersall *et al.* (2001), while Pedersen *et al.* (2003) utilized a sandwich ELISA to measure milk Hp. Hiss *et al.* (2004) also described an ELISA using purified bovine Hp and polyclonal antibody raised against the bovine Hp,

both assays had sensitivities of 11.5 µg/ml and 0.07 µg/ml respectively. ELISA however has the limitation of being time consuming, and the need for rapid on line diagnosis of udder infections is of paramount importance in its control.

A rapid format biosensor assay to determine Hp concentration in milk was described by Åkerstedt *et al*. (2006); the assay was based on the high affinity of Hp for haemoglobin and was a competitive (indirect) assay. Although not as sensitive as the ELISA, some level of success was achieved with this biosensor assay. However, this assay requires expensive machinery and well trained personnel to operate it.

Another attempt to adapt Hp determination to rapid field on line format explored the use of an amperometric immunosensor assay for detection of SM milk samples and had a sensitivity of 0.63 mg/l and agreed well with values for a commercial ELISA kit used to measure Hp in same samples, although above a certain concentration of Hp, the test became less reliable (Tan *et al*., 2012).

In addition to the need for an on farm format for measuring these APP, it is important to determine reference values of these compounds in milk under different physiological conditions and in health. Other factors that could influence variations in the concentration of these parameters in milk other than disease need to be identified.

C-reactive protein

For a long time, C-reactive protein has been categorized as a minor APP of the bovine species (Eckersall and Conner, 1988). In canine and porcine however, it is a major APP that has found wide usage for the diagnosis and monitoring of numerous inflammatory and infectious conditions (Eckersall and Bell, 2010; Petersen *et al*., 2004). It is also secreted from the liver in response to cytokine stimulation and known to play roles in activating phagocytosis by binding to phosphocholine portions of pathogens or dying cell membranes, complement activation, opsonisation of pathogens and binding to immunoglobulin receptors (Black *et al*., 2004).

C-reactive protein has a molecular weight of about 115 kDA and consists of 5 identical subunits linked together non-covalently (Black *et al*., 2004). It was first identified based on its ability to bind to pneumococcal phosphocholine. The

name C-reactive protein (CRP) was given to a pentameric protein which was found to increase greatly in plasma following inflammation and infections, bind to pneumococcal C-polysaccharide and was discovered by Tillett and Francis in 1930 (Clyne and Olshaker, 1999). Figure 1-3 is a model of human CRP showing its 5 subunits in the shape of a pentagon with a central core.

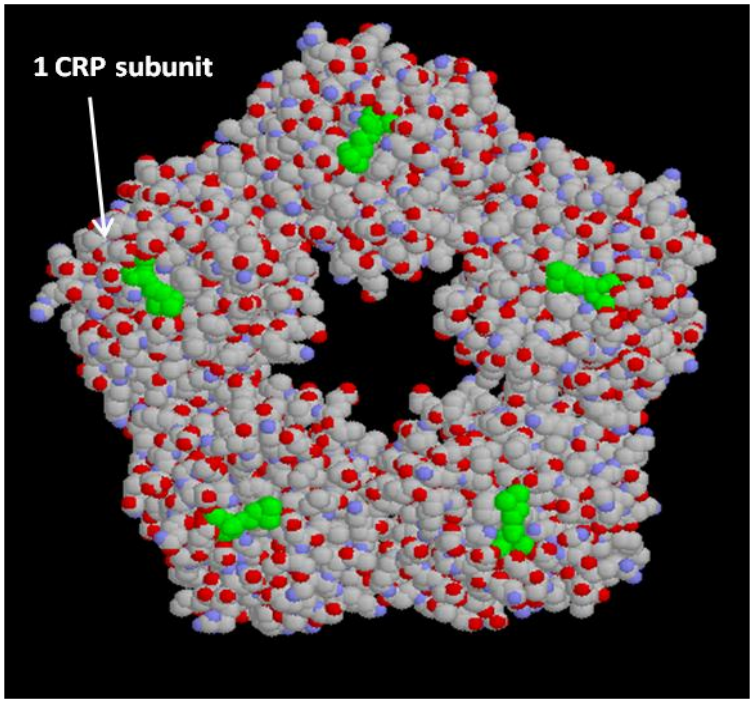

Figure 1-3: Model of C-reactive protein, showing the 5 subunits forming a pentamere
Source; protein data base www.pdb.org entry 1GNH for human CRP

It has since been established that CRP is an acute phase protein, which is a member of the pentraxin family of proteins, is a pattern recognition receptor (PRR) and is synthesized mainly in the liver (Darren *et al.*, 1999; Hirschield and Pepys, 2003). It is a cyclic pentamere (containing five identical subunits) with a core at the centre.

It is synthesized in response to cytokine (predominantly IL-6) stimulation, and has been shown to bind to phosphocholine (in a calcium dependent mechanism), one of the components of lipid bilayer of cell membranes, specifically in dying or dead cells leading to the activation of complement system needed for phagocytic clearing of cellular debris (Agrawal, 2005). It has also been demonstrated to be involved in the opsonisation of bacteria and modulation of platelet aggregation (Cheryk *et al.*, 1996).

C-reactive protein is a very important APP in humans and is frequently evaluated to monitor most inflammatory and infectious conditions as well as response to therapy (Darren *et al.*, 1999). CRP has been identified in several other body fluids including cerebrospinal and arthritic fluid, as well as in human breast milk and colostrum (Fetherston *et al.*, 2006). It is not however clear if human milk CRP is sourced locally as well as or entirely from the circulation. It is a very commonly used marker of inflammation in human medicine particularly also because of its short half-life.

In animals, CRP is a major APP in dogs and pigs. CRP is the most sensitive APP in dogs and frequently used in canine and porcine medicine for diagnosis and monitoring of inflammatory and infectious conditions (McGrotty *et al.*, 2004; Eckersall and Bell, 2010). Only a few studies have reported any relationship between bovine serum or milk CRP and any disease such as mastitis (Schrodl *et al.*, 1995; Hamann *et al.*, 1997; Kruger and Neumann, 1999; Lee *et al.*, 2003; Shcroedl *et al.*, 2003; Ozmen, 2009). However, Maudsley *et al.* (1987) were able to isolate two bovine serum pentraxins (confirmed by electron microscopic appearance) of which one was identified as bovine CRP, using a calcium dependent affinity chromatography. Furthermore, Morimatsu *et al.* (1989) were able to characterize bovine CRP after isolating it from a large quantity of bovine serum as being glycosylated and having a molecular weight of about 100,600 Daltons and having 23 kDa subunits.

Although, CRP is said to be a minor APP in the bovine species, some studies have demonstrated the presence of CRP in serum and milk of cows showing correlation with health conditions and even mastitis (Schrodl *et al.*, 1995; Lee *et al.*, 2003; Schroedl *et al.*, 2003). In the study by Lee *et al.* (2003) a correlation between bovine serum CRP and lactation status as well as body condition score, general animal health and the ability of CRP to identify inflammatory and stressing conditions by its increasing levels in serum was shown.

In earlier studies, Morimatsu *et al.* (1991b) showed an association between the levels of bovine serum CRP with lactation in Holstein cows. Schroedl *et al.* (2003) showed a significant rise in CRP in blood of calves one day after receiving colostrum, suggesting a passive transfer of CRP from colostrum (CRP was earlier demonstrated to be present in colostrum by the same researchers) into the

blood of calves and suggesting its role in the elimination of bacteria and importance in the innate immune protection of the new born.

C-reactive protein is typically measured using immunoassays such as ELISAs, immunodiffusion and agglutination tests and immunoturbidimetric assays (Kim CH *et al.*, 2013; Sarikaputi *et al.*, 1992; Deegan *et al.*, 2003). Immunoturbidimetric analysis such as nephelometry, are based on the reaction of CRP with antibodies and measuring of the turbidity of the resultant antigen-antibody complex. Recently Lin *et al.* (2013) demonstrated the success of using magnetic nanoparticles in combination with capillary electrophoresis (CE) and laser-induced fluorescence detection technique for CRP. The high affinity of CRP to phosphocholine has also been exploited as generic quantitative technique for assay of multispecies CRP by conjugating phosphocholine to bovine serum albumin and linking the conjugate to HRP, and this is then used in a CRP-ELISA or turbidimetric assay format (Deegan *et al.*, 2003). Recently the Life Diagnostics *Inc.* laboratories have produced a new sandwich ELISA for measuring bovine CRP, which is reported by the manufacturer to be sensitive enough to measure bovine milk CRP.

1.1.7.12 Other biomarkers for bovine mastitis

A biomarker is a molecule used to measure or indicate the effects or progression of a disease, condition, or treatment (Metzger *et al.*, 2009). Biomarkers have also been described as a measurable entity that sensitively, specifically and without bias indicates the presence, progression or absence of a disease (Leichtle *et al.*, 2013).The need for biomarkers in the management of bovine mastitis has become a necessity in order to complement the role of somatic cells in the diagnosis of bovine mastitis, particularly of the subclinical forms. In the search for biomarkers, major considerations include sensitivity, specificity, accuracy and avoidance of bias. Other characteristics of a good biomarker include ability to remain unchanged in other conditions not related to the disease and be reproducibly measured and quantified.

It has also been suggested that as well as a biomarker molecule, biomarker profile (s) of compounds may be harnessed for use as sensors for rapid detection of mastitis especially on line in an AMS farm (Boehmer, 2011).

Some examples of biomarkers which have been suggested for bovine mastitis includes the major APP (SAA and Hp) discussed above, and other milk compounds, for example lactoferrin (Lf). Lactoferrin is a soluble protein that plays a role in host defence against pathogens by facilitating the destruction of invading pathogens (Wellnitz and Bruckmaier, 2012). Lactoferrin is present in milk, it acts by sequestering iron and thus makes it (iron) unavailable to bacteria to use, hence its antibacterial action. It is also considered an APP released from PMN during inflammation and its usefulness in indicating metabolic stress in early post-parturient cows has been investigated by Hiss *et al*. (2009). Arnould *et al*. (2009) evaluated the genetic content of lactoferrin in milk and found some correlation between mid-infra-red spectrometry predicted Lactoferrin values and somatic cell scores.

In recognition of its potential as a marker of mastitis and also nutritional value of milk Soyeurt *et al*. (2012) went further to derive an equation to measure Lf using mid-infrared spectrometry in milk, more rapidly than using ELISAs or SRID. However the sensitivity and specificity of Lf for bovine mastitis still requires detailed studies and validation.

Interleukin 6 is another protein the potential of which as a mastitis biomarker has also been investigated (Sakemi *et al*., 2011). Milk cathelicidins have also been proposed as markers of bovine mastitis by Smolenski *et al*. (2011). These are a group of polypeptide markers of inflammation that have shown antimicrobial properties (antimicrobial peptides) and originate from lysosomes of macrophages, PMN and other cells. The authors did not however find a very good correlation between milk Cathelicidins and SCC in natural infections of mastitis as was in the case of experimental infections (Smolenski *et al*., 2011).

Several proteomic studies have been able to identify several low abundance proteins related to bovine mastitis (Danielsen *et al*., 2010, Boehmer *et al*., 2008, Boehmer *et al*., 2010, Smolenski *et al*., 2007 and Hogarth *et al*., 2004). For example, the study by Boehmer *et al*. (2010) identified some specific proteins, namely inter-alpha-trypsin inhibitor heavy chain$_4$ (ITIH$_4$) (heavy chain-4), apolipoproteins, kininogen-2 and clusterin, previously unreported in milk but which showed a typical biomarker trend with mastitis.

The need for early diagnosis of mastitis for prompt and efficient treatment to be effected cannot be overemphasized. Moreover, it has also been reported that treatment of cows or udders prior to development of classical signs of CM (given that occurrence of CM could be predicted by appropriate diagnostic indices in a rapid way), would drastically reduce the severity and duration of cases (Hillerton and Berry, 2005). There is therefore a need for more sensitive and robust markers of mastitis that may even enable pathogen specific identification of IMIs (Danielsen *et al.*, 2010). Advances in bioinformatics and mass spectrometry have enabled the development of new tools that can be used for biomarker discovery, together with the new 'omics' technologies such as peptidomics and metabolomics, as well as the earlier proteomics.

1.1.7.13 Proteomics

Proteomics is the comprehensive study of a specific proteome, including information on protein abundances, their variations and modifications, along with their interacting partners and networks, in order to understand cellular processes (NCI, 2014). The 'proteome' refers to the entire complement of proteins that can be found within a biological system.

Most disease states are associated with changes in the protein constituents and/or concentrations within a biological system. Thus comparing the protein profile in health and disease are potential sources of biomarkers and therapeutic targets (Scrivener *et al.*, 2003).

Protein analysis in samples has been carried out for decades using immuno and biochemical assays, but these assays are usually limited to detection of a particular protein or quantitative analysis such as total protein (Ceciliani *et al.*, 2014) and often require the use of antibodies. With the evolution of mass spectrometric techniques and advances in bioinformatics such as the sequencing of the whole genome of some species in the late 1990s, the next major omics technology emerged, which was proteomics (Vaudel *et al.*, 2014). This technology has enabled the characterization of protein profiles in various biological systems, under different conditions, helping to understand the role of complex proteins in cellular and molecular mechanisms; it has also facilitated the discovery of biomarkers for disease diagnosis.

Current approaches to proteomic studies include gel based and non-gel based methods and has additional advantages over the immuno and biochemical assay techniques in identifying a wider panel of proteins within a single experiment without the need for specific antibodies (Boehmer *et al.*, 2010). They have also enabled the elucidation of post translational modifications (PTMs) as well as quantification of proteins. Two fundamental principles underlie modern proteomics studies; these are the separation of individual proteins from a complex mixture of proteins (fractionation) and the identification or characterization of these proteins (Ceciliani *et al.*, 2014).

Gel based proteomics

The gel based methods are based on the use of an electric field (electrophoresis) for the separation of protein samples loaded onto gel matrix commonly sodium dodecyl sulphate (SDS) polyacrylamide gels followed by the application of an electrical field i.e. separation by electrophoresis. In such gels, molecules can be separated on the basis of either their molecular weight or isoelectric points (1 dimensional electrophoresis (1DE), isoelectric focusing (IEF) or based on both properties (2 dimensional electrophoresis (2DE) (Roncada *et al.*, 2013).

The 2DE was first described in the 1970s (O'Farrell, 1975), here samples are first separated based on their pI in a pH gradient on an IEF gel strip. This is then loaded onto a SDS-PAGE gel and separated according to mass, resulting in the generation of protein spot patterns which are visualized following staining with gels dyes (Hogarth *et al.*, 2004). One dimensional gel electrophoresis has poor resolution power although is robust, while 2DE has been able to resolve up to a few thousands of protein spots which can be excised, digested and characterized using mass spectrometry (MS). It has major advantages of being able to characterize some post translational modifications in proteins and represents the most powerful tool for separating complex mixtures of intact proteins (Roncada *et al.*, 2012).

Difference gel electrophoresis was first described by Unlu *et al.* (1997) and was developed in order to overcome the problem of lack of reproducibility in 2DE. It utilizes different fluorescent dyes to label proteins in different samples and an internal standard (pool of samples) also labelled with another dye; these samples are then electrophoresed in combination within a single 2D gel.

Dyes which were originally designed and used in DIGE comprise of cyanine dyes; propyl-Cy3-NHS and methyl-Cy5-NHS (Cy3 and Cy5) and an additional amine-reactive dye, Cy2 that was targeted to bind to lysine residues. Later dyes which targeted cysteine residues were produced (Minden, 2007). Lysine targeting dyes are used for minimal labelling strategies in cases of protein concentration >100 µg/sample while saturation labelling strategies utilize the cysteine targeting dyes, for low abundance protein samples.

After labelling samples with dyes, they are pooled and run according to a standard 2DE protocol. The gel is viewed using a florescent imager with different excitation wavelengths for the separate dyes, thus up to 3 images are produced which can be assessed for differences and proteins quantified relative to an internal standard (Minden, 2007).

Non-gel based proteomics

Non-gel based proteomics involves the use of non-gel methods such as liquid chromatography (LC) and capillary electrophoresis (CE) for separation of proteins in a complex mixture prior to mass spectrometry analysis. Liquid chromatography entails the migration of molecules in a liquid phase, the migration can be based on ion migration speed (size exclusion chromatography), electrical properties (ion exchange chromatography), hydrophobicity (reversed phase chromatography) and binding with ligands (affinity chromatography). Capillary (zone) electrophoresis entails the movement of molecules through a capillary tube filled with electrolytes upon the addition of an electric field.

Two different approaches may then be used for detection of proteins, firstly detection of intact proteins after ionisation (with the retention of all PTMs in the proteins) and secondly detection of peptides after tryptic digestion of the proteins also called bottom up proteomics. Proteolytic digestion of intact proteins to peptides can be coupled to on-line ionisation phase by a mass spectrometer (MS) for detection of peptide fragments. Over the years, many different approaches to the ionisation of separated molecules prior to detection on an MS machine have been developed. Some of these methods include electron ionisation (a gas phase ionisation), chemical ionisation (CI), electrospray ionisation (ESI), matrix assisted laser desorption ionisation (MALDI), nanospray ionisation (NSI) and atmospheric pressure chemical ionisation (APCI). Each of

these methods has their specific benefits and limitations and choice of ionisation methods to be used is usually based on type of molecule to be analysed. MALDI is commonly used in proteomics studies. Electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) are some of the most frequently used methods for ionising peptides in proteomic experiments and ESI is increasingly used for ionisation of peptides and intact proteins (Catherman *et al.*, 2014). Ionised peptides are then passed through a mass analyser where the various ions are separated based on mass and charge and subsequently detected

A secondary fragmentation of peptides can be carried out; tandem mass spectrometry (MS/MS) and this can be achieved through collision induced dissociation (CID) or electron transfer dissociation (ETD) and this permits protein sequencing. A multidimensional Protein Identification Technology (MudPIT) utilizes the separation of peptide content of complex protein samples in a liquid phase in two dimensions (2D-LC); by reversed phase and ion exchange chromatography in a micro-capillary column, followed by a MS/MS with secondary peptide fragmentation by CID and searching and matching with sequence databases (Delahunty and Yates III, 2007; Schirmer *et al.*, 2003). This method is sensitive, robust and valuable for both qualitative and quantitative proteomics.

Non-gel based methods are becoming increasing popular as they can be adopted into (by coupling the protein separation technique to mass spectrometric identifications) an on-line proteomics experiment (Roncada *et al.*, 2012), utilizing the high throughput separation procedures of LC and CE.

Database searching

Ultimately, detected ions are represented in a spectrum and converted from raw data to proteomic file formats (for example mzXML, mzML, mzData, dta, mgf and so on) that can be searched in databases (Swiss Institute of Bioinformatics (SIB); http://www.isb-sib.ch/aboutsib/mission.html, 2009).

Protein database search engines such as Mascot, SEQUEST, ProteinPIlot are applied for searching and matching of MS generated-peptide spectrums with that of databases for example UniProt, Swiss-Prot, Proteomics identifications database (PRIDE), Protein Data Bank (PDB). Protein scores are then computed for

every protein match and false discovery rate (FDR) is also assessed. It is also possible to search the spectra obtained from experiment through a library of mass spectra which has been made available (SIB, 2009).

A typical work-flow for proteomics analysis involves initial sample preparation and separation in gel electrophoresis, excision and digestion of proteins of interest by a known protease (usually trypsin) into peptides and the subsequent identification of peptides on a mass spectrometer. After ionisation of molecules in the mass spectrometer, mass to charge (m/z) ratio of the molecule is then measured and used to plot a mass spectra that is then searched in a relevant database to match obtained masses. Of late, however there has been a focus on non-gel based methods of separating the protein constituents of samples prior to mass detection on the spectrometer, with liquid chromatography tandem mass spectrometry (LC-MS/MS) being the most widely utilized means of proteomic analysis.

Quantification of proteins

In order to quantify detected proteins in a proteomics experiment, several approaches have been developed and utilized including labelling techniques and label–free approach. In the labelling approach, several approaches are possible such as, DiGE, a gel based method earlier mentioned, and that can be employed for quantification of intact proteins. Chemical labelling of peptides with isobaric tags (iTRAQ) or dimethyl groups, and metabolic labelling with stable amino acid isotopes (stable incorporation of labelled amino acids in culture- SILAC) are non-gel labelling approaches for protein quantification on MS (Lengqvist and Sandberg, 2013). Spectral counting is an example of a label-free approach to protein quantification in proteomics (Nissen *et al.*, 2013; Wong and Cagney, 2010).

Milk is composed of two major groups of proteins, the insoluble caseins (high abundance proteins) and soluble whey proteins. There are several types of caseins including α-caseins (α-CN), β-CN and κ-CN; all these constitute about 80 % of the total milk proteins. The remaining 20 %, whey proteins (low abundance proteins) are made up of β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin, lactoperoxidase as well as cytokines and other immune proteins (Pepe *et al.*, 2013). The low abundance milk proteins

present a repertoire of proteins from which likely biomarkers of disease conditions of the mammary gland can be found.

In order to overcome the effect of high versus low abundance protein, fractionation steps are often carried out on milk samples and can vary from centrifugation, acidification, and filtration to the use of peptide ligand libraries as well various precipitation methods to rid the samples of the high abundance proteins (Nissen *et al.*, 2013; D'Amato *et al.*, 2009). These recent advances in fractionation techniques have helped to resolve the limitation posed by presence of high abundance proteins (Boehmer *et al.*, 2010). Different fractions of milk proteins, for example whey, have been studied using proteomics. Another major fraction of milk that has been studied using proteomics is the milk fat globule membrane (MFGM) (Reinhardt and Lippolis, 2008) where new proteins not previously known to be in milk were identified.

Mass exclusion filters, 1DE and commercial depletion kits are also available for this purpose of fractionating milk proteins prior to proteomic analysis (Boehmer, 2011). In the study by Nissen *et al*. (2013), centrifugation at a very high speed compared other milk proteins fractionation techniques such as acidification or filtration, before carrying out a proteomics experiment, was found to be the most reproducible and robust method of obtaining the milk proteome.

Recently, the use of combinatorial peptide ligand libraries has been developed and was successfully employed for fractionation of peptides and identification of new proteins (D'Amato *et al.*, 2009). Enrichment for example by cysteine-tagging has also been used to enhance the identification of low abundance caseins in milk containing cysteine as against the abundant α- s_1 CN and ß-CN that do not (Holland *et al.*, 2006). Proteomics methodologies in recent years have been adopted for the discovery of biomarkers of bovine mastitis. Some measure of success has been achieved with up to about 80 different proteins identified that vary in milk in response of the host to pathogen infection of the mammary gland (Viguier *et al.*, 2009), although most of these have not been validated.

A recent application of proteomics has been established for non-culture identification of mastitis- causing bacteria in milk using a matrix assisted laser desorption ionisation – mass spectrometry (MALDI-MS) (Barreiro *et al.*, 2012). This method employs bacterial ribosomal proteins as fingerprinting markers to

identify specific micro-organisms, from a dedicated MALDI biotype reference library after a pre-concentration step. However, a high bacteria count is required for accuracy and only a few species of bacteria have been evaluated in milk using this method.

By and large, proteomics is a promising technique that can be used to identify, validate and screen biomarker candidates for bovine mastitis (Boehmer *et al.*, 2010; Lippolis and Reinhardt, 2010; Bendixen *et al.*, 2011; Eckersall *et al.*, 2012; Bassols *et al.*, 2014), but further work needs to be carried out using different models of mastitis, including both field and experimental models as well as mastitis caused by different pathogens, and representing diverse clinical phases.

1.1.7.14 Peptidomics

The peptidome, in analogy with the proteome, is the collection of all peptides within a biological system at a given time. Peptidomics is one of the newly emerged 'omics technologies and is a subset of proteomics. It is the detection, identification and quantification of all peptides with their post translational modifications within a cell, tissue organism or biological sample. Developments in mass spectrometry as well as high resolution separation techniques for biological sample constituents heralded this new technology. Due to the fact that peptides represent a very important class of biological compounds that have been demonstrated to play crucial roles in many processes in the body especially as neurotransmitters in the nervous system, peptidomics technology has proven useful in the areas of neuroendocrine research, biomarker and drug discovery (Menschaert *et al.*, 2010; Schulz-Knappe *et al.*, 2001).

Peptidomics primarily focuses on the simultaneous identification of endogenously derived peptides within a biological fluid/system, however often encompassing peptide products of protein degradation. It can be used to elucidate proteolytic regulation of bioactive peptides as a key to understanding the physiology and identifying possible drug targets of these peptides (Kim YJ *et al.*, 2013).

Peptidomic analysis depends largely on mass spectrometry techniques such as MALDI-time of flight (TOF) or electrospray ionisation (ESI) combined with tandem mass spectrometry (MS/MS). Separation technologies for peptides within

complex mixtures comprise chromatographic techniques such as liquid chromatography, reversed-phase high pressure liquid chromatography (HPLC), capillary electrophoresis and ion exchange chromatography (Schrader and Schulz-Knappe, 2001). Capillary electrophoresis- mass spectrometry (CE -MS) has been used for high throughput analysis of urine samples with successful turnover of biomarker candidates with high reproducibility (Soloviev and Finch, 2006; Balog *et al*., 2009). Surface-enhanced laser desorption ionization time- of-flight mass spectrometry (SELDI-TOF-MS) another commonly employed method in peptidomics has been specifically used for urine. Another method has also been used for peptidomics analysis is liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS), which was applied by Guarino *et al*. (2010) to detect adulteration of goat milk with sheep milk for making cheese, involving identification of a particular casein plasmin-hydrolysis peptide of sheep cheese, used as a marker.

Milk Peptidomics

A number of discoveries about peptides in milk have been made possible as a result of milk peptidomics. Antimicrobial peptides amongst other peptides exhibiting diverse properties such as immunomodulation have been identified following endogenous proteolysis of the major milk proteins (caseins and lactalbumin) in human milk (Dallas *et al*., 2013). In a similar study Wan *et al*. (2013) also elucidated the peptidome of human term and preterm milk, but found many peptides to be quantitatively diverse between the two classes of milk samples.

Several antimutagenic properties have also been associated with peptides obtained after hydrolysis of milk protein constituents such as caseins and lactalbumin (Larsen *et al*., 2010b).

Peptides in milk increase during episodes of mastitis, not least as a result of the action of proteases such as plasmin, elastase, cathepsins A and B. Aminopeptidases, in addition to these proteolytic enzymes may leak into milk from blood through a disrupted blood milk barrier, or be secreted into milk by somatic cells or mammary epithelial cells as a tool for killing bacteria, or arise from microorganisms metabolism. Proteases originating from leucocytes that

increase in the mammary gland during episodes of inflammation also abound and may be considered as endogenous non-native proteases that could account for most of the proteolytic activity in high somatic cell count milk (Napoli *et al.*, 2007). The proteolytic activities of enzymes in milk ultimately result in loss of milk caseins which compromises the quality and technological properties of milk such as in cheese formation (Larsen *et al.*, 2010b). Mass spectrometry based peptidomics has enabled the identification of a great number of peptides in milk samples from which biomarkers might be discovered.

Larsen *et al.* (2010b) utilized capillary reverse-phased–HPLC to study the peptide profile of milk following a LTA challenge of quarters; spectral peaks were subsequently identified using tandem MS, following ionisation by MALDI-TOF. In a recent study, Mansor *et al.* (2013), identified up to 31 polypeptides which could differentiate healthy from mastitic milk samples and up to 14 polypeptides that were able to distinguish between different pathogens responsible for infections.

1.1.7.15 Metabolomics

General Metabolomics

Metabolites are small molecules which are intermediates and products of metabolism, and exist in a dynamic state within the body. The word 'metabolome' was first used by Oliver *et al.* (1998) in relation to the metabolic analysis control aspect of the yeast genome sequencing, in reference to measurement of the change in the relative concentrations of metabolites as a result of the deletion or over-expression of a gene. Since then, the word has evolved to be used to describe the complete collection of all low molecular weight (MW) metabolites in a cell, which are involved in growth, maintenance and normal functioning of the cell (Dunn and Ellis, 2005).

Metabolomics is the study of the composition, relative abundances, interactions and dynamics of the metabolome in response to change of environment of metabolites within a biological system (Osorio *et al.*, 2012). It entails the use of sophisticated analytical techniques in non-biased identification and quantification of all metabolites in a biological system (Dettmer *et al.*, 2007).

Since the metabolites that can be measured, are directly responsible for health and changeable by physiologic or pathologic interventions; their qualitative and quantitative measurement within a biological system is a potentially useful tool for understanding the phenotype (Watkins and German, 2002).

The range of metabolites that can be analysed in a metabolomics experiment is remarkably diverse and cuts across all possible biochemical pathways within a living system, therefore analysis of molecules extending from very low MW-polar to much larger MW non-polar compounds is the norm in a typical metabolomics experiment (Dettmer *et al.*, 2007). Metabolomics procedures involve controlled sample collection and preparation, instrumental analysis, data processing and data interpretation (Dunn and Ellis, 2005).

A variety of samples can be used for metabolomics studies ranging from blood (serum or plasma), urine, cerebrospinal fluid, saliva, milk, organ effusions and even tissue (Dettmer *et al.*, 2010). Most metabolomics studies are carried out for either of or both of two major reasons; first to understand the biological process at any given time, and second to detect and identify biomarkers (Xia *et al.*, 2013). Three major approaches to metabolomics studies are metabolic profiling, metabolic fingerprinting and metabolic foot printing.

Metabolic profiling is usually hypothesis driven and involves the analysis of a given set (targeted) of metabolites based on class or pathway while metabolic fingerprinting does not target a particular set of metabolites but aims to identify all metabolites present within the given biological system as they relate to changes in genes or phenotype (Courant *et al.*, 2013). In metabolic foot printing naturally secreted or excreted biofluids such as urine, saliva and milk are studied to determine what is going on within the biological system under different conditions (Kell *et al.*, 2005). Also referred to as the ‘exometabolome’, metabolic footprints offer a convenient strategy to understand and characterize the cellular metabolic pathways and biochemistry. Metabolomics has the particular advantage over other omics technologies of being the most reflective of current status of the biological system and comparatively less expensive to perform.

Metabolomics can be carried out by the use of mass spectrometry (MS), Fourier transform infrared spectroscopy (FTIS) or nuclear magnetic resonance (NMR)

spectrometry. Mass spectrometry is usually combined with separation techniques such as liquid or gas chromatography (LC-MS; GC-MS) or less commonly capillary electrophoresis (CE-MS). Added resolution in the separation technique may also be achieved by the use of either high pressure liquid chromatography (HPLC) or ultrahigh pressure liquid chromatography (UPLC).

Mass spectrometry provides a more sensitive (than NMR) qualitative and quantitative identification of metabolites being able to assess to the picogram (pg) level (Atzori, *et al.*, 2009) and is the most commonly used technique in metabolomics. The basic principle in mass spectrometric analysis involves the ionisation of molecules, separation of these molecules based on mass to charge ratio within an electrical field and detection of the each separated ions, which is then used to generate a mass spectra.

Direct injection MS (DIMS), is an MS technique where no prior separation of samples is done before loading onto the mass spectrometer. This form of analysis is commonly used for screening as against quantitative analysis (Dunn and Ellis, 2005).

Several MS instruments are obtainable such as quadrupole, triple quads, ion traps, and time-of-flight mass analysers. An orbitrap analyser is another form of MS that is able to detect ion mass with greater accuracy (Gowda *et al.*, 2008).

Mass spectrometry has several drawbacks despite of its high sensitivity which includes the need for thorough sample preparation (especially in GC-MS) and is also time consuming to perform. In addition, with metabolomics experiments carried out using LC-MS, batch to batch variations of analytes are commonly encountered. This may occur as a result of variation in the extent of sample preparation or as a result of the instrument selectivity. New improvements in MS methods are also being introduced which tackle the problems of sample preparation and analysis time.

In a typical metabolomics experiment, hundreds and sometimes thousands of spectral signals can be generated representing hundreds and thousands of metabolites. This data needs to be properly processed in order for the results to be biologically interpretable. Briefly, data analysis entails file conversion of raw data, peak or feature detection, data alignment and then normalization (or data

binning and scaling), multivariate analysis, metabolite identification using standard (public) database searches and the use of calibrated data matrix for further data (statistical) analysis (Sugimoto *et al.*, 2012).

Statistical analyses using multivariate techniques are often employed in metabolomics as large data sets are generated from these experiments, in order to determine changes in metabolome between different sample-sources following generation of their metabolic profiles (Gowda *et al.*,2008; Courant *et al.*, 2013). These multivariate techniques include supervised approaches where prior knowledge of the sample category (e.g. control or diseased) is unknown; an example of this method includes principal component analysis (PCA) and hierarchical cluster analysis (HCA); and supervised approach, where the categories of samples are known, for example partial least squares discriminant analysis (PLS-DA), orthogonal signal correction (OSC) and random forest (RF) (Gowda *et al.*, 2008; Sundekilde *et al.*, 2013).

Metabolomics is useful for the discovery of biomarkers of diseases such as cancers and metabolic disorders like diabetes, determination of biochemical effects of various interventions such as drugs or environmental stressors, cellular characterisation and health assessment (Suhre, 2014; Nair *et al.*, 2014; Bonvallot *et al.*, 2013). It is being increasingly utilized in pharmacological studies, understanding neurological diseases (Zhang *et al.*, 2013), food and nutrition science, neonatal screening, clinical chemistry, and plant biotechnology (Okazaki and Saito, 2012) as well as in microbiology (Sundekilde *et al.*, 2013; Sugimoto *et al*, 2012).

The metabolome has been described to be the most predictive of the 'omics' technologies for the phenotype; hence a major advantage of metabolomics over proteomics, transcriptomics or genomics is that it reflects the state of the cell more accurately. Changes in function of the cells are also more amplified in metabolite profile, since these molecules are the products of on-going chemical reactions. Metabolomics is also said to be far less expensive than other 'omics technologies (Dunn and Ellis, 2005; Kell *et al.*, 2005). The first web human metabolome database was developed by Smith *et al.* (2005). Since then, the benefits of metabolomics in clinical diagnosis particularly in oncology have been emphasized (O'Connell, 2012).

Metabolomic investigations however have a few hurdles to overcome including lack of standardized procedures and bioinformatics modules especially for handling of the large quantities of data generated, as well as problems with identification of metabolites due to incomplete databases of many species. A number of limitations to biomarker discovery using metabolomics have encompassed issues of sensitivity, specificity and avoidance of bias as well as challenges with data processing (Leichtle *et al.*, 2013). Nevertheless, constant improvements in multivariate analysis, instrumentation as well as standardization of methodology and sample preparation techniques are showing promise for overcoming these limitations.

Metabolomics studies in Bovine species

Metabolomics has been valuable in several areas of study in the bovine species, in particular diagnostics of animal health and food safety as well as management practices geared to improvement of animal production. Numerous metabolomic studies have already been carried out in cattle and the bovine metabolome database (BMDB) is available on http://www.cowmetdb.ca/. This database comprises information on metabolites of dairy and beef cattle obtained by experiment on blood, meat, urine, milk and ruminal fluid (Hailemariam *et al.*, 2014; Wishart Research Group 1996 –2013).

Targeted evaluations of the metabolic profile (of known metabolites) in bovine samples such as urine, serum, plasma and milk have been frequently carried out. However untargeted approaches that aid in detecting new metabolites are gaining importance especially with new innovations in bioinformatics and mass spectrometric techniques.

Some metabolomic studies which have been conducted in the bovine species include studies by Rijk *et al*. (2009) who utilized an untargeted UPLC-TOF MS to identify biomarker-candidates for the use of anabolic steroid prohormones; dehydroepiandrosterone (DHEA) and pregnenolone in cattle urine. Similar studies were also carried out by Regal *et al*. (2013) for assessing two other anabolic steroids, estradiol-17β and progesterone, this time using serum samples. They utilized HPLC coupled to an Orbitrap spectrometer and found significant differences that discriminated use and non-use of these hormones. With the same focus, a targeted approach for the detection of markers of natural steroids

and 4-androstenedione abuse in urine of cattle was explored by Anizan *et al*. (2011). Phase II metabolites of steroid detoxification (glucuronides and sulphates) in urine were the focus of both experiments and a UPLC-MS/MS technique was applied. All these studies resulted in the detection of several compounds which were not previously recognized and which once properly validated could serve as markers for screening of animals for steroid abuse.

In trying to ascertain which blood sample type would be most suitable for assessing the blood metabolome, Dettmer *et al*. (2010) compared the metabolomic profiles of blood collected from bovine into; EDTA-plasma only and EDTA-plasma with acetyl salicylic acid, and blood collected and allowed to clot without addition of anticoagulants (serum) using a GC-TOF-MS. Differences were observed in the fingerprints from these three different types of blood sampling. Trabi *et al*. (2013) also used NMR based metabolomics to investigate the effect of long term storage on the metabolite profile of bovine plasma samples.

Bender *et al*. (2010) observed significant differences in metabolites in follicular fluid of heifers compared to those of lactating cows using GC-MS, and also between dominant and subordinate follicles; these discrepancies were suggested to be able to give an insight into increasing incidences of low fertility and variances in fertility level between these two groups of cows.

Additional metabolomics studies by Osorio *et al*. (2012) aimed at elucidating biomarkers that could be applied to discriminate between beef produced under different pasture and concentrate-based production systems; creatinine, glucose, hippurate, pyruvate, phenylalanine and phenylacetylglycine, were observed to have promise in differentiating beef cattle based on these parameters.

Metabolomics studies have also shown that differences in the concentration of up to 19 metabolites are potentially able to distinguish subclinical ketosis from normal serum samples, whilst up to 31 differentiated clinical ketosis from normal. Eight metabolites were also found to vary between subclinical ketotic and normal serum samples. These metabolites are thus potential biomarkers of ketosis in dairy cows (Zhang *et al*., 2013).

More research using metabolomics is still on-going in a variety of aspects dealing with cattle farming management and health. Thus the metabolomics approach is finding diverse and growing applications in cattle. The bovine ruminal fluid metabolome was elucidated by Saleem *et al.* (2013), using a combination of NMR spectroscopy and GC-MS, along with literature searches. A database containing the metabolites to use in this study has been made available at http://www.rumendb.ca.

1.2 Proposed research

Despite the enormous problem and economic impact of mastitis on dairy farming globally, there is as yet no definitive ‘parameter' the measurement of which in milk can be reliably used as a 'gold standard' for the confirmation of subclinical mastitis in a rapid on-farm tests (Uhler, 2009). The commonly used indicator, SCC, cannot be directly measured on the farm especially with the newer AMS in dairy farms. In addition, SCC has shown marked variation with other factors other than diseases of the mammary gland. This situation often results in false negatives that can subsequently lead to uncontrolled spread of contagious mastitis pathogens in a dairy herd.

Furthermore, a major limitation to the use of SCC is its inability to be measured directly rapidly on line, while indirect measurements of SCC are less sensitive.

Recently there has also been the focus on the need for pathogen-specific diagnosis of mastitis online to facilitate quick treatment intervention measures (Taponen *et al.*, 2006; Hettinga *et al.*, 2008). This would require markers specific for particular causative organisms, which as yet have not been reported for any of the major causative agents of bovine mastitis.

Also, other parameters that have been brought to the fore over the years for use in mastitis detection, for example the APP, but have not received justifiable investigation of their potentials in on-farm use whereas others have had several shortcomings or poor exploration and validation of their prospects.

Recent developments in technologies such as proteomics has allowed their use for better understanding of the mechanisms of disease well as in the discovery of marker compounds of bovine mastitis (Ferreira *et al.*, 2013). In the last decade,

more omics applications have played and continue to play a crucial role in biomarker discovery from biological samples.

However, there have been few studies focusing on the use of the newer omics (peptidomics and metabolomics) analyses for bovine mastitis biomarker identification, although more attention has been drawn to its use in characterizing the technological properties of milk such as SCC, which are related to mastitis. The few studies which have investigated the properties of milk in relation to disease using the omics technologies have shown the possibility of discovering new mastitis markers (Boehmer, 2011; Ferreira *et al.*, 2013).

1.3 Aims and objectives

It is the aim of this study to further elucidate and assess the potentials and applicability of the major bovine milk APP (Hp and M-SAA3) in specifying inflammatory conditions of the mammary gland. Reference levels, including baseline values in milk samples from uninfected quarters/cows, variations and dynamics of the APP under different physiological conditions affecting the dairy cow, in particular, the puerperal period, and other factors other than IMI that can alter APP levels will be explored. Characterization studies of Hp to determine its source in milk, from the mammary gland will be carried out. Analysis of these APP will also be carried out to further validate their levels of significant correlation with onset, progression, peak and resolution stage of an IMI using an experimental mastitis model. Furthermore how the major APP vary in milk with different mastitis causative pathogens will be examined.

Following previous reports on the mastitis biomarker potential of bovine milk CRP (discussed above) and with the new experimental evidence and validation of this erstwhile minor bovine APP as a possible marker of mastitis, studies in this work will attempt to determine the value of CRP as a mastitis indicator under various conditions as detailed above for Hp and M-SAA3.

In addition, because sample treatment or preparation is an important factor that can alter target indices within samples, analyses of the effects of some frequently used milk-sample storage-related treatment protocols; heat

treatment, preservation at different cold temperatures for various durations of time and use of chemical preservatives in milk samples, will be assessed.

Using current approaches to proteomics, peptidomics and metabolomics, another major objective of this study, will be to identify compounds, metabolites, traits or biological features and patterns of compounds in milk that are discrepant between health and disease states of the mammary gland. An aim to identify reliable markers which eventually can be applied or adapted to rapid on-line diagnosis of IMI will be targeted. Particular attention will be paid to detecting biomarkers which can sufficiently discriminate pathogen-specific causes of mastitis episodes. Adopting an experimental host-adapted *S. uberis* mastitis as a model, and covering a period of 0 to 312 h, which spans the pre-infection to resolution stage of the acute mastitis model, it is aimed that profile of the proteome, peptidome and metabolome of milk as it varies with the progress and remission of infection will highlight compounds that will be of value in discriminating mastitis generally and *S. uberis* mastitis specifically. This study will attempt to fill the gap in knowledge of mastitis resolution metabolic markers specific for *S. uberis*.

1.4 Justification

Justification for this study comes from the growing impact of bovine mastitis as an economic and welfare issue in the dairy industry, the recognition of major the limitations to its control and management being the lack of its rapid definitive and causal-specific diagnosis.

With the added complexity that AMS introduces to existing diagnostic measures such as CMT that would otherwise have been uncomplicated with conventional milking methods, the need for newer techniques or parameters whose measurements can be easily adjusted to work with the milking system, is warranted.

This PhD research is therefore channelled to addressing and attempting to answer some of these pertinent problems and questions, confronting the management (diagnosis) of bovine mastitis using the tools of acute phase proteins analyses, proteomics and metabolomics of milk samples.

2 Acute phase proteins in a commercial dairy farm

2.1 Overview

In this chapter, the development of an assay for measuring milk haptoglobin (Hp), its optimization and validation as well as its use in assaying the profile of Hp in milk from a dairy herd in the west of Scotland is described. Mammary associated serum amyloid A3 (M-SAA3) as well as C-reactive protein (CRP) were also measured using commercially available ELISA kits adapted for use in milk M-SAA3 and CRP assays respectively and are also reported. In addition, a range of sample treatment protocols were examined to determine their effect on milk Hp and M-SAA3. An in-house, relatively inexpensive assay was developed and validated for the measurement of milk Hp. All samples were analysed for milk Hp, whereas only herd composite samples and some of the sample treatment protocols were analysed for M-SAA3. For the same reason, only composite milk samples from the dairy herd were assayed for CRP.

2.2 Introduction

2.2.1 Milk haptoglobin

Haptoglobin can be assayed directly by antibody detection using immunoassays such as enzyme linked immunosorbent assay (ELISAs) and single radial immunodiffusion (SRID) as described by Morimatsu *et al.*(1992), as well as indirectly by measuring the peroxidase activity of the haemoglobin (Hb)-haptoglobin complex, exploiting the high affinity binding of Hp to free Hb (Eckersall *et al.*, 1999). Direct immunoassays using antibodies have an advantage of higher sensitivity than assays using Hb binding assays which are also subject to interference.

Haptoglobin has also been measured in bovine serum using high performance liquid chromatography (HPLC) as described by Salonen *et al.* (1996). In this method, Hp was first separated from serum by binding to cyanomethaemoglobin (HbCN) step after being depolymerised; gel filtration using the HPLC was then used to separate the HbCN. This method of assay was sufficiently sensitive to measure baseline levels of Hp.

A capillary zone electrophoresis method for measuring serum Hp was also described by PIrlot *et al*. (1999). This was also based on the capacity of Hp to bind to Hb, though the assay did not work well for bovine serum Hp.

Since the discovery of Hp in milk and the reports of its increases in relation to mastitis, the measurement of milk Hp as a potential tool for diagnosing mastitis has been explored by several researchers (Eckersall and Bell, 2010). The need for a corresponding assay specifically adapted to detect milk Hp has also been highlighted. It was recognized by Eckersall *et al*. (2001) that the presence of milk (lacto) peroxidase could interfere with the measurement of Hp using the peroxidase activity of an Hp-Hb complex, thereby ruling out the use of that biochemical assay for measuring milk Hp.

A number of commercial Hp assay kits exist for the measurement of Hp and a commercial SRID assay has also been used (Takahashi *et al*., 2007); however these kits are not be specifically optimized for measurement of milk Hp. Some workers have also developed assays specifically for milk Hp measurement (Hiss *et al*., 2004; Yang *et al*., 2011) whilst other workers have also attempted to adapt Milk Hp assays to rapid on-farm detection of mastitis methods, with varying levels of sensitivities (Åkerstedt *et al*., 2006; Tan, *et al*., 2012).

An immunoassay is a method of detection of a compound or macromolecule (usually proteins or polysaccharides which are immunogenic) by the use of immunoglobulins or antibodies. The underlying principle of all types of immunoassays is the inherent ability of antibodies to bind to specific antigen.

Engvall and Perlmann (1971) were the first to originally describe the enzyme linked immunosorbent assay (ELISA), one form of immunoassay that utilises the labelling of antibodies with enzymes and adsorption of analyte to a solid phase. The use of ELISAs became predominant in the seventies having evolved from radioimmunoassay (RIA). Since then, they have been found to be very useful for assay of large numbers of samples as they are reliable, sensitive and relatively inexpensive.

There are several formats of ELISAs including direct, indirect and sandwich or capture ELISA, but generally all formats involve the coating of a solid support with the antigen or analyte (directly or indirectly), blocking of unoccupied sites

on the solid phase with non-reactant protein or other molecules and washing off unbound compounds. Antigen/analyte specific antibodies are then used to detect or probe the antigen, with a signal based on enzyme-substrate interaction of enzymes tagged to either the antigen-specific-antibody or a secondary antibody (to the primary antibody). This is subsequently measured spectrophotometrically to quantify the product of the enzyme substrate reaction which is used to interpolate the concentration of the analyte in samples.

Competitive ELISAs utilize the principle of competition between reference antigens and the antigens in a test sample with a small amount of antibodies. In this format either the reference antigens or the antibodies may be labelled and the signal is inversely proportional to the amount of antigens in the test sample.

A number of ELISAs have been described for the assay of Hp in biological samples (Sheffield *et al.*, 1994; Young *et al.*, 1995; Saini *et al.*, 1998; Hiss *et al.*, 2004; Flanagan *et al.*, 2014) and there are also several commercial ELISAs for Hp now available (Wenz *et al.*, 2010; Giannetto *et al.*, 2011).

A sandwich or capture ELISA is one in which a primary or capture antibody is first immobilized onto the solid support phase (in a process called coating) in order to enhance the specific adsorption of the antigen of interest. The addition of antigens follows after washing off unbound primary antibodies and blocking of unoccupied sites in the wells.

A secondary or signal antibody which would bind to a different site on the antigen from the capture antibody is then incubated to detect or probe the antigen. Usually the signal antibody is tagged with an enzyme, the substrate for which is subsequently added to generate a signal that can be measured, based on chromogenic, chemiluminescent or fluorometric properties of the enzyme-substrate product.

Different antibodies, but which are specific to the analyte antigen, are often used for the capture (primary) and signal (secondary) in sandwich ELISAs. Usually monoclonal antibodies are preferred for coating and polyclonal antibodies for signal detection. It is also possible to use the same antibody type for both capture and signal (Leng *et al.*, 2008).

This is a highly specific ELISA format as only the specific antigen is 'captured' to the surface of the wells and no other sample proteins due to the presence of the coating/primary antibody. Sandwich ELISAs are useful to detect antigens which are present at a low level in samples or within a complex mixture (KPL ELISA technical guide, Thermo scientific, 2013).

2.2.2 Mammary associated serum amyloid A3

Due to its hydrophobicity and high affinity for the lipoprotein fraction in serum, SAA is difficult to purify from blood and this poses many technical problems in the production of its stable antibodies (Yamada *et al.*, 1999). In addition, the SAA molecule is poorly immunogenic (Wilkins *et al.*, 1994).

SAA is also known to adhere to the surface of plastic (sample) tubes leading to potential loss of SAA during sample concentration (Yamada, 1999), the reduction in alpha helical structure found in the last 1-11 amino acid residues of the amino terminal in A-SAA has been suggested to enhance the binding of SAA to polystyrene surfaces (Liang *et al.*, 1996). It also sometimes shows a high tendency to aggregate or form multimeres once elution buffer was removed and after episodes of freeze thawing or in the presence of calcium (human samples); thus immunoreactive bands at about 66 kDa were noticed in 1D SDS-PAGE, and these were thought to be 5-unit multimeres of SAA molecules (Molenaar *et al.*, 2009). This attribute is another contributing factor to the difficulty commonly encountered in developing assays for SAA. Also, because SAA is often found bound to HDL in serum, denaturing techniques need to be applied to the sample often in order to facilitate reaction of the molecule to its antibody (McDonald *et al.*,1991). Storage at different temperatures and conditions was recently shown to affect the concentration of SAA and its mammary associated isoform in serum and milk samples (Tóthová *et al.*, 2012), with a tendency to decrease with time of storage.

SAA has been purified from serum by isolating the HDL fraction and then performing ultracentrifugation and delipidation steps (Smith and McDonald, 1991), but very low recoveries were encountered. By using hydrophobic interaction chromatography, Smith and McDonald (1991) were able to get up to 56 % recovery of SAA. A number of other researchers have also been able to purify SAA and antibodies against it for use in SAA immunoassays. Horadagoda *et*

al. (1993) were also able to purify bovine SAA and applied it in an indirect ELISA for the quantification of SAA in serum of diseased calves.

Serum amyloid A is measured predominantly using immunoassays particularly ELISAs and less frequently western blotting (Weber *et al*., 2006; Wells *et al*., 2013), radial immunodiffusion assay (Chambers and Whicher, 1983) and radioimmunoassay (Eriksen and Benditt, 1986). A dot-blot immunoassay was also described (Ogata, 1989) and is based on the reaction of samples with a dot of commercially available hyper immune serum to SAA.

McDonald *et al*. (1991) developed a sandwich ELISA using monoclonal antibodies from rat raised against human SAA, and this assay formed a basis for the development of a commercial SAA multispecies sandwich ELISA kit by Tridelta Development Co. (Kildare, Ireland) which is the most frequently used method of assay for SAA in animal studies.

In the present study, several attempts were made to develop an ELISA for measuring M-SAA3 with antibody previously produced in the laboratory (Prof Eckersall, pers. comm.) but were not successful; hence the commercial ELISA kit by Tridelta Development Co. (Kildare, Ireland), was used for measuring M-SAA3.

2.2.3 C - Reactive protein

Generally, CRP has received very little attention as an APP in serum or milk of the bovine species that may be useful for diagnosis or prognosis of any inflammatory condition. The use of CRP as a parameter for mastitis has not been the subject of much investigation, even though there has been a small number of earlier reports of its potentials in this regard (Schrodl *et al*., 1995; Kruger and Neumann, 1999; Lee *et al*., 2003). A probable reason for this could be due to the narrow range of CRP in milk as reported in one study, where CRP was not found useful in differentiating milk from healthy and SM milk samples, therefore lacking adequate sensitivity (Hamann *et al*., 1997). Similarly in human milk, CRP was reported to have a narrow range, thus it was not feasible to utilize it to make a differential diagnosis of infective and non-infective forms of mastitis (observed in the human subjects of that study) (Fetherston *et al*., 2006).

Recently, studies at the Life Diagnostic *Inc*. laboratory (West Chester, USA), using a newly developed ELISA for measuring bovine CRP, that utilized antibodies

raised specifically for cow CRP, showed that a range of 3.1 ± 2.8 ng/ml existed in normal milk (n=17), while a range of 4218 ± 2658 ng/ml existed in milk from mastitic cows (cow C-reactive protein, Life Diagnostics ELISA kit instruction manual, 2014). This new and promising report in the range and sensitivity of CRP in healthy and mastitis milk for diagnosis of mastitis may have resulted from the use of a more sensitive assay, which was able to measure a wider range of CRP, even in normal (healthy) milk samples.

In view of the overall objective of this PhD research to identify new and more reliable biomarkers of bovine mastitis, the value of bovine milk CRP, which has only been reported in a few studies as yet, was investigated as a useful parameter of bovine mastitis.

2.2.4 Study of acute phase protein profiles on farms

The importance of APP for herd diagnosis of various disease conditions have been shown (Humblet *et al.*, 2006). There are numerous reports of the profile of APP in bovine serum and their value in recognizing disease conditions on a herd basis (Murray *et al.*, 2014; Abuelo *et al.*, 2014; Trevisi *et al.*, 2014).

There are also a few reports on the profile analysis of milk APP in samples encompassing entire dairy farms (Petersen *et al.*, 2005 and Åkerstedt *et al.*, 2007). Petersen *et al.* (2005) examined the MAA profile in five herds, and correlated findings with the SCC, presence or absence of clinical mastitis and bacteriological agents. They found no correlation between SCC and MAA.

Åkerstedt *et al.* (2007) looked at samples from healthy cows from three different herds and related the APP profiles to the type of milk sample (quarter, composite or bulk tank milk), and to the SCC level. The dairy herds were of the Swedish dairy herd breeds and the two major APP, Hp and SAA were evaluated, and significant correlations discovered between SAA, Hp and SCC in quarters and composite milk but only between SAA and SCC in the bulk tank. The finding of this correlation between APP and SCC in samples from apparently healthy cows indicates that APP cut off points maybe adapted to determine mastitis status of cows or herds. Therefore it would be useful to evaluate the APP profile in a dairy herd, with the aim of determining cut off values for each in relation high

and low SCC levels. Such knowledge would enhance their potential for their future use on farms.

2.2.5 Stability of acute phase proteins in milk under different sample treatment conditions

Samples for diagnostic procedures often require some form of preparation, preservation or safe handling for transportation prior to analysis in the laboratory. This is especially true for milk samples which frequently need the use of preservatives to retain milk composition as close to freshly collected samples as possible. Chemical preservation is used to avoid cellular degradation prior to somatic cell count or progesterone analysis. Another form of treatment is milk fat and casein removal to expose 'milk serum' preceding some analytical techniques such as proteomics, and where samples are not to be immediately assayed, storage at freezing temperatures of between -20°C and -80°C is common.

In addition, as a requirement for the safe transfer of biological samples across international borders, some national regulations that demand heat treatment of liquid samples in order to inactivate harmful pathogens in the samples have been put in place (Council directive 82/894/EEC of 21 December 1982, under the European Communities Act 1972). However, high temperature is known to denature proteins and to influence the concentration and immunologic activities of some serum proteins (Hausen *et al.*, 2012; Ahmed and Saunders, 2012). Heat treatment has also been shown to result in irreversible changes in the structure of milk proteins (Raikos, 2010).

In the handling of milk samples for chemical analyses, the use of preservatives has become a requirement for some tests due to distances of laboratories from the farms where samples are obtained. Potassium dichromate ($K_2Cr_2O_7$) is a corrosive and toxic biocide which is often used in the preservation of milk samples in order to maintain milk composition from time of collection to analyses. It is known to be effective as a milk preservative (Barbano *et al.*, 2010), and is used to maintain progesterone levels in milk for pregnancy diagnosis. Other preservatives that have been used in milk samples include Mercury chloride ($HgCl_2$), Sodium azide and Bronopol ($C_3H_6BrNO_4$; 2-bromo-2-nitro-1, 3 propanediol) (Luck, 1975; Kvapilik and Suchanek, 1974). Unlike

potassium dichromate, Bronopol is reported to have no harmful effects and is often used in milk preservation (Chalermsan *et al.*, 2004), particularly for preserving SCC prior to analysis through its action as an antimicrobial agent.

However it is not known if such commonly used milk preservatives or heat treatment used to inactivate pathogens before international transit, have an effect on the concentration of milk APP but it is important to be aware of such effect if milk APP are to be performed on samples treated with these methods.

One of the aims of this study was to evaluate the effect of some sampling and storage variables on milk Hp concentration. This consisted of the assessment of milk Hp following the use of milk preservatives such as potassium dichromate (Lactab Marks III; Thompson & Capper Ltd, Cheshire, UK) and Bronopol (Broad spectrum Microtab® II; Advanced instruments, *Inc.*, Massachusetts, USA), which are used to preserve milk composition from collection to analysis of for example, SCC or progesterone for pregnancy diagnosis.

This study also explored the possible effect on milk APP when samples were heated to pathogen destroying temperatures. Storage of samples at different durations of freezing temperature, on milk APP profile was also examined.

2.2.6 Study objectives

The objectives for this chapter were the following;

- The first aim of this study was to develop, optimize and validate an immunoassay of a sandwich ELISA format which would be sensitive and reliable for the measurement of milk Hp.

- Studies were carried out to evaluate the effect of sampling and storage variables on milk Hp and M-SAA3 concentration. This consisted of the assessment of milk Hp and M-SAA3 following the use of the milk preservatives; potassium dichromate (Lactab® Marks III) and Bronopol (Broad spectrum Microtab® II) for milk Hp only, which are used to preserve milk composition from collection to analysis (to preserve milk meant for somatic cell counts (SCC) and progesterone measurement respectively). This study also explored the possible effect on the milk APP, when samples were heated to pathogen destroying temperatures.

Storage of samples at different temperatures for different durations of time was also examined in relation to the APP.

- To evaluate the profile of milk Hp, M-SAA3 and CRP in samples from a commercial dairy herd and to compare the results with some commonly measured milk traits such as SCC and cow factors such as parity and stage of lactation of each cow.

2.3 Materials and methods

2.3.1 Reagents

General chemicals were obtained from Sigma-Aldrich, Poole, UK, except where otherwise stated. In all experiments, milli *Q* water was used.

2.3.2 Haptoglobin ELISA development

2.3.2.1 Assay Protocol

Antibody conjugation

Purified rabbit anti bovine haptoglobin (RABHp) (*Life Diagnostics*, West Chester, USA) was conjugated with alkaline phosphatase (Innova Biosciences, Cambridgeshire, UK) according to manufacturer's instruction. An aliquot of 3.7 µl of LL-Modifier was added to lyophilized product (alkaline phosphatase-AP) in the vial and gently mixed by pipetting up and down. 37 µl of the RABHp IgG (immunoglobulin G) (0.1 mg) was then added into the vial, gently mixed and left at RT overnight. 3.7 µl of LL-Quencher was added the next day to stop the reaction.

The conjugate was ready for use after 30 minutes (min). 200 µl of phosphate buffered saline (PBS) was added to the conjugate to give a final volume about 244 µl, and an antibody concentration of 0.4 mg/ml. This was stored at 4^{o}C until used. The success of the conjugation of the rabbit anti bovine Hp to alkaline phosphatase was assessed by western blotting (of SDS-PAGE of milk and serum samples with varying concentration of Hp) incubation with the conjugated antibody and staining with 1-Step™ NBT/BCIP (alkaline phosphatase substrate for immunoblotting; Thermo scientific, Rockford, USA).

Sandwich ELISA procedure

Unconjugated rabbit anti bovine Hp was diluted to give a series of concentrations of 1 - 0.125 µg/ml in coating buffer (0.05 M $NaHCO_3$ pH 9.6). 100 µl aliquots were dispensed into individual wells of Nunc-Maxisorp 96 MicroWell™ plate (Nunc International, Rochester, New York, USA) and incubated at 4^{o}C overnight. After discarding the antibody coating solution, each well was washed using 250 µl wash buffer; 250 µl of 0.02 M Tris-HCl containing 0.05 % (v/v) Tween-20 (pH 7.4), per well, four times. Unoccupied binding sites were blocked by adding 250 µl of 10 % (w/v) Marvel milk protein in wash buffer, per well and incubated at 37^{o}C for 60 min.

After washing, standard bovine Hp (1.64 mg/ml, Life Diagnostics Inc., West Chester, USA) was diluted to a concentration of to 1025 ng/ml in wash buffer and then double dilutions made from 1025 - 8 ng/ml, in order to generate a standard curve. Milk samples were also diluted in wash buffer (1:200, 1:400, 1:800, 1:1600 or 1:3200). 100 µl of each standard bovine Hp and diluted milk samples were added into duplicate wells and incubated at 37 °C for 60 min with gentle shaking.

Wells were washed, and 100 µl of the alkaline phosphatase-conjugated antibody (section 2.1.1) diluted 1:1000, 1:5000 and 1:10,000 in wash buffer, were dispensed into each well of the ELISA plate and incubated at room temperature (RT) for 60 min with gentle shaking. After washing, substrate solution, BluePhos® Microwell phosphatase substrate system by KPL (KPL laboratories, *Inc.*, Maryland, USA) was made up according to manufacturer's instruction and 100 µl was added into each well for colour development taking approximately 10 min. APstop™ solution (KPL laboratories, *Inc.*, Maryland, USA) was used by adding 100 µl per well, to stop further colour development after the optimum was reached.

The absorbance was read at 595 nm using FLUOstar OPTIMA plate reader (BMG Labtech Ltd, Bucks, UK) and the results analysed and calculated using the associated MARS (Optima) data analysis software (BMG LABTECH, program 2.40) with a 4 parameter logistics (4PL) standard curve plotted on a log-linear scale.

In order to obtain a better intra assay coefficient of variance (CV) per ELISA plate (where the intra assay CV was above 15 % probably due to instrument error), absorbances for some plates were also read using the built-in Triturus® ELISA plate reader at wavelength of 600 nm. Better intra and inter assay CVs were obtained using this reader. So for subsequent readings of samples described in chapters 4 and 5 of this thesis, the Triturus® reader was used. A 4PL standard curve was selected and concentration and validation calculations were carried out by the Triturus® software.

2.3.2.2 Assay validation

Precision

Precision of the assay was determined by calculating the intra assay and inter assay coefficient of variance (CV). Intra assay was determined by the mean CV of 40 duplicate samples while inter-assay was calculated by the mean CV of repeating 2 different samples (high and low Hp milk) in 10 different assays.

Limit of detection

Minimum detection limit was taken as the haptoglobin concentration of mean of absorbance reading 4 blank samples plus 3 standard deviations (SD) being the concentration that could be differentiated from zero.

Specificity

Specificity of the assay was assessed by western immunoblotting of milk samples containing varying concentrations of Hp and samples of commercial milk spiked with known concentrations of purified bovine Hp with the alkaline phosphatase labelled anti bovine Hp as antibody (the western blotting procedure is described in details in Section 3.3.1.3.2).

Accuracy

Accuracy was determined by percentage recovery of Hp concentration, from spiked milk samples, using the ELISA assay. Spiked samples were prepared from separate aliquots of a commercial milk (with no measurable level of Hp) to which standard bovine Hp (Life Diagnostics *Inc.*, West Chester, USA) was added

to gain a final concentration of 100 μg/ml, 50 μg/ml, 25 μg/ml and 0 μg/ml (not spiked), followed by gentle mixing at 4°C overnight.

2.3.3 Mammary associated serum amyloid A3 assay

2.3.3.1 ELISA kits

Tridelta Development Ltd supplied the Phase™ Range SAA ELISA kit (sandwich ELISA kit for measuring multispecies SAA, Phase™ Range by Tridelta Development Ltd (Kildare, Ireland).

2.3.3.2 Reagent preparation

Diluent buffer and wash buffer (1x) were prepared from the stock of 10x and 20x respectively using milli Q water, according to the manufacturers' instructions. The calibrator for the assay was reconstituted by adding 1 ml of 1x diluent buffer to the lyophilized SAA standard and vortexed vigorously. Aliquots of the top calibrator were made and stored at -20ºC. Serial dilutions of the top standard were made to achieve 6 standards per assay (300 – 0 ng/ml), which were run in duplicates. Milk Samples were diluted in 1x diluent buffer.

2.3.3.3 M-SAA3 assay protocol

Samples were diluted to 1:50 or 1:500 and 50 µl of each sample, as well as of the standards (calibrators), were dispensed into wells of a pre-coated 96-well plate to which 50 µl of anti SAA-HRP conjugate had been previously added. ELISA plates were gently tapped to mix the contents and then incubated at 37°C for 60 min.

Wells were washed four times with 1x wash buffer, after the last wash the plate was tapped dry on absorbent paper. Substrate for HRP; tetramethyl benzidine (TMB) solution, was added (100 µl/well) and incubated for 15 min at RT in the dark. The reaction was stopped by adding 100 µl of the stop solution provided in the kit, and absorbance read at 450 nm using FLUOstar Optima ELISA plate reader at wavelength of 450 nm. A 4-parameter logistics (4PL) standard curve was used to plot the standards; sample concentration interpolations were calculated using the MARS (Optima) analysis software (v. 2.40, BMG Labtech).

Concentrations of samples were interpolated from the linear portion of the standard curve generated.

2.3.3.4 M-SAA3 assay validation

The limit of detection (LOD) was determined from the mean plus 2 standard deviations of 4 blank samples while the specificity and accuracy of the assay were based on the data supplied by the manufacturer (Phase™ Range, Instructions for use, 2014).

Intra assay precision was evaluated from the mean coefficient of variance (CV) for 42 samples ran in duplicates, while inter assay precision was calculated from the mean CV of 5 repeats of high and low quality control samples.

2.3.4 CRP assay

2.3.4.1 ELISA kits

Cow C-reactive protein (CRP) ELISA kits for assay of milk CRP were supplied by the Life diagnostics *Inc*. (West Chester, USA). The assay was based on solid phase sandwich ELISA format, and comprised of a primary anti-bovine CRP antibodies immobilized to the wells of a 96-well microtitre plate and secondary antibodies against the anti-bovine CRP conjugated to HRP.

2.3.4.2 Reagent preparation

Diluent buffer and wash buffer were prepared from the stock of 10x and 20x solution respectively, using milli Q water according to the manufacturer's instruction.

CRP standard was reconstituted by adding 1 ml of the 1x diluent buffer into the vial of lyophilized standard and vortexed vigorously. 14.25 µl of the reconstituted standard was added to 485 µl of diluent buffer to give the top standard with a concentration of 62.5 ng/ml. The top standard was then serially diluted to give 6 other standards with concentrations ranging to 0.98 ng/ml. Plain diluent buffer was used as the blank (0 ng/ml). Milk samples were diluted initially at 1:250 in 1x diluent buffer, but for samples with higher CRP concentrations, a dilution of 1:2000 was re-used and for sample with very low CRP concentration a lower dilution of 1:5 was used.

2.3.4.3 CRP assay protocol

Diluted samples and standards were mixed thoroughly and 100 µl of each sample or standard was dispensed into duplicate wells of the 96-well microtitre plates provided. This was then incubated on an orbital microplate shaker at 150 revolutions per minute (rpm) at RT for 45 min. Contents of the wells were then discarded and wells washed five times each using 1x wash buffer. After ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, 100 µl of the secondary antibody-HRP conjugate was then dispensed into each well and incubated on the shaker at RT for 45 min. The wash step was repeated and 100 µl of TMB reagent (HRP substrate) was dispensed into wells and colour development was allowed to proceed for 20 min on the shaker at RT. The reaction was stopped by adding 100 µl of stop solution per well into the wells. Absorbance was read using a FLUOstar Optima plate reader at 450 nm within 15 min of stopping the reaction. A four parameter logistic curve (4PL) was used to plot the standard curve, and concentrations of samples were interpolated from the linear portion of the standard curve.

2.3.4.4 CRP assay validation

The limit of detection of the CRP ELISA was determined as the mean plus 3 standard deviations of the concentration value of 4 blanks. The specificity and accuracy of the assay was based on the data supplied with the kit (Life diagnostics, *Inc.*, catalog number 2210-8, instruction manual). Intra assay and inter assay precision was calculated from the mean CV of 40 samples ran in duplicates and that of 5 repeats of 2 QC samples (high and low CRP concentration) respectively.

2.3.5 Assayed samples

2.3.5.1 Effect of sample treatment on milk APP

Milk samples with a varying range of APP concentration were obtained from the University of Glasgow Cochno Research Farm dairy herd and from Veterinary Diagnostic Services (VDS), University of Glasgow. Several 500 µl aliquots were made from each sample with aliquots treated to assess for;

- Effect of heat treatment by subjecting one set of aliquots (500 µl per aliquot) to heat treatment (56°C, for 30 min in a water bath); Hp (n=38), M-SAA3 (n=20).
- Effect of use of preservatives by adding and mixing to one set of aliquot each;
    - 1 tablet (30 mg) of potassium dichromate (Lactabs® Mark III, Thompson and Capper Ltd, Cheshire, UK) per 10 ml of milk samples; Hp (n=35), M-SAA3 (n=8);
    - 1 tablet (8 mg Bronopol + 0.30 mg Natamycin) per 40 ml of milk sample of Bronopol (Broad spectrum Microtabs® II, Advanced instruments, *Inc.*, Massachusetts, USA); Hp (n=33)
- For evaluation of effects of different storage temperatures and duration on milk APP, separate sets of aliquots of fresh milk samples (n=6) collected from Cochno Dairy, were stored at 4°C for 24 h; -20°C for 7 days and -20°C for 21 days, after which they were all assayed for Hp and M-SAA3. Control aliquots of the samples were analysed for Hp and M-SAA3 on the day of collection, before any form of storage.

2.3.5.2 Milk Samples from cows at Cochno dairy

Composite and quarter milk samples were collected during the morning (6:00 am) and afternoon milking (3:00 pm) respectively, from all lactating Holstein-Friesian cows at Cochno dairy farm, Glasgow between the period of September and October 2012. Sample collection was carried out with the assistance of Mr Ian Cordner (Cochno Dairy farm, University of Glasgow) and Mary Waterston (Institute of Infection, Immunity and inflammation, University of Glasgow).

The health statuses of the cows were confirmed by routine veterinary records maintained for each cow. Cows were milked twice daily, housed in pens and fed a standard diet. Just before application of milking machine clusters to the udder, teats were wiped with an iodine based disinfectant, the first few jets of milk were discarded, and approximately 50 ml milk per quarter was expressed into sterile Falcon tubes and labelled. Composite milk samples were obtained after application of the milking machine cluster to the teats of functional quarters of each cow, ~50 ml of sample was then collected from a milk tube

linked to the claw of the milking machine, into sterile tubes. Samples were later aliquoted (~15 ml, 3 aliquots) and stored -20°C until analysed.

One hundred and forty nine (149) quarter and fifty four (54) composite milk samples collected from Cochno Dairy farm were assayed for Hp, while only composite milk samples (n=54) were analysed for both M-SAA3 and CRP. Samples were thawed at RT, thoroughly mixed by vortexing, and diluted in diluent/wash buffer. They were then assayed using the respective sandwich ELISAs for each of the APP as described above.

Data of SCC, percentage fat and percentage protein in milk samples as well as lactation number (number of times cow had calved) and days in milk (DIM) of the cows were obtained from farm records (SCC, fat and protein tests were carried out by the National Milk Records Company (NMR Co.), Hillington-Park, Glasgow.

Somatic cell counts were categorized into high (>200,000 cells/ml) and low (≤200,000 cells/ml), based on suggestions by Pantoja *et al*. (2009). Cut off values for SCC to determine subclinical mastitis have been a subject of debate (Åkerstedt *et al*., 2011), therefore in this study a second categorisation level for SCC was used based on suggestions of Schwarz *et al*. (2011) and Berglund *et al*. (2007) (healthy samples- SCC<100,000 cells/ml; subclinical mastitis (SM)-SCC 101,000 -200,000 cells/ml; clinical mastitis (CM)-SCC>200,000 cells/ml). The APP distributions were compared between these various SCC categories.

Lactation stage was determined from DIM as: 0-60 days= early lactation, 61-240 days=mid lactation; 241-305 days=late lactation; 306 and above=dry period) and distribution of each APP was across lactation stages were compared. Correlations between the APP, lactation number, percentage (%) fat and % protein were examined.

2.3.6 Statistical analyses

Results obtained were initially stored in Excel (Microsoft 2007) and simple descriptive statistics were obtained. Data were later exported to IBM statistical package for social sciences (SPSS) statistical software; version 21 (IBM Corporation, 2012) for further analysis.

Tests for normality were run on the APP values using Kolmogorov–Smirnov and Shapiro–Wilk test along with normal probability plots and quantile-quantile (Q-Q) plots. To analyse effects on milk APP of heat treatment, Lactab® and Microtab® use, different storage conditions and milk defatting and casein removal, the Wilcoxon signed ranks test for non-parametric data was used to compare the two groups (treated or untreated), significance level of P was taken as <0.05. Non-parametric correlations test (Spearman's rho) was carried out to determine correlations between APP in the different groups.

For variables which were not normally distributed, non-parametric tests (Mann-Whitney's test for two groups and independent Kruskal-Wallis test for more than 2 groups) were used to evaluate the differences between groups (SCC categories and lactation stage). Bivariate correlation analyses (Spearman's rho for non-normally distributed data) were carried out between each APP, SCC, lactation number, % fat and % protein of Cochno composite milk samples. P-value was considered significant at <0.05. The Stata® statistical package (version SE/12.1, StataCorp, USA) was used to calculate reference values for the APP in relation to high and low SCC categories (>200,000 and <200,000 cells/ml) of the composite milk samples from Cochno dairy farm.

2.4 Results

2.4.1 Milk haptoglobin

2.4.1.1 Hp ELISA optimisation and validation

Coating Antibody

A final concentration of 0.125 µg/ml of the primary antibody in coating buffer (100 µl/well) was used as the optimized capture solution. This value was selected as it produced the best signal to noise ratio absorbance on the ELISA plate wells and contributed to standard curve with the lowest blanks and sufficiently steep, after trying concentrations of 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml and 0.125 µg/ml. Figure 2-1 shows standard curves obtained using different concentration of coating antibody.

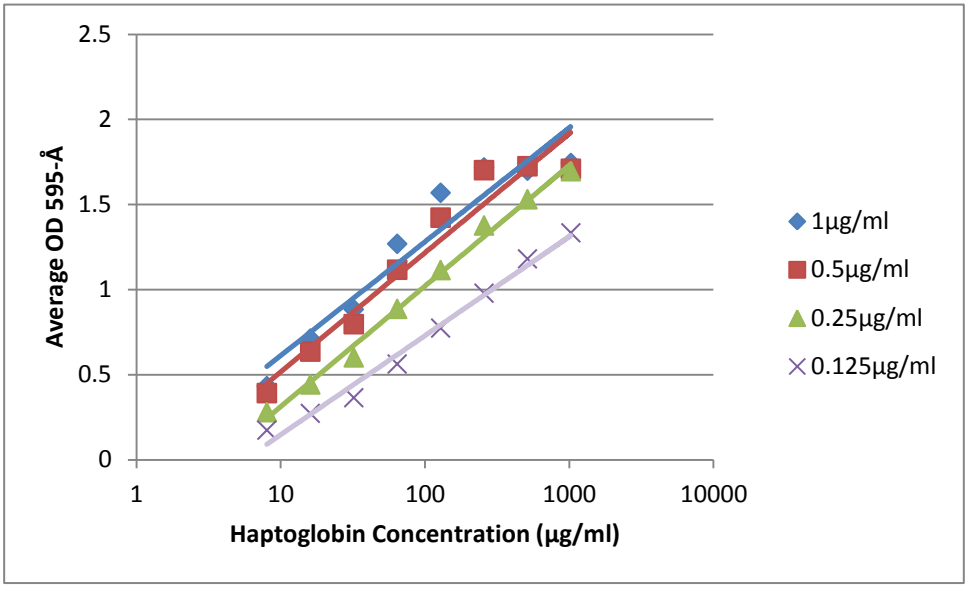

Figure 2-1: Optimization of the coating antibody solution for the ELISA showing curves obtained for various coating antibody concentrations.
The 0.125µg/ml solution gave the steepest curve with lowest blank and was therefore adopted for the ELISA.

Standards and sample dilution

A range of concentrations of bovine Hp standard from 1025 ng/ml double-diluted to 8 ng/ml (in wash buffer) gave the range of steepest 4PL curve (on the log-linear scale as seen in Figure 2-2).

Samples were diluted to 1:400, 1:800, 1:1600 or 1:3200 in wash buffer depending on the concentration of Hp in order for the measured value to fall within the linear portion of the standard curve.

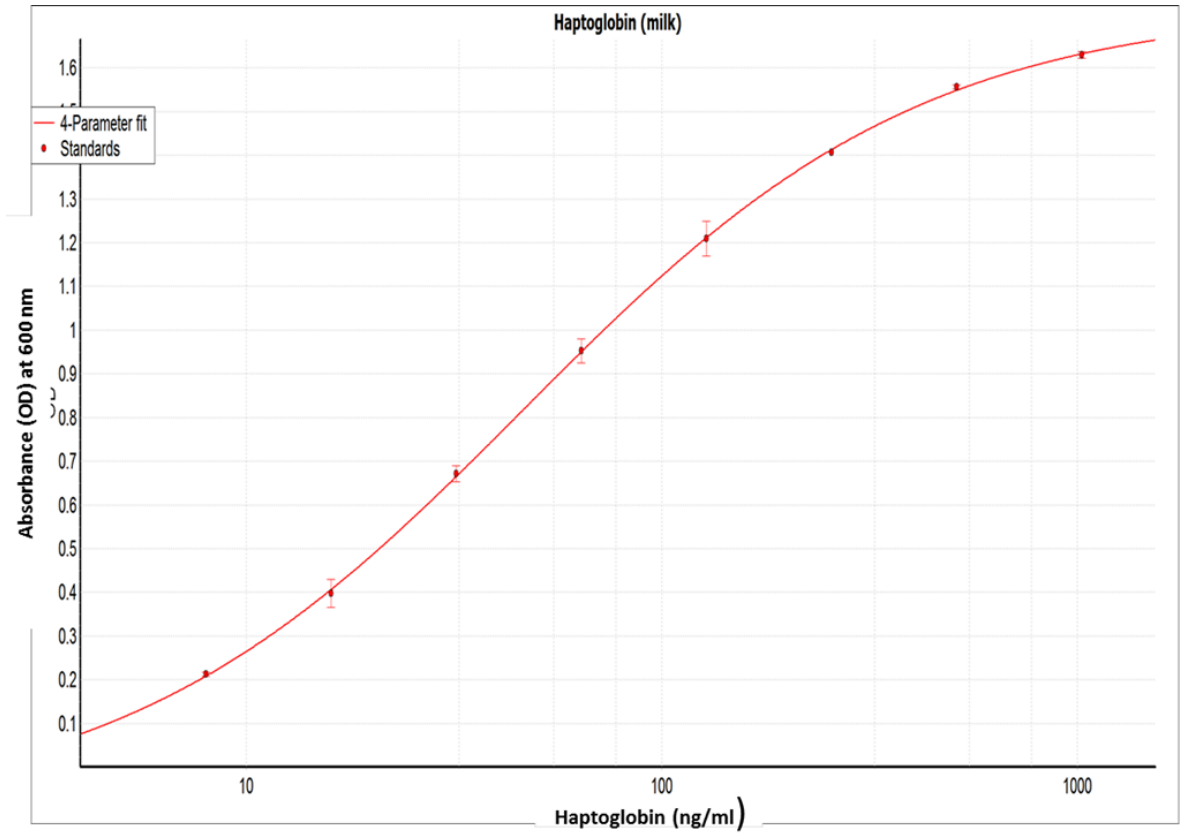

Figure 2-2: Example of 4PL standard curve generated for standard bovine Hp.
Dilutions were from 1025-8 ng/ml, from which sample concentrations (n=21) were interpolated. Points are mean ± standard deviation (SD) of standards.

Signal antibody

A final dilution of 1:10,000 of the conjugated antibody (alkaline phosphatase labelled RAB-Hp) in wash buffer (0.4 μg/ml) gave the optimum concentration of signal for the determination of Hp levels in the milk samples compared to 1:1000 and 1:20,000 and was thus adopted as the optimized concentration of signal antibody for the assay. Figure 2-3 shows standard curves generated for the three different signal antibody concentrations used.

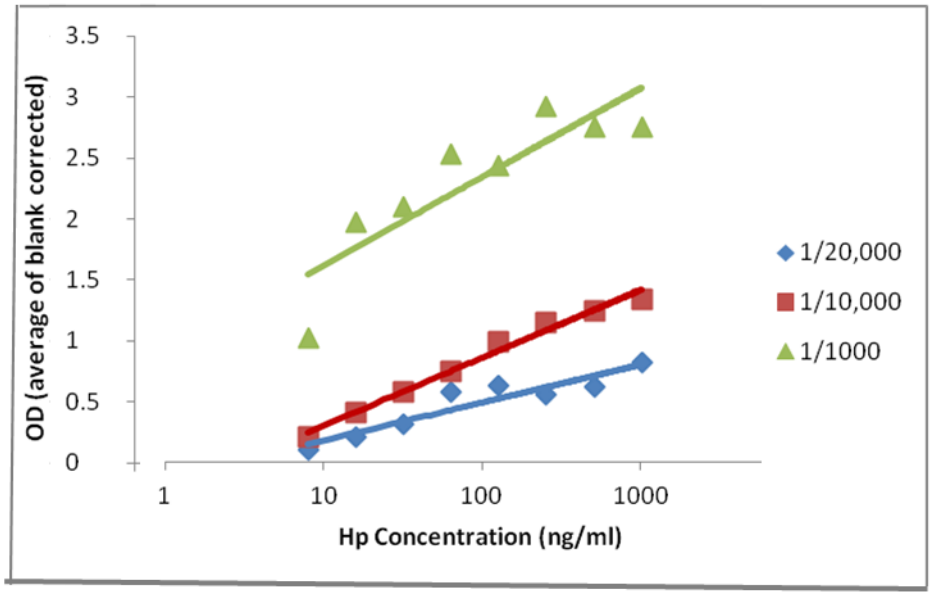

Figure 2-3: Optimization of signal antibody (RAB-alkaline phosphatase conjugate) for developed ELISA.
Standard curves generated using signal antibody concentrations of 1:1000, 1:10,000 and 1:20000. The 1:10,000 signal Ab concentration was choosen because it had a lower blank and at the same time suffienctly steep curve than the other concentrations.

Precision

The intra assay CV was 5.74 %, while inter assay CV was 26.92 %. Table 2-1 shows the concentration (mean of duplicates) and calculated standard deviations (SD) and CV used to arrive at the inter assay precision.

Table 2-1: Haptoglobin concentrations determined by the developed ELISA in two quality control (QC) samples
Samples 97FL (high Hp) and 43FL (low Hp) in 11 different plate assays, read using the FLUOstar optima plate reader, an inter assay precision of 30 % and 23 % for the high and low QCs respectively was obtained.

	Hp (µg/ml)	
TEST	High QC	Low QC
1	30.2	3.67
2	21.3	5.65
3	18.58	7.33
4	17.86	7.31
5	33.68	7.17
6	34.4	8.69
7	23.74	9.06
8	25.1	7.07
9	17.22	5.85
10	15.05	6.95
11	16.26	4.97
Mean	**23.04**	**6.70**
SD	**7.01**	**1.57**

% CV 30.44, 23.4

Average % CV (interassay CV) =26.92 %,

In order to further optimize and improve the precision of the Hp ELISA, standard dilutions were later made from 512.5 ng/ml to 4 ng/ml and assays were run on an automated ELISA equipment robot (Triturus® ver. 4.01 by Diagnostic Grifols, Spain).

Absorbances were then read using the built-in Triturus® ELISA plate reader at wavelength of 600nm. Standard curve plotting, concentration calculations and validation calculations were carried out by the Triturus® software. Calculated intra assay CV was 1.6 % (mean of CVs of 40 samples run in duplicates) and inter assay CV (mean) was 15.6 % after these adjustments. Table 2-2 shows the mean and calculated SD and % CV of 10 repeats of the controls used to determine the inter assay CV.

Table 2-2: Haptoglobin concentrations of 2 QC samples (high and low Hp milk) in 10 repeats.
Determined using the developed ELISA after modifications on standards' concentration range and by the use of Triturus® ELISA plate reader. 10 different ELISA analyses were carried out giving an inter-assay precision of 14 % and 17 % for the high and low Hp milk respectively.

TEST	High QC	Low QC
1	18.8	4.6
2	15.56	5.28
3	14.36	4.88
4	21.84	6.84
5	18.08	6.04
6	16.48	5
7	16.08	4.68
8	21.84	5.8
9	16.7	5.9
10	17.04	7.52

Mean 17.67, 5.65
SD 2.51, 0.96
CV 0.14, 0.17
% CV 14.22, 17.03
AVERAGE % CV=15.62 %

Limit of detection

The limit of detection (LOD) which was determined from the concentration of the mean of four blank samples plus 3 standard deviations was 2 ng/ml (0.002 μg/ml) for milk samples after dilution which allowing for the minimum dilution of 1:200 gave a limit of 0.4 μg/ml for the lowest concentration that could be measured in milk.

Accuracy

The calculated accuracy of the assay was determined from recovery concentration of spiked samples and this was 96 %, shown in Table 2-3.

Table 2-3: ELISA determination of haptoglobin concentrations in Hp-spiked milk samples;
Used to evaluate the accuracy of the ELISA

Samples	Hp Spike (μg/ml)	Hp value (ELISA, μg/ml)	Recovery %
C100	100	76.17	76
C50	50	44.04	88
69BR	10	10.63	106
69BR	40	37.51	94
69BR	20	23.86	115

Mean Recovery = 96 %

Accuracy was also determined by evaluating the linearity of 3 different milk samples of varying Hp concentration after serial dilutions of the samples at 1:400, 1:800 and 1:1600, Figure 2-4 is a chart showing the linearity of the samples.

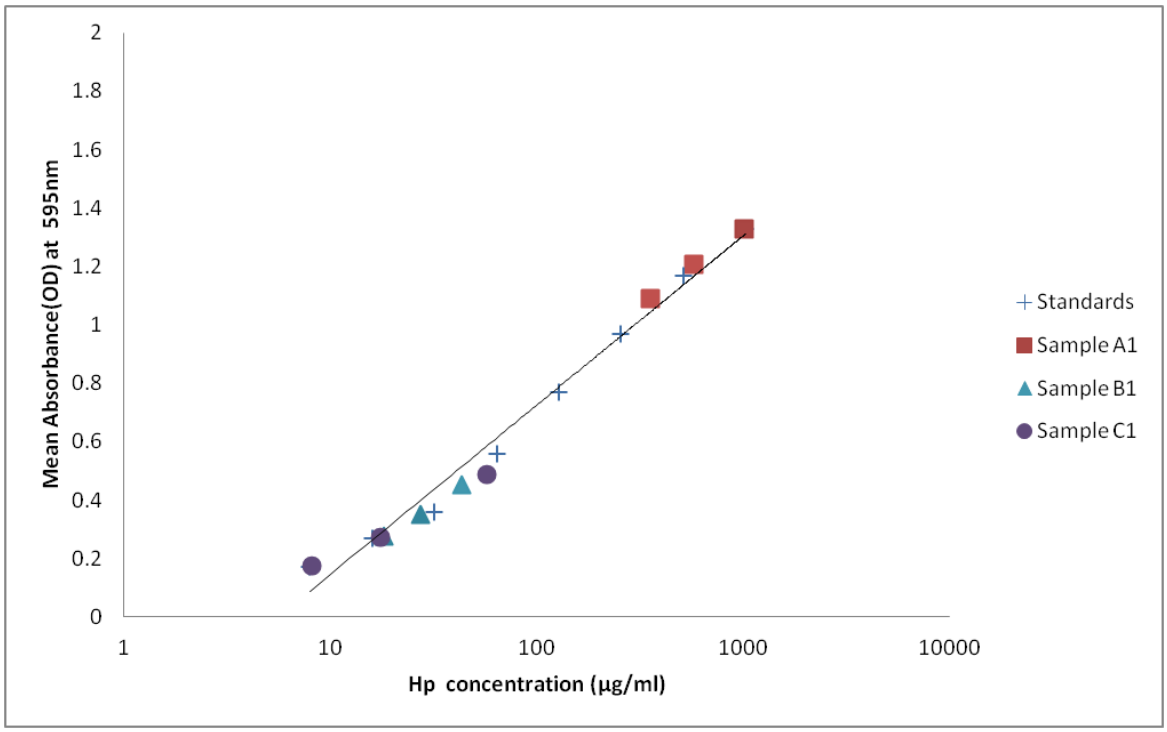

Figure 2-4: Linearity of 3 milk samples' (A1, B1 and C1) Hp after dilution of the samples at 1:400, 1:800 and 1:1600

Specificity

The western blot assessment of the specificity of the assay using milk samples spiked with known concentrations of standard bovine Hp and is shown in Figure 2-5. Figure 2-6 shows a western blot of serum and milk samples having naturally high or low haptoglobin and shows bands developed in high Hp containing samples. In both spiked samples and those with naturally elevated Hp, the ß-chain of Hp around 40 kDa reacted with the labelled antibody used in the Hp ELISA and no other protein.

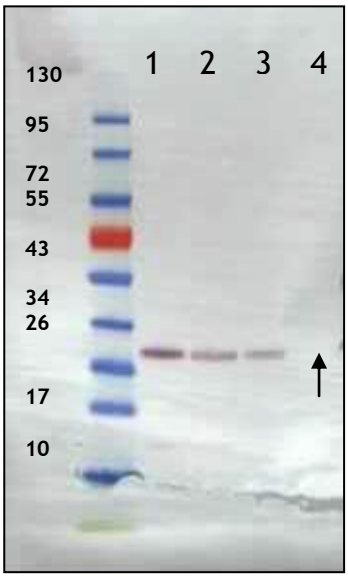

Figure 2-5: Western immunoblotting of milk samples spiked with Bovine Hp.
Lane 1, 2 and 3 having samples spiked with 100 µg, 50 µg and 25 µg Hp respectively showing increased intensity of bands with higher Hp concentration) and lane 4 not spiked and having no detectable level of Hp and no Hp β-band formed (arrow).

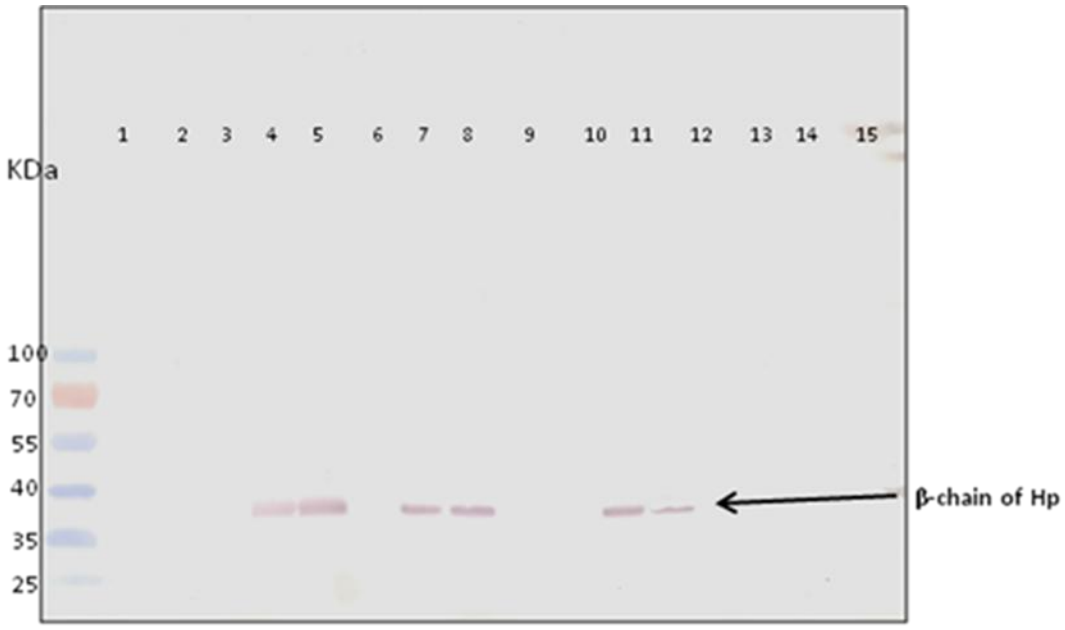

Figure 2-6: Western blot of serum and milk samples using RABHp with varying Hp concentration.
Serum samples (1-9) with low (1-3, 6 and 9) and high Hp (4, 5, 7 and 8) and milk samples (10-15) with low (12, 13 and 14) and high Hp (10 and 11), showing the β-chain band of Hp in the high Hp samples.

2.4.1.2 Effect of different sample treatment on milk haptoglobin.

Significant differences were found in the milk Hp levels of heat treated and non-heated samples (P=0.03). Heated samples (56ºC for 30 min) showed an average 11 % reduction in milk Hp compared with unheated samples. However there was a high correlation between the heated and unheated samples (r = 0.97). Table 2-4 shows the related samples Wilcoxon signed ranks test of comparison between the two sets of samples (heated and unheated), and in Figure 2-7 is a scatter plot of the heated and unheated samples is depicted.

There was no significant difference in milk Hp with use of the preservative, potassium dichromate (Lactabs® Mark III) compared with controls (P=0.08, r=0.98) (scatter plot shown in Figure 2-8). No significant differences were also observed for use of Bronopol (Broad spectrum Microtabs® II) (P=0.796, r=0.88) (scatter plot shown in Figure 2-9) compared to controls. Samples stored at different storage temperatures ranging from 4ºC for 24 h and -20ºC for 21 days did not show any significant difference in their Hp levels (supplementary data).

Table 2-4: Wilcoxon signed ranks test of comparison of milk Hp in heated and unheated samples

Negative ranks indicate samples in which heated samples had Hp concentration less than in unheated samples.

Ranks

		N	Mean Rank	Sum of Ranks
heated - unheated	Negative Ranks	26[a]	17.17	446.50
	Positive Ranks	9[b]	20.39	183.50
	Ties	3[c]		
	Total	38		

a. heated < unheated
b. heated > unheated
c. heated = unheated

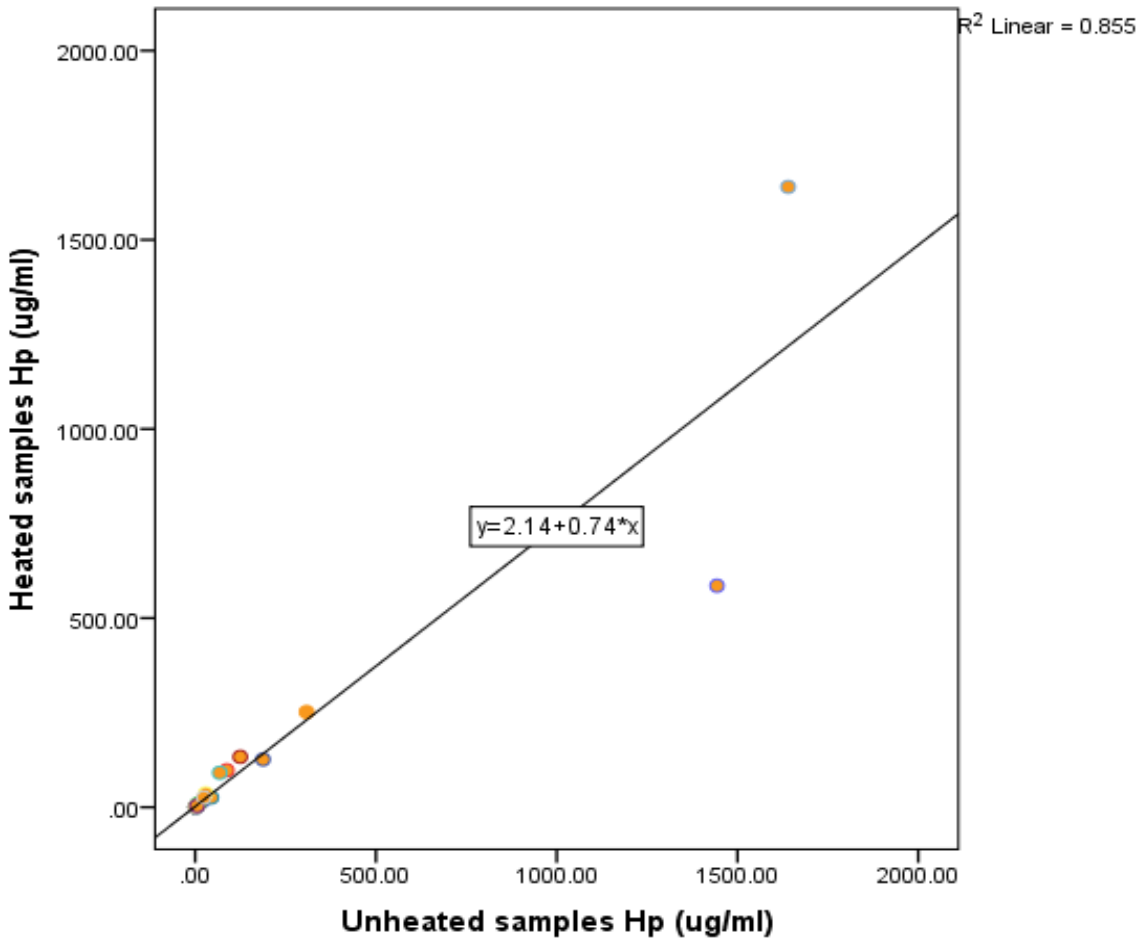

Figure 2-7: Scatter plot of heated versus unheated milk sample Hp concentration (n=38).

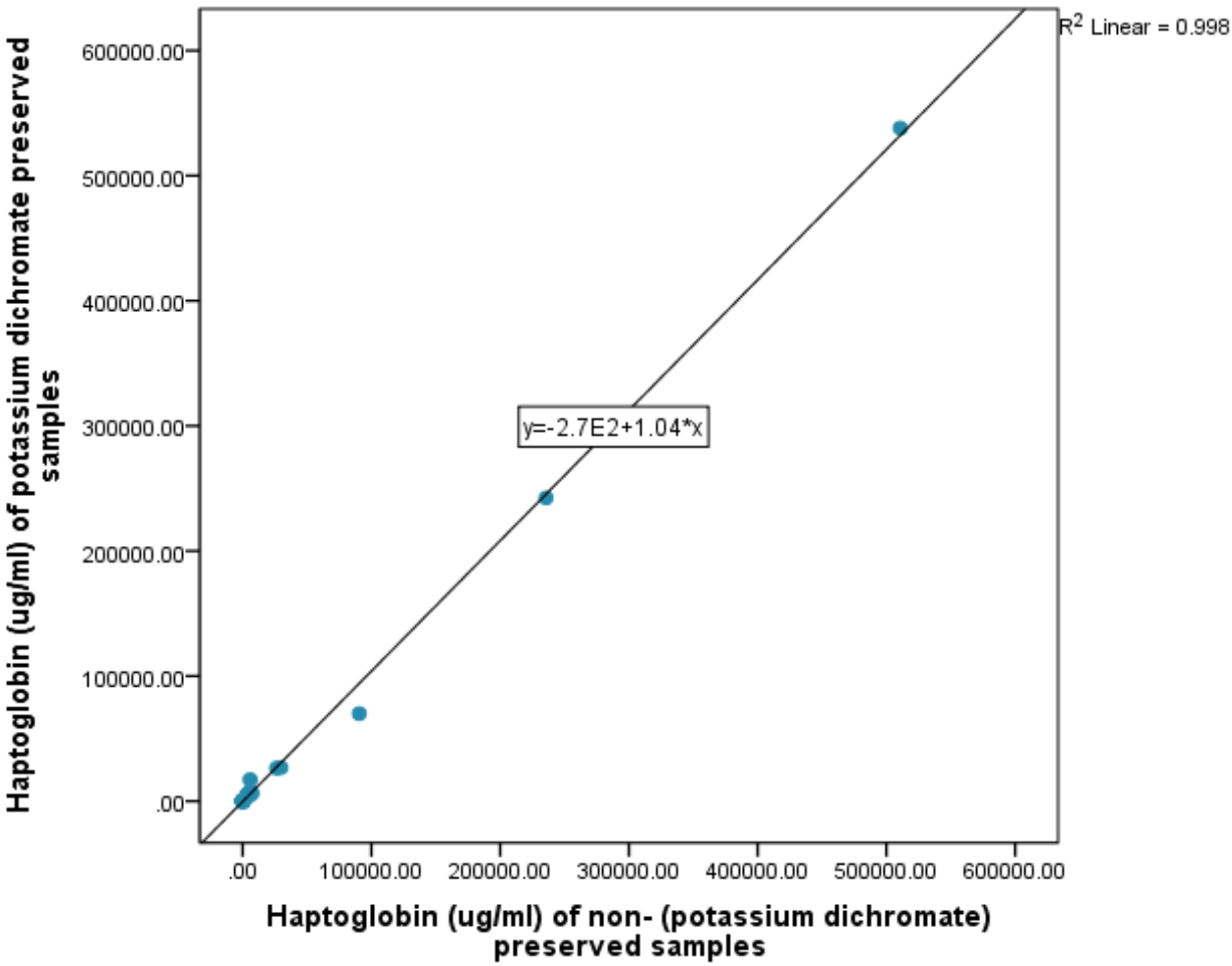

Figure 2-8: Scatter plot of samples preserved and non-preserved with Potassium dichromate
(Lactab® Marks III, n=35)

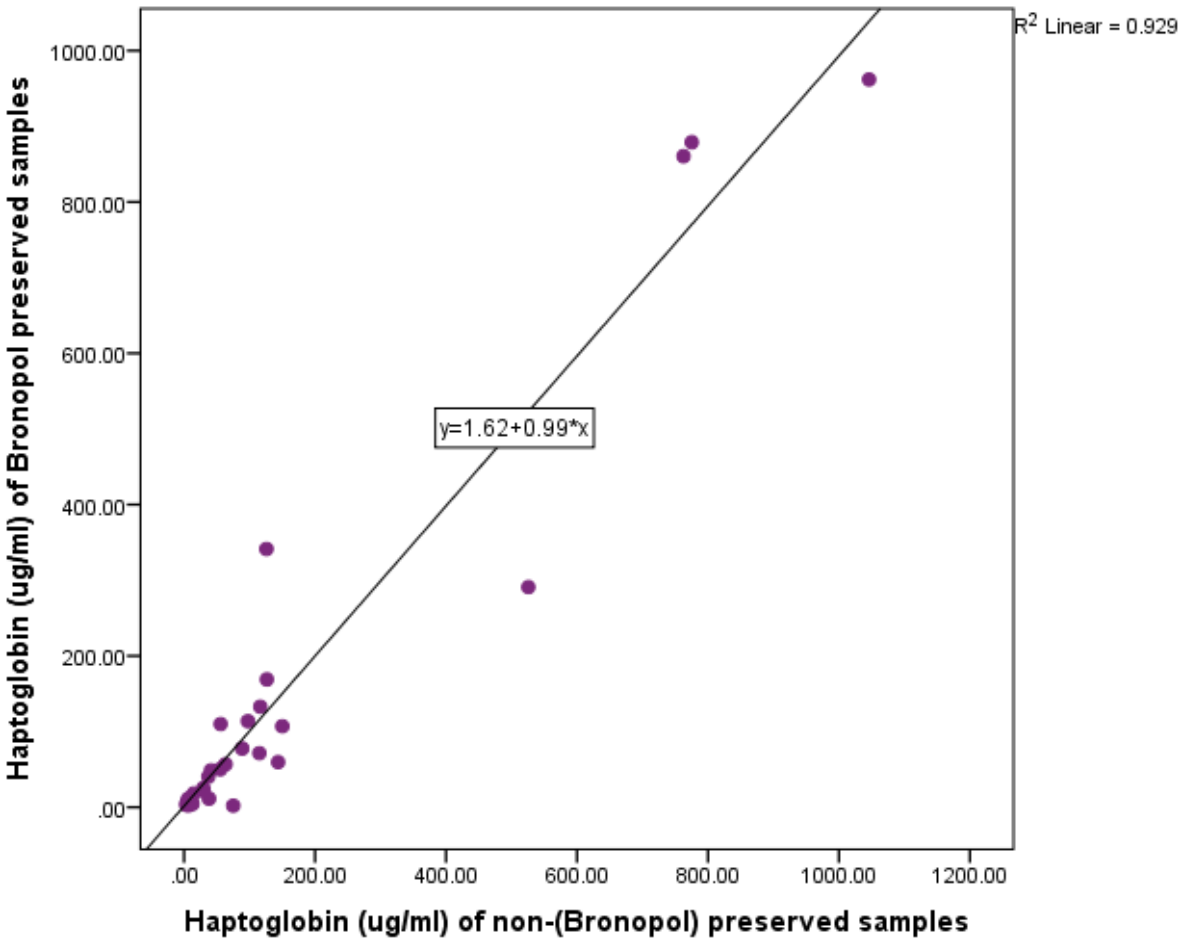

Figure 2-9: Scatter plot of samples preserved or not preserved with Bronopol (Microtabs, n=33)

2.4.1.3 Haptoglobin profile of Cochno dairy farm milk samples

Quarter milk samples

Haptoglobin concentration in quarter milk samples (n=149) collected from Cochno Farm ranged from <0.4 μg/ml to 420 μg/ml and had a skewed rather than normal distribution (Figure 2-10). The median was 36 μg/ml. Figure 2-11 shows the distribution of quarter samples having Hp below 100 μg/ml.

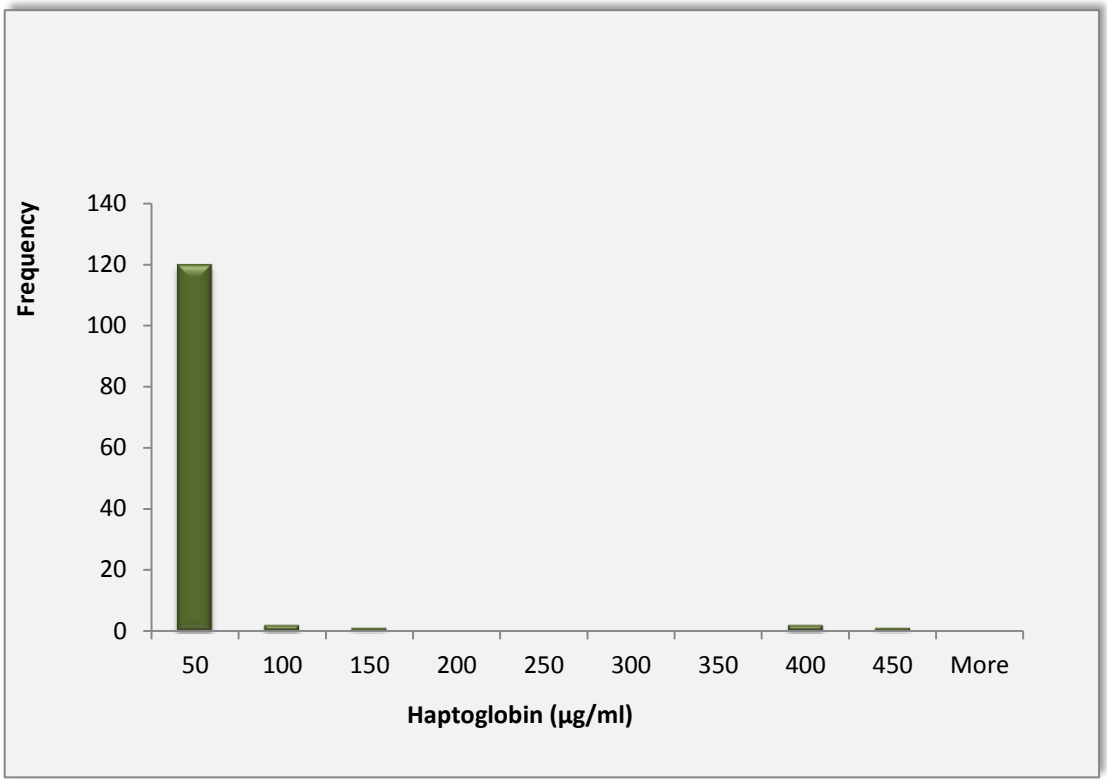

Figure 2-10: Distribution of all quarter milk samples' Hp concentration showing range for all samples (n=149).

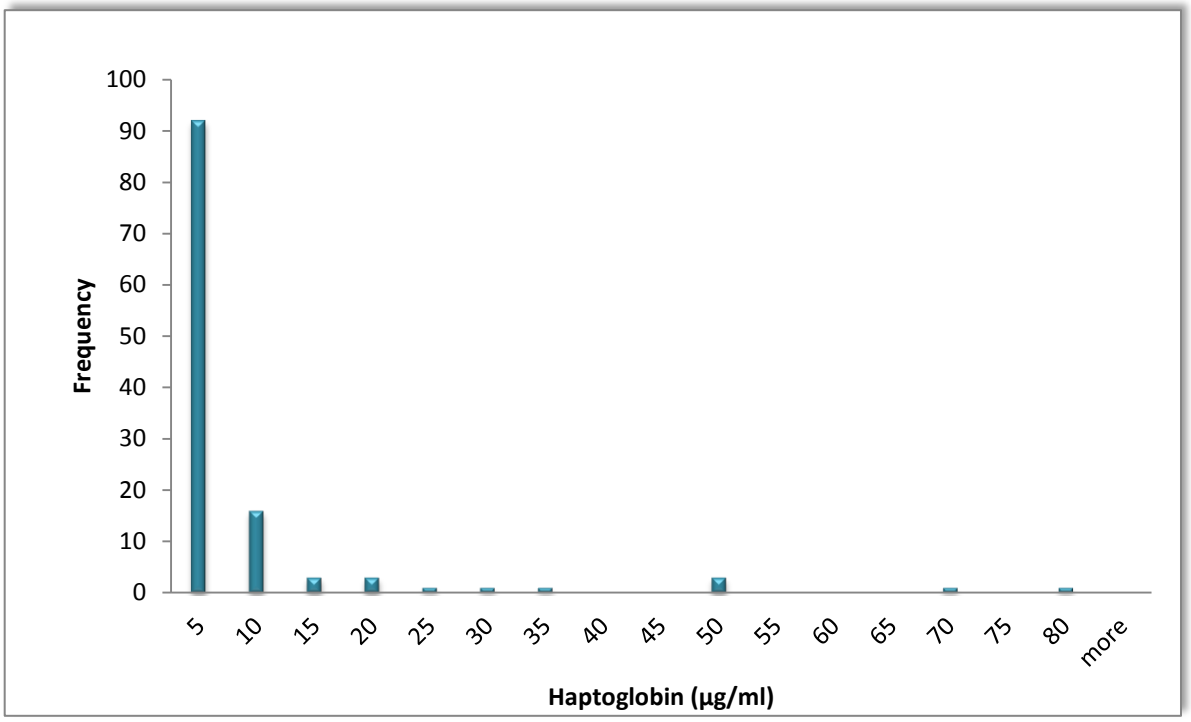

Figure 2-11: Distribution of quarter milk samples with Hp concentration of ≤100 µg/ml (n=145).

Composite milk samples Haptoglobin

Descriptive statistics of Hp and relevant data from the NMR Co. in composite milk samples are presented in Table 2-5, while the frequency distribution is shown in Figure 2-12. Hp concentration ranged from 0.4-55.46 µg/ml and a median of 3.4 µg/ml.

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Table 2-5: Descriptive statistics' of composite milk samples APP (n=54).

	Hp (µg/ml)	M-SAA3 (µg/ml)	CRP (ng/ml)	SCC cells/ml	Parity	% fat	% Protein	DIM (days)
Mean	6.97	3.87	32.64	485	3	11.75	3.47	222.20
*SEM	1.47	1.08	5.00	159	0.28	7.55	.06	20.11
Median	3.46	1.17	24.56	96	3	4.28	3.44	188.50
SD	10.82	7.95	36.76	1170	2	55.50	.41	147.79
Minimum	<0.4	<0.6	<1.80	9	1	2.79	2.71	11.00
Maximum	55.46	50.13	172.47	6154	10	412.00	4.84	565.00

***SEM-standard error of mean**
SD-standard deviation
LN-lactation number/parity
M/yield-milk yield
DIM-days in milk

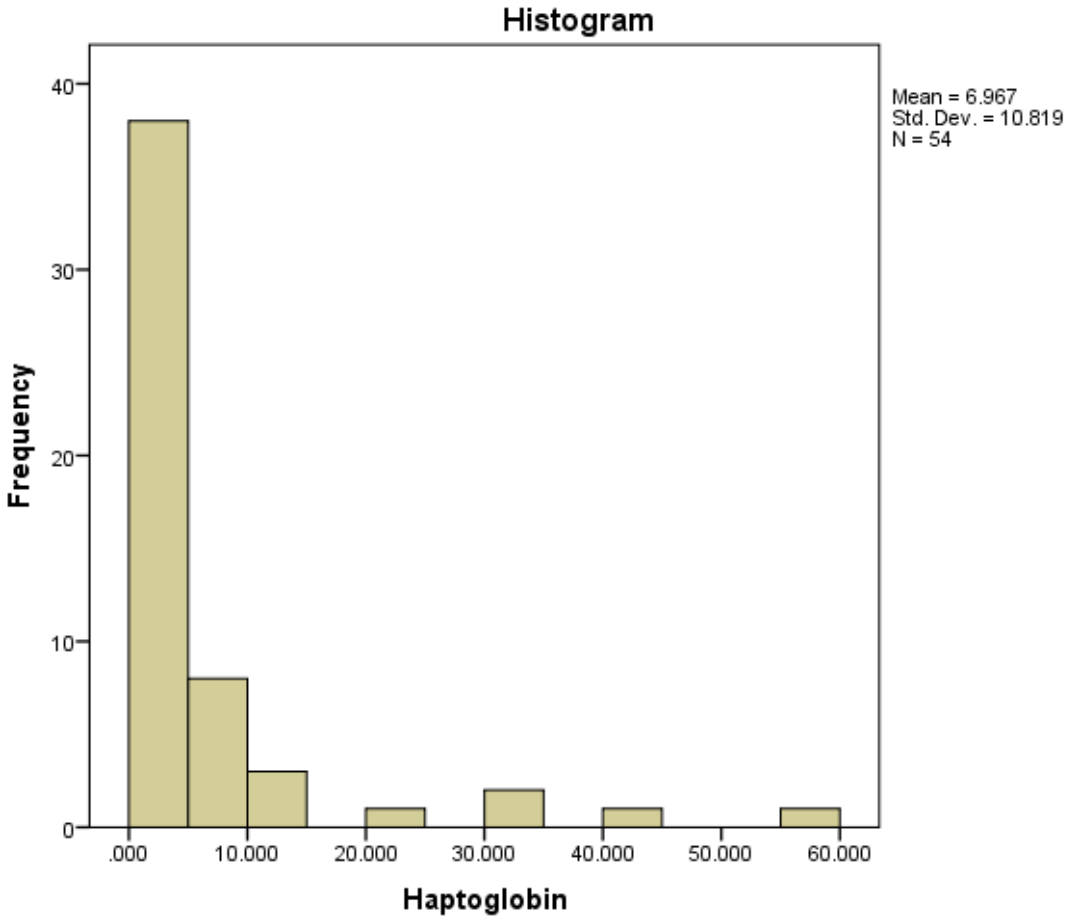

Figure 2-12: Frequency distribution chart of Haptoglobin in composite milk samples on Cochno dairy farm (n=54).

Data were not normally distributed. Therefore non-parametric tests were used.

There were significant correlations between Hp concentration and SCC (P<0.01) and Hp and lactation number (P=0.009). SCC and lactation number were also significantly correlated (P<0.05). Significant differences were observed in the Hp concentrations of the SCC categories (high and low; P=0.001). Figure 2-13 shows the box plot of Hp concentration in the high and low SCC categories. Table 2-6 gives the median and range values of Hp concentration in the two SCC categories. There was however no significant difference between the Hp concentrations of the second set of SCC categories of healthy (≤100,000 cells/ml), SM (101,000-200,000 cells) and CM (>200,000 cells/ml). No significant difference was observed between Hp of the different stages of lactation, or SCC and stage of lactation (P>0.05). No correlations were also found between Hp and M-SAA3, Hp and CRP, Hp and percentage fat, and Hp and percentage protein (Table 2-13).

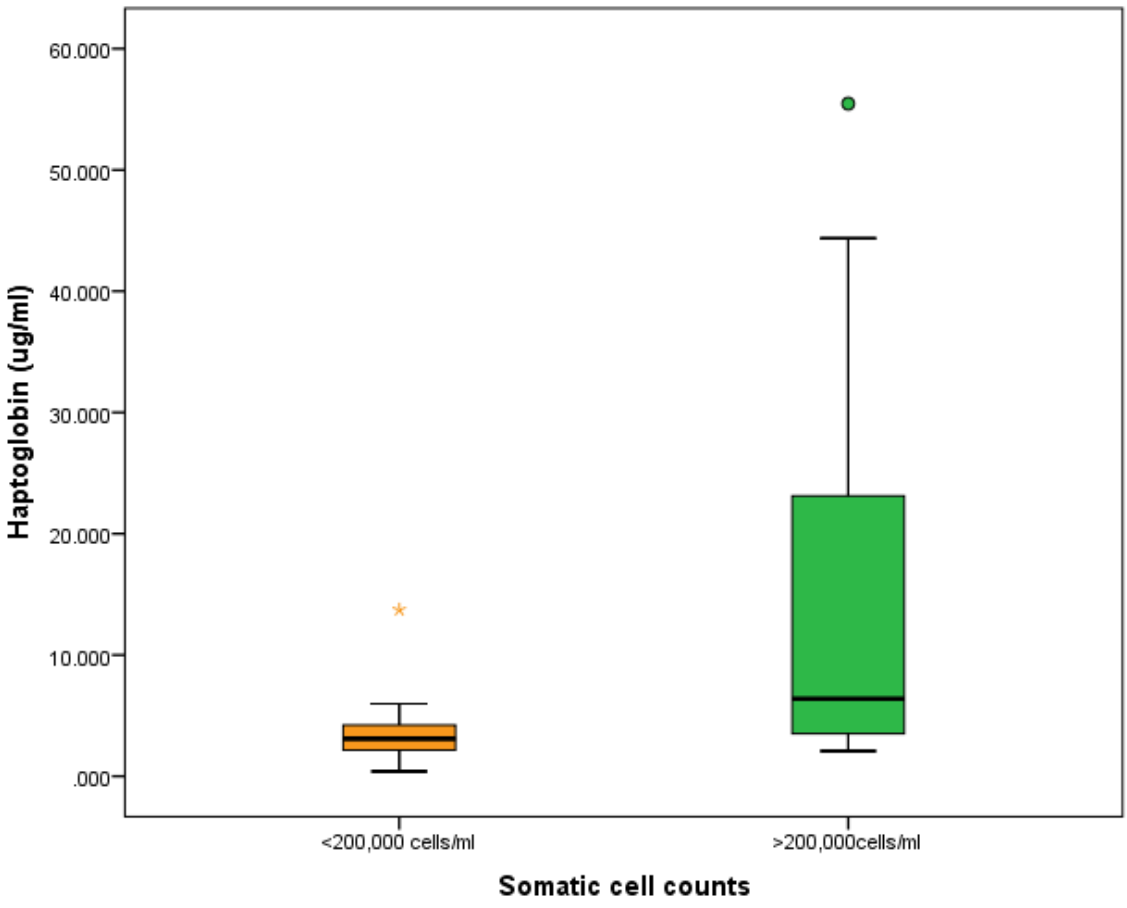

Figure 2-13: Box plot showing two categories of SCC and the Hp concentrations (bars) of composite milk samples

* indicate extreme values, ° indicates outlier subject

Table 2-6: Showing values for Hp in two different categories of SCC, high (>200,000cells/ml) and low (≤200,000 cells/ml)

SCC	Haptoglobin (µg/ml)				
(cells/ml)	Mean	Median	Minimum	Maximum	N
Low (≤200 x 10^3 cells/ml)	3.60	3.08	<0.40	13.74	37
High (>200 x 10^3 cells/ml)	15.04	6.40	<0.40	55.46	17

In order to determine reference values for Hp in composite milk samples from healthy, subclinical mastitis cows and clinical mastitis, SCC was further categorized into healthy (<100,000 cells/ml), subclinical mastitis (101,000-200,000 cells/ml) and clinical mastitis (<200,000 cells/ml) based on another suggested SCC cut off values by Berglund *et al*. (2007) and Madouasse *et al*. (2010), and the median and range values of Hp in these categories are shown in Table 2-7.

Following the use of the Stata® package (version SE/12.1, StataCorp, USA), the area under curve (AUC) of receiver operating characteristic (ROC) for Hp optimal cut off was determined to be 7.92 µg/ml (AUC=0.78, P=0.001) with a specificity of 94.59 % and a sensitivity of 52.94 % based on high and low SCC categories (>200,000 and <200,000 cells/ml).

To obtain reference values based on SCC >100,000 cells/ml, an AUC of 0.75 was obtained for Hp with cut off between 3.33 to 3.52 µg/ml (sensitivity of 74 % and specificity of 69.44 %).

Table 2-7: Showing median and range of haptoglobin in healthy, SM and CM range of SCC in composite milk

SCC	Haptoglobin (µg/ml)		
Category	N	Median	Range
Healthy (SCC<100 x 10^3 cells/ml)	29	2.96	<0.4-13.74
Subclinical mastitis (SCC=101 x 10^3-200 x 10^3 cells/ml)	8	4.02	<0.4-5.28
Clinical mastitis (SCC>200 X 10^3 cells/ml)	17	6.40	2.08-55.46

2.4.2 Mammary associated serum amyloid A3

2.4.2.1 M-SAA3 Assay validation

Sensitivity of the SAA ELISA determined from the LOD (calculated from mean of four blanks plus 2 standard deviations) was 0.012 µg/ml and to allow for a minimum dilution of 1:50, the LOD is 0.6 µg/ml. The calculated intra assay precision (mean CV of 40 samples assayed in duplicate) was 7 %. Inter assay precision (mean CV of 2 QC samples in 5 different assays) was 33 %, values of each QC sample per assay, used to calculate the inter assay precision (CV) as 33 % (Appendix Chapter 2).

2.4.2.2 Effect of different samples treatment on milk M-SAA3

For heat treatment effect, twenty pairs of samples were assayed; however, the results of one pair were excluded from further analysis because the difference between the two Hp values was more than 100 % indicating experimental error. There was a significant difference observed in the median M-SAA3 of heated milk samples and unheated milk samples (Wilcoxon signed rank test of related samples, P= 0.000), but the two groups of samples (heated and unheated) were significantly correlated (r=0.87). M-SAA3 concentrations in heated samples were approximately 36 % lower than those recorded from the unheated samples. Table 2-8 displays the ranks for statistical comparison of M-SAA3 in heated and unheated samples using the Wilcoxon signed ranks test; N-samples with negative ranks represent samples in which unheated samples had a higher M-SAA3 value than in heated pair while positive ranked samples indicate the number of samples with M-SAA3 lower in unheated than in heated. Figure 2-14 displays the scatter plot of M-SAA3 in the heated versus unheated samples and shows the equation and R^2 of the curve.

Table 2-8: Wilcoxon Signed Ranks Test showing ranks of heated versus unheated milk M-SAA3

Negative ranks indicate samples in which unheated samples had higher M-SAA3 than in the heated pair, while positive ranks are samples in which the unheated samples had less M-SAA3 than in their heated pairs.

Ranks		N	Mean Rank	Sum of Ranks
Unheated - heated M-SAA3	Negative Ranks	2[a]	4.50	9.00
	Positive Ranks	18[b]	11.17	201.00
	Ties	0[c]		
	Total	20		

a. Unheated M-SAA3 < heated M-SAA3
b. unheated M-SAA3 > Heated M-SAA3
c. Unheated M-SAA3 = heated M-SAA3

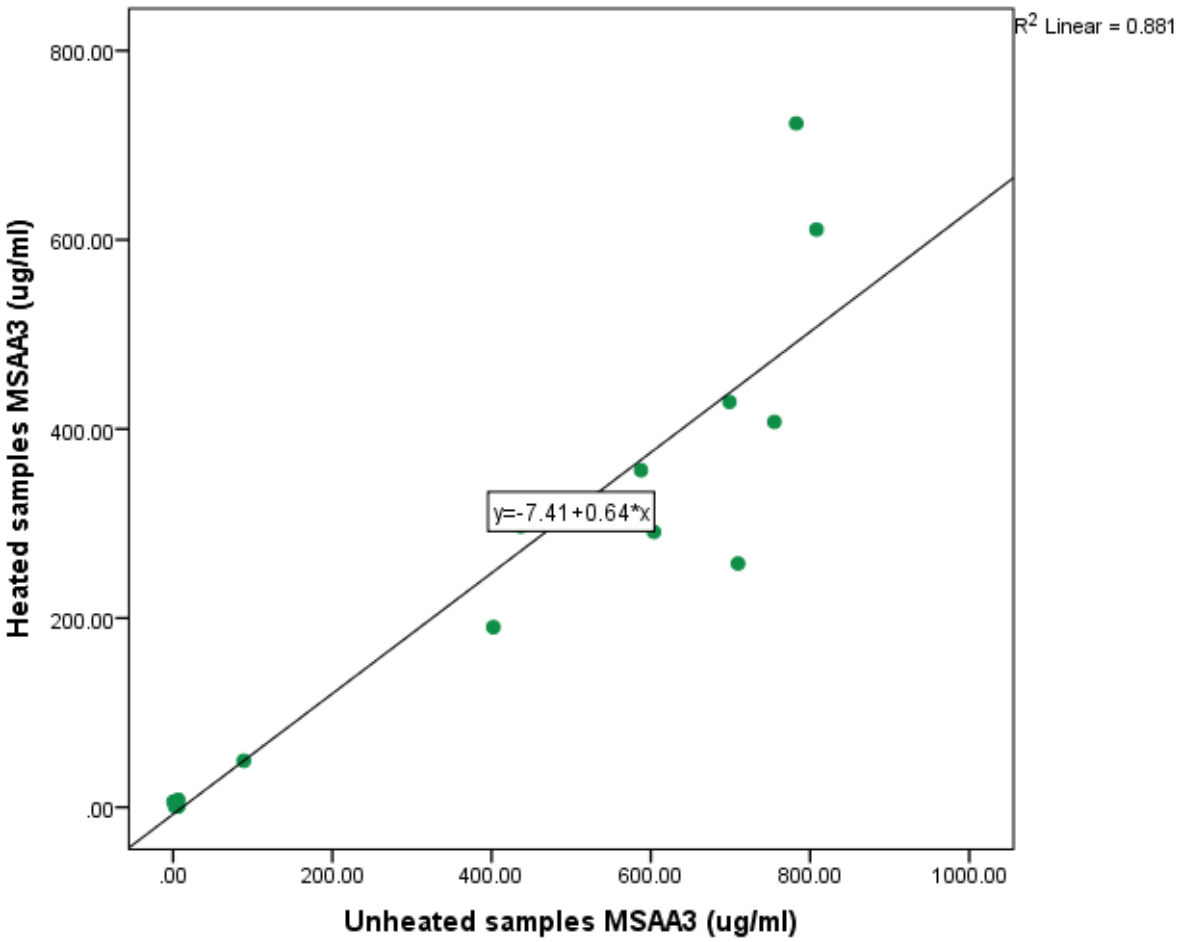

Figure 2-14: Scatter plot of heated versus unheated milk sample M-SAA3 concentration.

There was no significant difference in M-SAA3 between the samples preserved with potassium dichromate (Lactabs® Marks III) and those not preserved (P=0.75,

correlation coefficient (r) = 0.97). A scatter plot comparing M-SAA3 in potassium dichromate preserved and unpreserved samples is shown in Figure 2-15.

No differences in M-SAA3 distribution were seen between samples frozen for 7 or 21 days and the non-frozen samples (supplementary data).

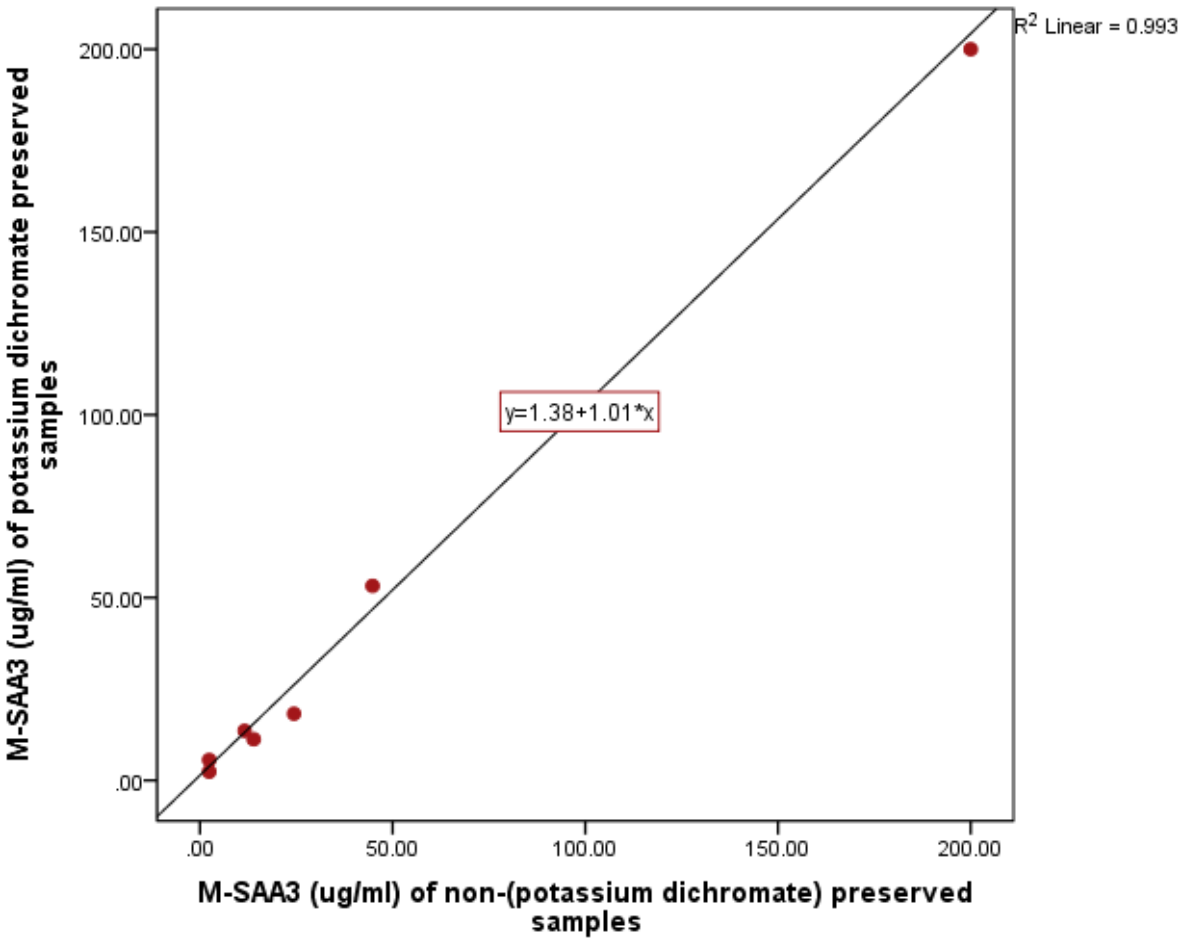

Figure 2-15: Scatter plot of M-SAA3 in potassium dichromate preserved and unpreserved milk samples.
N=8

2.4.2.3 Cochno composite milk M-SAA3

The frequency distribution of M-SAA3 concentration in Cochno composite milk samples from 54 cows is shown in Figure 2-16. Values were skewed to the right with a range of <0.6 -50.13 µg/ml and a median of 1.17 µg/ml. There was no significant difference observed between the M-SAA3 of the high and low SCC categories (categories defined in Section 2.3.5.2) (P=0.174). There was also no

significant difference in M-SAA3 concentrations between the healthy, SM and CM categories of SCC values (categories defined in Section 2.3.5.2) of composite milk (P=0.166). Furthermore no correlation existed between M-SAA3 and Hp or M-SAA3 and any of the other composite milk or cow factors considered. The descriptive values of M-SAA3 concentration in the high and low SCC categories are shown in Table 2-9.

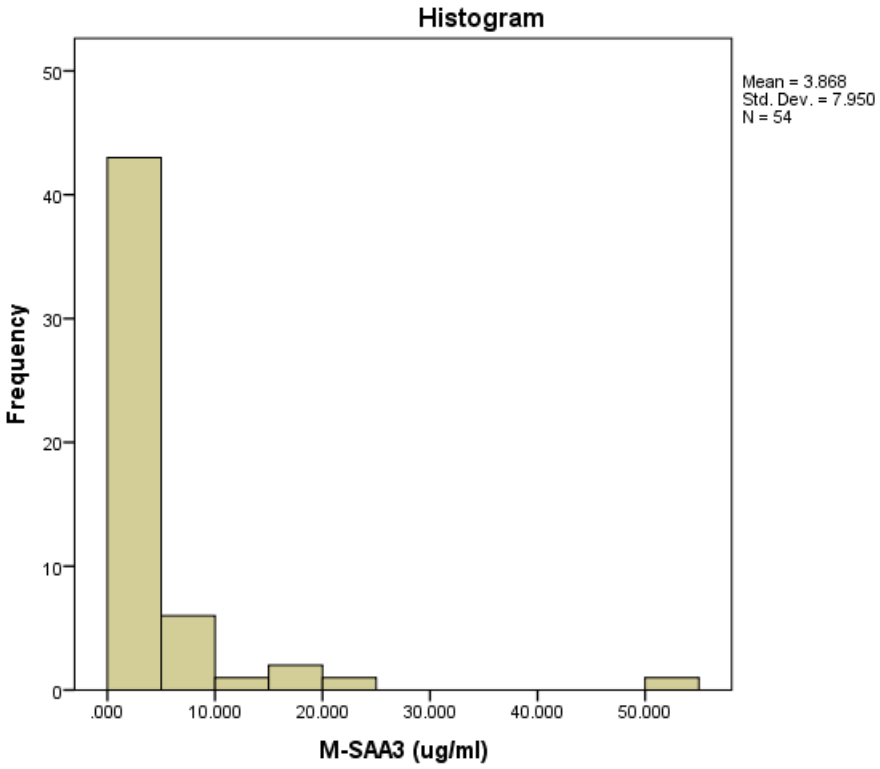

Figure 2-16: Frequency distribution histogram of M-SAA3 in Cochno Dairy composite milk samples (n=54).

Table 2-9: Descriptive values of M-SAA3 for two different categories of SCC.
High (>200,000 cells/ml) and low (≤200,000 cells/ml).

SCC	M-SAA3 (µg/ml)				
(cells/ml)	Mean	Median	Minimum	Maximum	N
Low (≤200X10^3 cells/ml)	3.85	0.96	<0.6	50.13	37
High (>200X10^3 cells/ml)	3.90	1.42	<0.6	24.81	17

The AUC of the ROC for M-SAA3 SCC level (>200,000 cells/ml) was not significant (AUC=0.57, P= 0.180), therefore cut off values could not be obtained. Further categorisation of milk into healthy, SM and CM samples based on SCC levels (defined in Section 2.3.5.2), did not result into any significant difference in the M-SAA3 across the categories, the median and range values of M-SAA3 in these categories are shown in Table 2-10.

Table 2-10: Median and range of M-SAA3 in healthy, SM and CM range of SCC milk.

	M-SAA3 (µg/ml)	
Somatic Cell Counts	Median	Range
Healthy (<100 x 10^3 cells/ml) n=29	0.60	0.60-50.13
SM (101 x10^3-200 x 10^3 cells/ml) n=8	0.60	0.60
CM (>200 x 10^3 cells/ml) n=17	0.60	0.60-24.81

2.4.3 C-reactive protein

2.4.3.1 Assay validation

The limit of detection of the assay was calculated to be 0.18 ng/ml (mean +3 SD of 4 blank samples), and allowing for a minimum dilution of 1:5, the lowest detectable CRP concentration in milk (different from zero) was 1.8 ng/ml. Intra assay precision (mean CV of 30 samples ran in duplicates on a single ELISA plate) was 4 % while the inter-assay precision calculated from 5 repeats of two QC samples (high and low CRP concentration) was 7 %.

2.4.3.2 Cochno composite CRP

The range of concentration of CRP in composite milk from the dairy farm was <1.8 to 172.47 ng/ml with a median value of 24.56 ng/ml (Histogram showing the frequency distribution of CRP in Cochno composite milk is shown in Figure 2-17). A compilation of some descriptive statistics of these samples, categorized based on SCC categories described in Section 2.3.5.2, are shown in Tables 2-11

and 2-12. There were no differences observed in the median CRP for the various SCC categories.

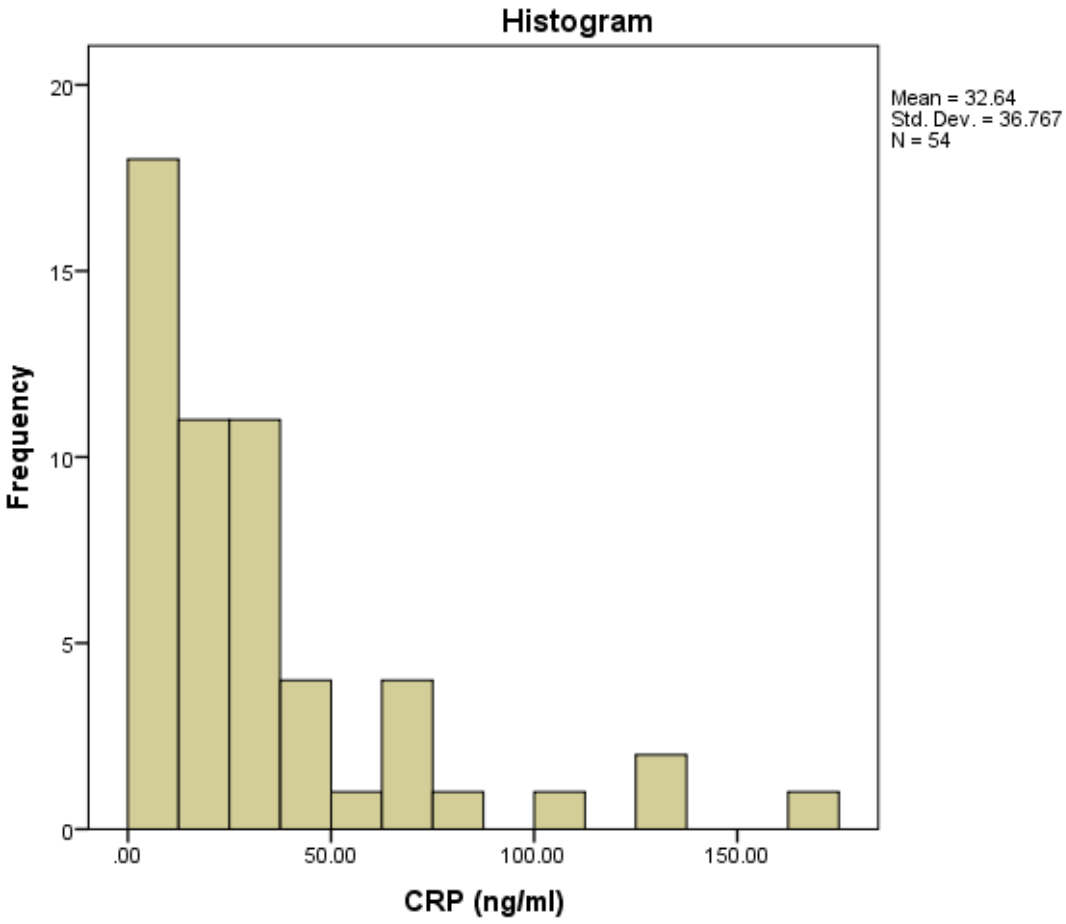

Figure 2-17: Frequency distribution of CRP in Cochno dairy composite milk samples (n=54).

Table 2-11: Descriptive values of CRP for two different categories of SCC.
High (>200,000cells/ml) and low (≤200,000 cells/ml)

CRP (ng/ml)					
SCC	Mean	Median	Minimum	Maximum	N
Low (≤200X10^3 cells/ml)	27.68	22.40	<1.80	136.73	37
High (>200X10^3 cells/ml)	43.42	27.12	6.44	172.46	17

Concentrations of CRP across the high (>200, 000 cells/ml) and low (<200, 000 cells/ml) SCC categories and across the healthy, SM and CM SCC categories were

not statistically different (P=0.133 and 0.272 respectively). The AUC of the ROC for CRP for the different SCC levels was not significant (AUC=0.63, P= 0.078).

Table 2-12: Median and range of CRP in healthy, SM and CM range of SCC milk.

	CRP (ng/ml)	
Somatic Cell Counts	Median	Range
Healthy (<100 x10^3 cells/ml) n=29	22.40	<1.8 - 136.73
SM (101 x 10^3-200 x 10^3 cells/ml) n=8	30.63	<1.8-108.84
CM (>200 x 10^3 cells/ml) n=17	27.11	6.44-172.46

SM-subclinical mastitis, CM-clinical mastitis, SCC-somatic cell counts.

There was no correlation between CRP and Hp or between CRP and M-SAA3. Neither was there any correlation between CRP and SCC, nor CRP and parity. However significant correlations were found between CRP and percentage fat and protein contents of the milk samples. Table 2-13 shows the results of tests for correlation of the dependent variables (APP, SCC and lactation number), with the significant correlations highlighted in light blue.

Table 2-13: Tests for correlation between APP and other variables.
Variables with significant correlation highlighted in light blue.

			M-SAA3	CRP	Hp	SCC	Lactation number	% fat	% protein
Spearman's rho Correlations	SAA	Correlation Coefficient	1.000	.228	.062	.244	.205	.054	.089
		Sig. (2-tailed)	.	.098	.658	.075	.137	.698	.523
	CRP	Correlation Coefficient	.228	1.000	.178	.247	.123	.414**	.422**
		Sig. (2-tailed)	.098	.	.198	.072	.377	.002	.001
	Hp	Correlation Coefficient	.062	.178	1.000	.456**	.352**	.164	.186
		Sig. (2-tailed)	.658	.198	.	.001	.009	.236	.178
	SCC	Correlation Coefficient	.244	.247	.456**	1.000	.335*	-.021	.090
		Sig. (2-tailed)	.075	.072	.001	.	.013	.883	.517
	Lactation number	Correlation Coefficient	.205	.123	.352**	.335*	1.000	-.046	-.038
		Sig. (2-tailed)	.137	.377	.009	.013	.	.741	.786
	% fat	Correlation Coefficient	.054	.414**	.164	-.021	-.046	1.000	.512**
		Sig. (2-tailed)	.698	.002	.236	.883	.741	.	.000
	% protein	Correlation Coefficient	.089	.422**	.186	.090	-.038	.512**	1.000
		Sig. (2-tailed)	.523	.001	.178	.517	.786	.000	.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

2.5 Discussion

2.5.1 Haptoglobin

2.5.1.1 Milk haptoglobin ELISA

A reliable and specific assay was developed and validated and shown to be sufficiently accurate and reproducible to measure the concentration of Hp in milk from cows in a dairy farm.

Using the FLUOstar Optima ELISA reader, deviations were observed in readings of between replicates of sample and standard (duplicate or triplicate) leading to a higher inter and intra assay precision (CVs). This was suspected to be an instrument based error; hence an alternative ELISA reader was utilized in order to check/correct for this variation. The Triturus® (ver. 4.01 by Diagnostic Grifols, Spain) was utilized 10 ELISA plates with varying concentration of milk samples and standards. It was observed that with readings by the Triturus®, intra assay CVs (between sample duplicates or triplicates) fell within a range of 0-5 % and only rarely (very few samples) went above 5 % and such samples were repeated and subsequently gave a lower CV. When the same ELISA plates were read on the 2 different ELISA readers (FLUOstar Optima and Triturus®) within 10 min of each reading, it was also observed that considerably higher variations in the replicates existed in the absorbance readings of the FLUOstar Optima (>5 %) compared to the Triturus® reader (<5 %). After this observation, it was concluded that the Triturus® reader was more reliable for reading the absorbances of the Hp ELISA plate and was used for all remaining ELISA Hp absorbance readings. However the data generated in the ELISA plates read by the FLUOstar Optima were included in the study (for examples analysis of quarter milk samples from Cochno (149) because intra-assay CVs calculated was below 20 % and considered sufficiently low for an immunoassay.

The ELISA is a direct assay of milk Hp requiring little sample preparation. Hiss *et al*. (2004) in their study demonstrated an ELISA that was developed to measure milk Hp from healthy quarters (basal levels) with sensitivity (limit of detection) of 0.07 µg/ml. The ELISA developed in the present study had a sensitivity of 0.002 µg/ml in the diluted samples and was able to determine milk Hp concentrations in many milk samples from healthy quarters (low SCC). The

present assay utilizes bovine specific anti-Hp antibodies conjugated to the enzyme alkaline phosphatase (AP), a previously reported enzyme-based bovine milk Hp ELISA (Hiss *et al.*, 2004) and commercial ELISAs (Life Diagnostics *Inc.*, West Chester, USA; Tridelta Development Limited, Kildare, Ireland) have utilized the horse radish peroxidase enzyme system or Hb-Hp peroxidase activity for substrate detection. Yang *et al.* (2011) in their study also used an AP enzyme system, from their results and results of the present study it can be concluded that the alkaline phosphatase system is an equally suitable enzyme-antibody conjugation system offering optimum sensitivity and specificity in milk Hp ELISA.

ELISAs are generally known to be cost effective and easy to perform, and sandwich ELISAs have the added advantage of being more specific and sensitive than other immunoassay formats such as SRID. However, ELISAs are time consuming and difficult to adapt to an on-farm format. A biosensor assay that utilizes the Hp binding to haemoglobin principle has been described by Åkerstedt *et al.* (2006). Although a rapid method of testing for milk Hp, it also requires technical equipment, namely an optical biosensor.

More recently an immunosensor assay for detection of Hp milk was also described by Tan *et al.* (2012). The assay was reproducible and has the potential for on farm measurement of milk Hp for detection of mastitis. The detection of Hp was coupled to generation of an electrical response following the reaction of milk Hp with anti Hp antibodies mounted on an electrode, and though a sensitivity of 0.63 mg/l was reported, a more sensitive assay would be desirable to distinguish subclinical from healthy milk samples.

Given the sensitivity of the ELISA in the present study it is anticipated that a lateral flow immunoassay format of the assay would be equally as sensitive.

2.5.1.2 Effect of different Sample treatments on milk haptoglobin

The significance of the findings that use of two common milk preservatives did not affect milk Hp in this study is that these sample preservation methods can be used in milk without confounding the values obtained for milk Hp assay. Therefore samples being collected for SCC or for progesterone analysis can also be used for determination of Hp concentration.

However in this study, an extended time lapse (post preservation) was not employed and may become an important factor that could affect milk composition preserved using such chemicals as observed by Chalermsan *et al*. (2004). Therefore, it is recommended that samples needing to be transported over long distances without any means of cold-chain storage, can be preserved using either potassium dichromate (Lactabs® Mark III) or Bronopol (Broad spectrum Microtabs® II), but should be analysed as soon as possible. It would be interesting to examine the effect of prolonged storage of milk with preservatives on milk APP for example Bronopol is said to be able to preserve milk samples at RT for up to 14 days. Since storage at different temperatures and for different durations did not affect the Hp concentration, this would allow for posting with an ice pack from farms to laboratories and will keep Hp without loss.

Heating caused a significant decreased in Hp concentration of milk; this is probably as a result of a denaturation effect of heat on the protein moiety (Hausen *et al.*, 2012) and/or structure of Hp that could probably affect its antibody binding ability. This finding should therefore be taken into consideration when assaying for Hp in samples which have undergone heating as a result of international transfer regulations involving heat treatment of biological samples. In such studies, control samples should be treated in the same manner as experimental samples or a correction factor, for example 26 % reduction in the mean Hp concentration, based on the findngs from this study can be applied.

2.5.1.3 Haptoglobin profile of Cochno dairy milk Samples

Haptoglobin from individual quarter milk samples of cows in the herd were not normally distributed, but skewed to the right with a higher percentage of samples falling within the low Hp category. Many factors such as lactation stage, stressing conditions, presence or absence of mammary infections/inflammation; stage and severity of such infections, pathogenic factors related to mammary infections, and other physiological factors may contribute to differing Hp levels in milk.

Somatic cell count values for each quarter were not obtained as only composite sample SCC were possible, therefore it was difficult to relate the quarter Hp values to SCC (composite), bearing in mind the dilution effect that often results

from the combination of milk from uninfected quarters (with low Hp concentrations), on high Hp containing milk quarters. Hp range in quarter milk (<0.4-420 μg/ml) was higher than composite milk (0.4-55 μg/ml) presumably also due to this dilution effect (Reyher and Dohoo, 2011).

At the time of sampling, all quarters were considered healthy with no signs of inflammation (the cows were under veterinary and handler observations and considered apparently healthy enough for commercial milk production) but from the SCC of the composite sample, it can be inferred that subclinical mastitis existed in the herd as some composite milk samples showed levels far above 200,000 cells/ml, the suggested cut off for discriminating healthy milk samples from mastitis samples (Pantoja *et al.*, 2009; Dufour and Dohoo, 2012). The SCC cut off points for SCC to determine mastitis has been a subject of debate, with other SCC cut-off values being advocated to distinguish healthy, subclinical and clinical mastitis samples (Schwarz *et al.*, 2011; Berglund *et al.*, 2007). In this study, no bacteriological tests were carried out to confirm SM, therefore SCC was used as the sole indicator of an IMI, and 2 different cut off ranges for SCC were assessed in order to cover discrepancies that may result from use of different SCC cut off values.

The percentage of composite samples with SCC below 200,000 cells/ml out of the total number of sampled cows (n=54) was 69 % indicating a prevalence rate of 31 % of subclinical mastitis on the herd. There was a significant correlation between SCC and Hp concentration in the composite samples; which agrees with studies by Thielen *et al.* (2005) and Lai *et al.* (2009) who demonstrated that Hp in milk could originate from neutrophils (major component of SCC) and mammary epithelial cells during mastitis. Therefore it is expected that as SCC increases, Hp in milk would also increase, although the possibility of direct Hp leakage from serum into milk exists. The correlation of Hp with SCC and mastitis, as is our observation in this study agrees with a number of other studies, which highlights the promise of Hp in diagnosis of IMIs (Eckersall *et al.*, 2006; Hiss *et al.*, 2004; Hiss *et al.*, 2007; Pyörälä *et al.*, 2011; Medvid *et al.*, 2011 and Kalmus *et al.*, 2013 amongst others).

Significant correlations were also observed between parity (lactation number) and milk Hp, with multiparous cows tending to have higher Hp values. SCC values

in healthy quarters have been suggested to be higher in multiparous cows which may have been exposed to previous episodes of mastitis compared to primiparous or first lactation cows (NMC, 2001) presumably due to carryover effect. The observation of correlation between parity and Hp as well as parity and SCC in this study may be a reflection of this effect. In a study by Kalmus *et al*. (2013), that compared APP profile, in samples from different pathogen-caused mastitis, with other cow factors, no association was observed between APP profile and parity of cows. Given that a larger sample size was used in that study (n=253) our observations in the current study may have been influenced by the smaller sample size (n=54).

2.5.2 Mammary associated serum amyloid A3

2.5.2.1 Effect of different sample treatment on milk M-SAA3

Heat treatment

Treatment with heat at 56ºC for 30 min was found to have a significant reducing effect on the apparent M-SAA3 concentration in milk samples. This may be due to the denaturing activity of high temperature on protein structure (Hausen *et al.*, 2012). Values of Hp in the heated and unheated samples were however significantly correlated.

The significance of this finding, similar to that of Hp, applies for diagnostic or research milk samples that need to be transported across international borders and require mandatory heat treatment for inactivation of pathogens that maybe contained within such biological samples, a prerequisite specified in the regulation concerning such samples' transfer for example the; European Communities Act (1972). It is therefore imperative that the effect of heat treatment be taken into account and corrected for using a factor of 36 % as observed in this study or by treating controls the same way as experimental samples, in M-SAA3 analysis of heat treated samples.

Preservative treatment

Preservation with Lactabs® Marks III did not affect milk M-SAA3, therefore it can be concluded that samples preserved with this reagent can be reliably assayed

for M-SAA3. Effect of Bronopol on milk M-SAA3 have not been performed in this study and would require experimental verification.

Duration of storage

Contrary to the findings of Tóthová *et al*. (2012), no differences were observed in the present study between samples analysed on collection day and those stored at -20°C for either 7 days or 21 days. Reasons for this discrepancy is not known, but could likely be due to the freeze-thawing cycles, which the samples stored at -18°C had to undergo at 2, 7, 14 and 21 days after storage in the Tóthová *et al*. (2012) study. In the present study, separate aliquots were stored for 7 days, and 21 days, thus re-freezing samples to analyse at a later date was not required, and this may have contributed to retaining the levels of M-SAA3 concentration of milk in the separate sets of aliquots. Generally, however, the sample size of milk samples (n=6) used in both studies can be considered small and may not give a very accurate picture of response of M-SAA3 to duration of freezing storage.

2.5.2.2 M-SAA3 in Cochno composite milk

Åkerstedt *et al*. (2007) reported the profile of M-SAA3 (as well as Hp) in quarter, composite and bulk tank milk samples and demonstrated the correlation of quarter, composite and bulk tank M-SAA3 to SCC. In the present study, we found no significant correlation between the composite milk M-SAA3 and the SCC. This observation may be due to the sample size (n=54) as against n=165 used in the Åkerstedt *et al*. (2007)'s study and may also be due to the fact that SAA was divided into two broad categories (detectable and undetectable) in that study. These observations could also suggest that SCC assay may not be consistent between laboratories even if the same commercial ELISA was used in both locations.

There was no correlation observed between the Hp and SAA in composite milk samples. This could be due to the fact that the two different APP, Hp and M-SAA3 have been shown to be produced from different sites in the mammary gland (Molenaar *et al*., 2009; Hiss *et al*., 2004; Thielen *et al*., 2007). Differences in the site of production and portal of release of these APP into milk have the potential of influencing APP concentration in milk at any specific time point.

Furthermore, depending on which mastitis-causing- bacteria is responsible for an IMI, levels of secretion into milk of the different APP may vary as different virulence factors can stimulate different cytokine responses, and also cause tissue injury to the mammary gland that is different in natures and extent.

The range of M-SAA3 values observed for these composite milk sample can be considered as low when compared to values obtained from individual mastitis affected quarters in the challenge study and even values recorded in colostrum (day 1 post-calving). This may be due to dilution effects.

2.5.3 CRP in Cochno composite milk

From this study, a range of 1.8 (lower limit of detection of assay) to 172 ng/ml was shown to exist in the composite milk samples from the dairy farm. This confirms the presence of detectable levels of CRP in bovine milk as reported by (Schrodl *et al.*, 1995; Lee *et al.*, 2003). In the herd examined, there was no reported case of clinical mastitis as at sampling time, therefore the maximum value seen in this study may only be reflective of the CRP values during SM. Although, milk samples with SCC of above 200,000 cells/ml were categorized as 'clinical mastitis', no clinical signs of mastitis was seen in the milk or cows, but classification was just for the purpose of identifying cut off range as suggested by Berglund *et al*. (2007) and Madouasse *et al*. (2010).

In this study, a minimum dilution of 1:5 was used for assaying samples of low CRP concentration. Milk CRP showed no correlations with SCC, Hp or with M-SAA3, but significant correlations with fat and protein content of milk. The reason for is correlation with fat and protein is not known and could be a subject of future investigation. Non-significant correlations seen between the three APP may be due to the individual dynamics of each of the APP. Most samples had low levels of CRP (< 50 ng/ml), which may reflect the general low mastitis incidence on the farm, if CRP can be considered a reliable indicator of IMI. However the lack of correlation between the APP may increase the value of assays for the entire APP as they may reflect differing stages of mastitis or differences in the pathogens causing the disease.

In an early study by Hamann *et al*. (1997), milk CRP compared to the SCC and health status in 47 cows over time, showed a negative correlation between SCC

and CRP (r=0.32) and a threshold value of 123 ng/ml of CRP in normal milk. These findings are similar to the results of the present study. Further classification of samples based on CRP concentration into different health status (healthy, mastitis or subclinical infection) in the Hamann *et al*. (1997) study using an SCC cut off of >100,000 cells/ml produced a poor sensitivity (55 %), as also observed in this study. Therefore it can be inferred that the secretion of CRP into milk does not follow similar dynamics with SCC or Hp, and could suggest local production of CRP in the mammary gland just as M-SAA3, under the influence of explicit signalling pathway(s). This should be subject of future evaluation.

It is probable that the observed correlation between CRP and fat content of milk samples may be due to the binding afintiy of CRP to phosphocholine which forms a major component of the lipid constituent of membranes. It is not however clear why a significant correlation existed between CRP % protein of samples.

No clear conclusions can be drawn from the observed profile of CRP from this study as sample size was small, but there appears to be some prospects for its use as a marker of mammary inflammation. Evaluation of a larger set of samples would be of additional value to give better understanding of the worth of CRP and its association with other common mastitis defining parameters.

2.6 Conclusion

2.6.1 Haptoglobin

A robust and reproducible ELISA for measuring milk Haptoglobin was developed. Studies have demonstrated that Hp is a reliable marker for bovine mastitis through good correlations between milk Hp and SCC. Parity of cows was seen as a possible confounding factor in an on farm diagnosis of mastitis using Hp. High temperature was recognized to have a significant reducing effect on milk Hp.

2.6.2 Mammary associated serum amyloid A3

This study has shown the significant effect that high temperature has on the concentration of M-SAA3 in milk samples; in addition the profile of M-SAA3 in composite milk from a commercial dairy herd was demonstrated.

2.6.3 C-Reactive protein

C-reactive protein has shown some potentials as an acute phase protein in bovine milk, able to differentiate between milk with high SCC and low SCC, further investigations of this potential in actually discriminating mastitis (clinical and subclinical) from non-mastitic, and clinical from subclinical mastitis samples, needs to be carried out.

3 Investigations of acute phase proteins in periparturient milk and in the mammary gland.

3.1 Overview

In this chapter, acute phase proteins and proteomic investigations on milk samples collected within ten days post-partum from calving cows, in a dairy farm are described. Antibody to bovine Hp was used for western blot analysis of these samples. Furthermore, studies with the same antibody in immunohistochemical studies of Hp in healthy versus mastitis bovine mammary glands are reported.

3.2 Introduction

3.2.1 Post-calving milk APP

The periparturient period is one of the most critical periods, health wise, in the productive life of a dairy cow characterized by an increased susceptibility to diseases (Trevisi *et al.*, 2010). This has been attributed to negative energy balance (NEB) and the associated immune suppression at the puerperal period (Waldron and Revelo, 2008; Hiss *et al.*, 2009). Related to this periparturient immune suppression is the occurrence in serum of increased levels of metabolic and endocrine markers such as prostaglandins (Yuan *et al.*, 2014), cortisol, ketone bodies (for example α-butyric acid) and non-esterified fatty acids (NEFA). Hypoglycaemia and hypocalcaemia have also been defined as markers of metabolic stress during this period in dairy cows (Esposito *et al.*, 2013; Waldron and Revelo, 2008). Some studies have also shown inflammatory markers such as the acute phase proteins, Hp and SAA to be increased in serum during the periparturient period (Trevisi *et al.*, 2012). This has been attributed to diseases of inflammatory aetiology characteristic of the period.

In addition, Morimatsu *et al.* (1991b) demonstrated an increase in bovine serum CRP in association with onset of lactation in Holstein cows and Schrodl *et al.* (1995) also showed the presence in bovine colostrum and milk of the APP CRP, and this was suggested to be passively transferred to colostrum fed calves (Schroedl *et al.*, 2003). In humans CRP has been shown to be increased in serum

at the post-partum period and this has been attributed to the trauma associated with childbirth, with concentration dropping back to baseline values by the 5th day post-partum (Fetherston *et al.*, 2006).

It is well established that colostrum and milk in the immediate post-partum period contains a large repertoire of immunological proteins including predominantly IgG and other Igs. Recently, the APP, M-SAA3 and AGP have been observed to be high in milk during the immediate post-partum period (McDonald *et al.*, 2001 and Ceciliani *et al.*, 2005) and this has been suggested to be due to physiological roles of these proteins in conferring immunity to the new-born. Hiss *et al.* (2009) also showed from their study that Hp is high in milk of metabolically stressed transition dairy cows. In that study weekly milk samples were used to determine the profile of Hp in the periparturient cow milk for up to 12 weeks post-partum.

Mastitis is one among many conditions in which new infections frequently arise during the periparturient period more than during any other period of a diary cow's life (Waldron and Revelo, 2008). With APP in milk gaining prominence as markers for mastitis diagnosis in dairy cows, it would be important to evaluate their usefulness in recognising new mastitis developing in the periparturient period. Due to the fact that there is the general physiological increase in inflammatory markers in the circulation during this period, and also the fact that post parturient milk (colostrum especially), contains high immune protein concentration, there is the challenge of readily diagnosing new infections in the mammary gland with regards to differentiating the physiological from the pathological increases of marker proteins. Obtaining reference values that can differentiate the physiological increases from pathological ones, during the periparturient period would be of immense value in readily identifying new mastitis conditions developing in post-partum cows in order to promptly institute treatment.

3.2.2 Immunohistochemical localization of Hp in the bovine mammary gland

Immunohistochemistry (IHC) is a method applied for the localisation of antigens of interest in body tissues based on the principle of the reaction of such antigens with their specific antibodies. Enzymes are usually conjugated to a primary or secondary antibody for chromogenic detection after substrate reaction or by fluorescent detection when a fluorophore is conjugated to the antibodies, under microscopy (Brandtzaeg, 1998).

The bovine mammary gland becomes fully developed during gestation, when mammary epithelial cells grow extensively into a network of ductules that terminate as alveoli (Sobolewska *et al*., 2011). The mammary gland alveolus is the basic functional unit of the gland; milk is produced by the epithelial cells, passes into the lumen of the alveolus from where it is propelled by contractile activity of the myoepithelial cells into collecting ducts of the gland (Alkafafy *et al*., 2012). The gland cells typically undergo periods of proliferation, differentiation and regression, corresponding to the period of preparation for lactation (pregnancy), onset of lactation and cessation of lactation (dry period) of a cow, all tightly regulated by growth factors and hormones.

The IHC technique has been used in a number of studies involving the bovine mammary gland; localization of protothecal mastitis (Corbellini *et al*., 2001), localization of immune cells and their characteristic distribution during a chronic *S. aureus* mastitis (Leitner *et al*., 2003). Also, localization of growth factors such as activin-like protein (Bloise *et al*., 2010) and in studies of the different stages of cellular maturation of the gland during lactation (Hodgkinson *et al*., 2007), distribution of adhesion molecules in the gland in mid-lactation cows (Simon *et al*., 2007) and even in the general assessment of the structural proteins of the gland (Alkafafy *et al*., 2012).

Immunocytochemical (ICC) localization and differential expression of the APP M-SAA3 was also carried out on bovine mammary gland after an experimentally induced subclinical *S. aureus* mastitis (Eckersall *et al*., 2006). In that study it was observed that M-SAA3 was located most in the secretory mammary epithelial cells (MEC), gland cistern, and, to a lesser extent, teat canal.

Another APP, α-acid glycoprotein (AGP), a lipocalin, was immunohistochemically identified in all areas of a section of normal bovine mammary gland and it has been suggested production occurs in the alveoli (Ceciliani *et al.*, 2007).

Noteworthy is the fact that while IHC deals with localization of proteins in whole tissues, maintaining as much as possible the normal architecture, ICC is distinguished by dealing with cells, although these two terms are often used interchangeably.

The liver has long been established as the major site of serum Hp synthesis in ruminants (Eckersall and Bell, 2010). However, the study of Lavery *et al*. (2003), suggested that Hp is constitutively expressed by the female reproductive organs in the bovine species. Hp found in milk was initially thought to be from extravasation from serum due to compromised blood milk barrier during mastitis (Eckersall *et al*., 2001), however in studies by Hiss *et al*. (2004), the qualitative and quantitative detection of Hp mRNA in mammary gland tissue indicated that milk Hp was probably sourced from the mammary gland (alone or in combination with serum seepage of Hp). It was later confirmed by immunohistochemical analysis of mammary gland sections following experimental mastitis, that Hp was synthesized in portions of the mammary gland (Hiss *et al*., 2005), although in that study, a significant difference between control and infected glands in the IHC staining of Hp, was not found. Further studies by Lai *et al*. (2009), using ICC along with RT-PCR, demonstrated that neutrophils and mammary epithelial cells (MEC) were major sites of Hp synthesis.

3.2.3 Proteomics

Proteomics methodologies and its applications in the search for bovine mastitis biomarkers has been discussed in the introductory chapter of this thesis and furthermore in section 5.2.3.

3.2.4 Objectives of the study

The studies carried out in this chapter had several objectives which includes;

- To determine the profile of Hp, M-SAA3 and CRP immediately following parturition and to investigate the potential of these APP for detecting new IMI during the periparturient period. The APP were measured in daily

milk (from the first to the tenth day post-partum) in calving cows of the Cochno dairy farm (University of Glasgow) using ELISAs. A western immunoblotting procedure specific for milk Hp was also used. All cows calving between January and June, 2013 on the dairy farm were evaluated.

- To determine quantitative and qualitative changes in milk proteins following parturition using one and then two dimensional gel electrophoresis (1DE and 2DE).

- To characterize the source of milk Hp, during mastitis by immunohistochemical localization (IHC) of Hp in the bovine mammary gland.

3.3 Materials and methods

3.3.1 Acute phase proteins in post-calving milk

3.3.1.1 Reagents

General chemicals were obtained from Sigma-Aldrich, Poole, UK, except where otherwise stated. In all experiments, milli *Q* water was used.

3.3.1.2 Samples

Daily quarter milk samples were collected from the first to the tenth day (morning milking, 6:00 am daily) following parturition, from all calving-cows in Cochno dairy herd which calved between January and June, 2013. Approximately 15 ml milk was collected after discarding the first strips of milk following teat disinfection. Three aliquots of ~5 ml each were prepared from each sample and stored at -20°C until analysed.

In total twenty four (24) cows were sampled. However 2 cows' samples were excluded from further analyses because they became lethargic following an acute metritis infection in one cow and an undiagnosed illness in the other, therefore samples could not be collected after the 6th day post-calving. In all, 575 quarter milk samples (functional quarters) obtained over ten days (average of 81 samples daily, from 22 cows) were analysed. All samples were thawed at

RT, thoroughly mixed by vortexing and diluted in the respective assay/wash buffer for Hp or M-SAA3 analysis.

3.3.1.3 Haptoglobin in post-calving milk

ELISA

Haptoglobin was analysed in all quarter samples collected from the post calving cows. The analysis was carried out as described in Section 2.3.2. To obtain daily (composite) values for post calving milk Hp, the mean of Hp concentration from all daily quarters' samples per cow was computed and designated daily composite Hp value of each cow.

Western blotting for Hp in Post-calving milk

An equal aliquot from daily quarter milk samples from 3 cows were pooled to obtain daily composite milk for each of the cows for the 10 days of sampling.

These composite samples were then each subjected to a western blotting analysis for the characterization of Hp variation in daily milk. 10 µl of daily composite sample was diluted to a final concentration of 2 mg/ml µg first in milli Q water and then in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 25 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol Blue) (1:1 with sample). 10 µg of each sample (10 µl) was then loaded into separate wells of precast gels and the electrophoresis ran. After electrophoresis, the gels were carefully removed from cassettes and put onto a blotting sandwich comprising of suitably sized nitrocellulose membrane (NCM) (0.45 µm; Bio-Rad laboratories *Inc.*, Germany), blotting pads and paper, fitted into a gel-holder cassette soaked with transfer buffer (6.06 g Tris-HCl, 28.8 g Glycine in 1600 ml milli *Q* water and 400 ml methanol).This was then placed into the Blotter tank (Bio-Rad Criterion, USA) filled with an adequate volume of transfer buffer and a cooling block. Electro-blotting was carried out at 70 V for 60 min. NCM was removed and immersed in blocking solution (10 % non-fat dry milk) at RT on a rocker overnight.

Membranes were then washed by gentle rocking in wash buffer (10 % Tween-20 in Tris buffered saline (TTBS) for 10 min. Wash was discarded and washing was repeated two more times, before addition of the primary antibody (conjugate of a rabbit anti-bovine conjugated to alkaline phosphate) diluted in 10 % non-fat

dry milk (1:5,000) which was allowed to incubate for 60 min on a rocker at RT. Washing was repeated 3 times, after which membranes were incubated with an alkaline phosphatase chromogenic substrate (PIerce™ NBT/BCIP, Thermo Scientific, UK) solution for 10-15 min to allow for colour development on Hp bands. Western blot images were scanned on Umax powerlook III (Hamrick software, USA). Images were further analysed using ImageJ software (National Institute of Health, Maryland, USA).

3.3.1.4 M-SAA3 in post-calving milk

For analysis of M-SAA3, daily composite milk samples from cows were used. Ten cows were selected from the 22 calving cows, and from each quarter collected daily per cow, a pool of composite milk was made by mixing aliquots of 200 µl of each quarter milk samples together. This was done for each of the cows, for 10 days. Daily composite samples were then assayed for M-SAA3 using ELISA as described in Section 2.3.3.

3.3.1.5 CRP in post-calving milk

For analysis of CRP, daily composite milk samples from ten cows were used. Aliquots (200 µl) of the daily samples (all quarters) were pooled to give a composite for each day per cow. Daily composite samples were then assayed for CRP using ELISA as described in Section 2.3.4.

3.3.1.6 Proteomic analysis of post-calving milk

1DE SDS-PAGE of post-calving milk

As a first step to characterizing the protein changes in milk from the first to tenth day post calving, 1DE electrophoresis of daily (composite) milk was carried out. Prior to gel electrophoresis, protein concentration was determined in the daily composite (pooled daily quarters) milk samples (of 3 cows) using Bradford reagent (Sigma-Aldrich, USA) as described in Appendix Chapter 4. Bovine serum albumin (BSA) was used for the standard. Samples were diluted in milli Q water and then mixed at a ratio of 1:1 with Laemmli sample buffer to which 5 % (v/v) of ß-mercaptoethanol had been added and thoroughly mixed, to achieve a final concentration of 2 mg/ml per sample (Appendix Chapter 4). This mixture was then heated at 95ºC for 4 min. After assembly of the 18-well comb precast gels

into the gel running tank (Bio-Rad, USA), and addition of sample loading buffer (1x Tris /Glycine/SDS buffer, Bio-Rad GmbH Ltd, UK) into the tank, 10 µl of heated samples (~10-25 µg protein/well) or pre-stained protein ladder (10-170 kDa, PageRuler prestained protein ladder, Thermo Scientific *Inc.*, USA) was pipetted into wells of precast gels. Gel electrophoresis was then run at 200 V for 40-45 min.

Gels were removed from the cassette and then stained for 1 hour in colloidal solution of Coomasie brilliant blue stain G-250 dye 0.1 % (w/v), 10 % (v/v) acetic acid, 40 % (v/v) ethanol; (Invitrogen, Manchester, UK) after which stain solution was discarded and de-staining carried out overnight using a solution of 10 % (v/v) acetic acid and 25 % (v/v) methanol. Image of gels were scanned using a UMAX Power Look III scanner and software (Hamrick software, USA).

2DE SDS-PAGE of post-calving milk

One representative day 1 and day 10 post-partum composite samples were subjected to 2DE SDS-PAGE in order to further identify key changes in the proteome of colostrum (day 1) compared to milk after 10 days post calving.

After determination of protein concentration in each sample (using Bradford assay- Appendix Chapter 4), samples were diluted in rehydration buffer (RHB) (Bio-Rad ReadyPrep™ rehydration/sample buffer) containing 8 M Urea, 2 % (w/v) CHAPS, 50 mM DTT, 0.2 % (v/v) Bio-Lyte® 3/10 ampholyte, 0.001 % (w/v) bromophenol blue (BioRad, Hemel Hempstead, UK) so that 200 µg of sample was in a final volume of 200 µl for application to an 11 cm IPG strip (BioRad, Hemel Hempstead, UK) according to manufacturer's instructions, of pH range 3-10.

Rehydration and Isoelectric Focusing

The prepared sample(s) was then applied on a gel focusing tray to a pH 3-10 IPG strip (11 cm, non-linear, Bio-Rad Lab, UK) and covered with 1 ml of mineral oil (Sigma-Aldrich, Dorset, UK). Active rehydration and then isoelectric focusing were carried out on a Bio-Rad Protean IEF cell using the protocol below;

Focus temperature 20°C;
Voltage intervals:
500 V for 1 hour,

1000 V for 1 hour,
2000 V for 2 hours,
4000 V for 4 hours,
8000 V for 12 hours.

Focused IPG strips were then either stored with the gel side up in a well-covered IPG tray at -20ºC or used immediately for SDS-PAGE.

Second Dimension SDS-PAGE

Equilibration buffers (EB) I and II were prepared (Appendix Chapter 5). Focused IPG strips were then incubated for 15 min in EB I with gentle shaking, washed in running buffer and incubated in EB II for another 15 min.

Equilibrated IPG strips were then carefully inserted horizontally on to the IPG well of the pre-cast IPG+1well comb which had been assembled in the gel running tank with added sample loading buffer. 10 µl of pre-stained protein ladder was added into the extra well. Electrophoresis was run at 200 V for 40-45 min at RT. Gels were carefully removed from the gel cassette and then stained and de-stained as described for 1DE in section 3.3.1.6 above.

3.3.2 Immunohistochemical localization of Hp in bovine mammary gland

Samples

Tissue sections from the mammary glands of two cows were examined histologically using H & E and Hp IHC. The cows were a 10 year old beef suckler cow with clinical mastitis and an age matched non-lactating beef suckler cow with no clinical or histological evidence of mastitis. Sections were supplied by VDS, University of Glasgow.

Tissue Sectioning

Tissues were fixed in 10 % neutral buffered formalin and embedded in paraffin wax (FFPE). They were then cut using a microtome to a thickness of 2.5microns and affixed onto a slide. Slides were baked at 57°C for 1 h prior to staining.

H&E staining

Sections of both healthy and mastitis mammary gland were stained with the H&E stain as described below.

First, the sections were brought to distilled water through graded alcohols and stained with Gill's haematoxylin prepared in-house, for 5 min after which they were rinsed in running tap water. Differentiation was carried out briefly with 10 % acid alcohol and once again sections were rinsed in tap water. Sections were then counterstained with eosin for 5 min after which sections were dehydrated, cleared and mounted.

Antibody Staining

Prior to staining, tissue slides were completely deparaffinised and rehydrated by performing several incubation steps in xylene and different concentrations of ethanol solution and finally running under water.

No antigen retrieval was required and the IHC was carried out using an automated immunohistochemistry system by Dako (Agilent technologies, UK). All procedures were carried out at RT. Tissue sections were placed on to a Dako autostainer and then rinsed with buffer (Tris buffer pH 7.5 + Tween) after which sections were blocked for 5 min with Dako REAL™ peroxidase blocking solution. Sections were buffer rinsed for 5 min and the primary antibody, rabbit anti-bovine IgG (*Life diagnostics*, USA) diluted to an optimum of 1:800 (1.72 μg/ml) in Dako universal diluent was applied for 30 min.

Subsequently two 5 min buffer washes were carried out and then sections were incubated with a secondary antibody (Dako Envision system HRP labelled polymer anti-rabbit (Dako UK Ltd, Cambridgeshire, UK). Two 5 min buffer washes were performed and the substrate, diaminobenzidine (DAB) (Dako DAB, K5007) was applied for 10 min. After 3 rinses with tap water, sections were counterstained for 27 seconds using Gills haematoxylin prepared in-house and finally sections were washed in water, dehydrated, cleared and mounted (DC&M) in synthetic resin.

Image Capture

Images of slides were acquired and analysed using an Olympus™ BX51 microscope (Olympus Life Science, Hamburg Germany) and were processed using a Cell^D imaging software (Electro Optics, Cambridge, UK). The images were captured using an Olympus DP71 digital camera. Sections were examined at magnifications of x100 and x200.

Histological sectioning and IHC staining for Hp was carried accomplished with the help of Ms Marion Lynn Stevenson of the Veterinary diagnostic services unit, University of Glasgow. Advice on interpretation of histological images was carried out by pathologists Dr. Pamela Johnston, Dr. Hayley Haining (Veterinary Diagnostic Services, School of Veterinary Medicine, University of Glasgow) and Dr Hal Thompson (formerly of the University of Glasgow).

3.3.2.1 Statistical analysis

Tests for normality were carried out on all APP data. A related samples Friedman's two-way analysis of variance by ranks test was run on results of daily post-calving milk Hp, M-SAA3 and CRP. Non-parametric pair-wise comparisons (related samples Wilcoxon signed ranks test) of daily samples were carried to determined days where significant variation occurred. Non-parametric correlation test (Spearman's rho) was used to assess the correlation of the daily APP in the same cows. P-value was considered significant at <0.05.

3.4 Results

3.4.1 Acute phase proteins in post-calving milk

3.4.1.1 Milk haptoglobin in post-calving cows

Milk Haptoglobin ELISA

The mean Hp concentration in milk decreased with the days post-calving (Figure 3-1) from a mean of 46.50 ± 7.94 (SEM) µg/ml on day 1 (colostrum) (n=83 quarter-milk samples; n=22 cows) to a mean of 6.31 ± 4.01 µg/ml (SEM) on day 10 (n=75 quarter-milk; n=22 cows). P-values to show pair-wise significant differences in Hp between days post-calving are shown in Table 3-1.

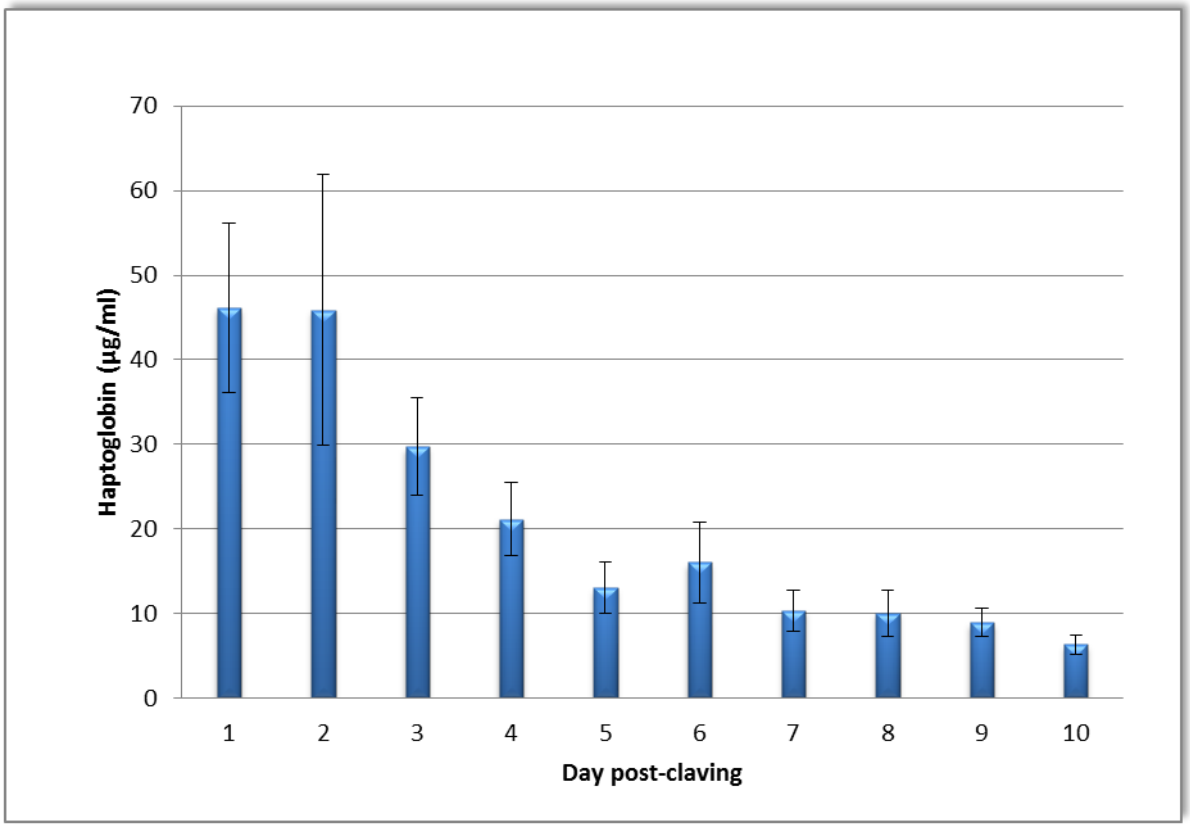

Figure 3-1: Histogram of mean± SEM of daily composite milk Hp from day 1 to 10 (n=22 cows)

Range of Hp in all samples (day 1-10) was <0.4 - >1250 µg/ml and median was 4.86 µg/ml.

Table 3-1: P-values of differences in daily milk Hp across 10 days post-calving.
Days of significant difference in Hp are highlighted in brown

Haptoglobin P-value of significant difference with days post calving.										
Day	1	2	3	4	5	6	7	8	9	10
1	*	0.170	0.008	0.000	0.000	0.001	0.000	0.000	0.000	0.000
2	0.170	*	0.027	0.006	0.001	0.006	0.002	0.001	0.004	0.001
3	0.008	0.027	*	0.002	0.002	0.014	0.001	0.001	0.001	0.002
4	0.000	0.006	0.002	*	0.012	0.064	0.002	0.014	0.011	0.003
5	0.000	0.001	0.002	0.012	*	0.117	0.064	0.044	0.059	0.048
6	0.001	0.006	0.014	0.064	0.117	*	0.027	0.025	0.084	0.073
7	0.000	0.002	0.001	0.002	0.064	0.027	*	0.748	0.184	0.057
8	0.000	0.001	0.001	0.014	0.044	0.025	0.748	*	0.199	0.044
9	0.000	0.004	0.001	0.011	0.059	0.084	0.184	0.199	*	0.013
10	0.000	0.001	0.002	0.003	0.048	0.073	0.057	0.044	0.013	*

Western blotting for Hp

The Hp western blot of daily composite milk samples from one representative calving cow is shown in Figure 3-2 and shows the antibody reacting with the α and β chains of bovine Hp at 15 kDa and 40 kDa respectively in milk from day 1 to day 4 post-calving. This blot also shows non-specific reaction with higher Mw protein of 100 kDa and 70 kDa which are likely to be lactoferrin and albumin. The reaction to these proteins, which persisted till the day 10-sample varied between blots (compare to the blot in Figure 3-3) and are likely to be due to differences in the blocking reactions with normal dried milk.

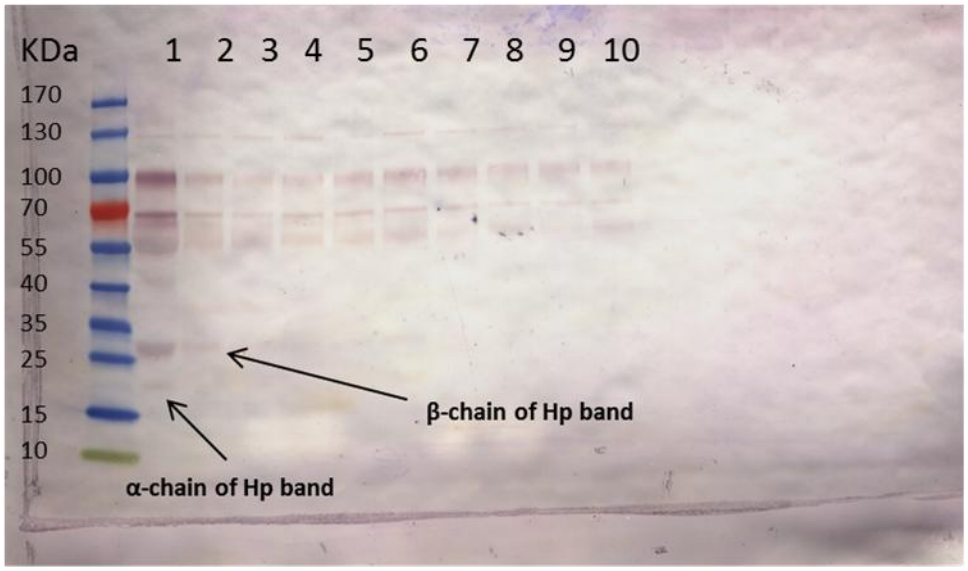

Figure 3-2: Western blot for Hp in 1DE of post-calving milk from day 1 to 10.
There is decreasing intensity of the Hp β by day 2, a very faint α-chain band on day 1 and both bands absent from day 3 to 10.

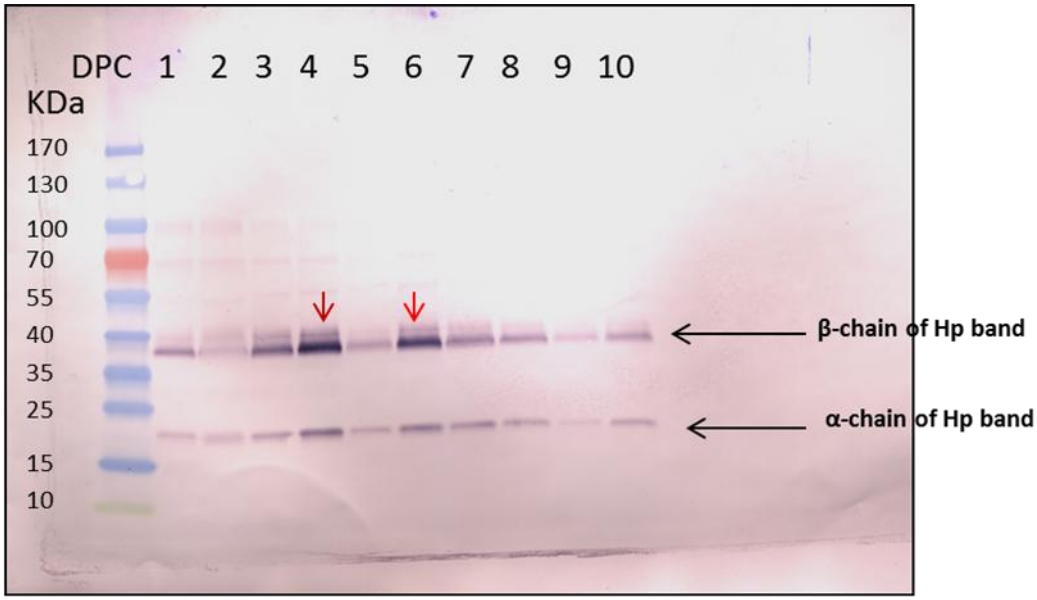

Figure 3-3: Example of irregular fluctuation in Hp concentration from day 1 to 10 a post-calving cow's milk.
Greater intensity of Hp bands are seen on days 4 and 6, while days 2 and 9 had lower intensities, these intensity fluctuations agree with the measured Hp concentration for each day in the particular cow.

3.4.1.2 M-SAA3 in post-calving milk

A moderately high mean M-SAA3 concentration was observed for day 1 and 2 post-calving (colostrum) samples (427 and 238 μg/ml respectively), which fell as

the days progressed reaching basal or undetectable levels (4.5 - <0.6 μg/ml) by day 4 to 5 (Figure 3-4). Range of M-SAA3 in all post calving samples was <0.6 μg/ml (LOD) to >1500 μg/ml (highest limit of detection at 1:5000 dilution). P-values to show pair-wise significant differences in M-SAA3 between days post-calving are shown in Table 3-2.

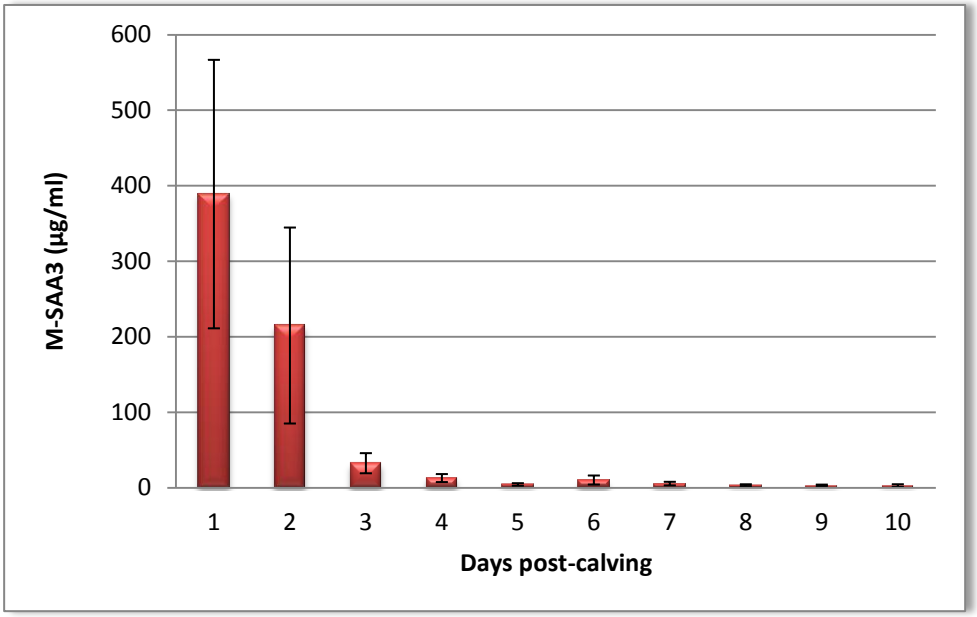

Figure 3-4: Concentrations of daily M-SAA3 (mean ± SEM) from day 1-10 post-calving composite milk samples (n=10).

Table 3-2: P-values of differences in daily milk M-SAA3 across 10 days post-calving.
Days of significant difference in M-SAA3 are highlighted in brown.

M-SAA3 P-value of significant differences between days post calving.										
Day	1	2	3	4	5	6	7	8	9	10
1	*	0.012	0.025	0.038	0.025	0.036	0.036	0.012	0.017	0.012
2	0.012	*	1	0.237	0.091	0.091	0.091	0.028	0.069	0.018
3	0.025	0.012	*	0.091	0.043	0.069	0.093	0.018	0.028	0.018
4	0.038	0.237	0.091	*	0.043	0.401	0.093	0.043	0.123	0.043
5	0.025	0.091	0.043	0.043	*	0.08	0.345	0.465	0.5	0.465
6	0.036	0.091	0.069	0.401	0.08	*	0.465	0.043	0.173	0.043
7	0.036	0.091	0.093	0.093	0.345	0.465	*	0.225	0.463	0.043
8	0.012	0.028	0.018	0.043	0.465	0.043	0.225	*	0.715	1
9	0.017	0.069	0.028	0.123	0.5	0.173	0.463	0.715	*	1
10	0.012	0.018	0.018	0.043	0.465	0.043	0.043	1	1	*

3.4.1.3 CRP in post-calving milk

Concentrations of CRP were moderately high in colostrum for 8 out of the 10 composite samples of day 1 post-calving assayed, while 2 out of 10 composite colostrum samples had undetectable levels of CRP. In one cow, CRP was undetectable from day 1 (colostrum) up until day 10, except for day 4, which showed detectable but basal concentration. When compared with other APP, the

same samples with undetectable CRP also had low or undetectable Hp and M-SAA3 concentration across the 10 days. Furthermore, some irregular fluctuations were observed across the days in some samples for example in 2 samples, the highest CRP concentration occurred on day 2. In 2 cows, CRP had low to moderate concentrations from day 1 to 8, but spiked in concentrations on day 9, but dropped again on day 10.

Figure 3-5 shows the mean and SEM of daily milk CRP concentrations in composite samples assayed for day 1 up until day 10 post calving. The range of CRP in all the post-calving milk was <1.8 to 607 ng/ml. Table 3-3 displays the P-values of pair-wise significant difference in CRP values across the 10 days.

The profile of Hp, M-SAA3 and CRP when compared in the ten cows (which were examined for the 3 APP) showed significant correlations (shown in Table 3-4).

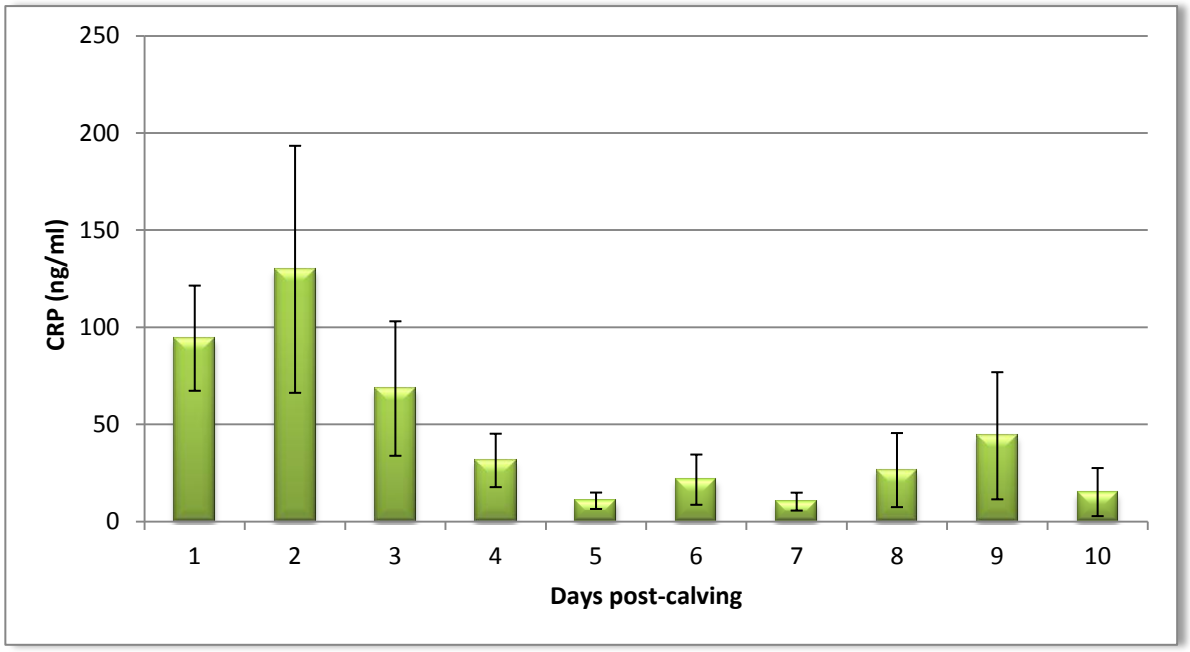

Figure 3-5: Concentrations of daily CRP (mean ± SEM) from day 1-10 post-calving composite milk samples (n=10).

Table 3-3: P-values of differences in daily milk CRP across 10 days post-calving.
Days of significant difference in CRP are highlighted in brown

CRP P-value of significant difference with days post calving.										
Day	1	2	3	4	5	6	7	8	9	10
1	*	0.594	0.401	0.021	0.012	0.051	0.021	0.011	0.086	0.012
2	0.594	*	0.018	0.011	0.012	0.017	0.012	0.017	0.208	0.012
3	0.401	0.018	*	0.401	0.091	0.263	0.017	0.069	0.208	0.018
4	0.021	0.011	0.401	*	0.046	0.499	0.043	0.398	0.889	0.116
5	0.012	0.012	0.091	0.046	*	0.6	0.753	0.6	0.463	0.715
6	0.051	0.017	0.263	0.499	0.6	*	0.043	0.5	0.6	0.345
7	0.021	0.012	0.017	0.043	0.753	0.043	*	0.345	0.173	0.893
8	0.011	0.017	0.069	0.398	0.6	0.5	0.345	*	0.345	0.043
9	0.086	0.208	0.208	0.889	0.463	0.6	0.173	0.345	*	0.043
10	0.012	0.012	0.018	0.116	0.715	0.345	0.893	0.043	0.043	*

Table 3-4: Correlation of Hp, M-SAA3 and CRP in the same cows (n=10)

			M-SAA3	CRP	Hp
Spearman's rho	M-SAA3 (µg/ml)	Correlation Coefficient	1.000	.548**	.660**
		Sig. (2-tailed)	.	.000	.000
		N	100	100	100
	CRP (ng/ml)	Correlation Coefficient	.548**	1.000	.661**
		Sig. (2-tailed)	.000	.	.000
		N	100	100	100
	Hp (µg/ml)	Correlation Coefficient	.660**	.661**	1.000
		Sig. (2-tailed)	.000	.000	.
		N	100	100	100
**. Correlation is significant at the 0.01 level (2-tailed).					

3.4.1.4 Proteomic analysis of post-calving milk

1DE SDS-PAGE

Figure 3-6 is the 1DE gel electrophoretogram of day 1 to 10 composite milk samples of a representative calving cow from Cochno Dairy farm. The labelling of bands and spots were carried out by inference using extrapolations from data obtained after MS identification of similar samples by Mansor (2012) (PhD thesis, University of Glasgow) and Henderson (2013) (MRes dissertation, University of Glasgow) see Appendix Chapter 3.

Between day 1 and day 10, there was a decrease in high abundance protein, especially IgG heavy and light chains while milk protein such as lactoferrin (Lf) or alpha-lactalbumin (α-LA) were more consistent between days.

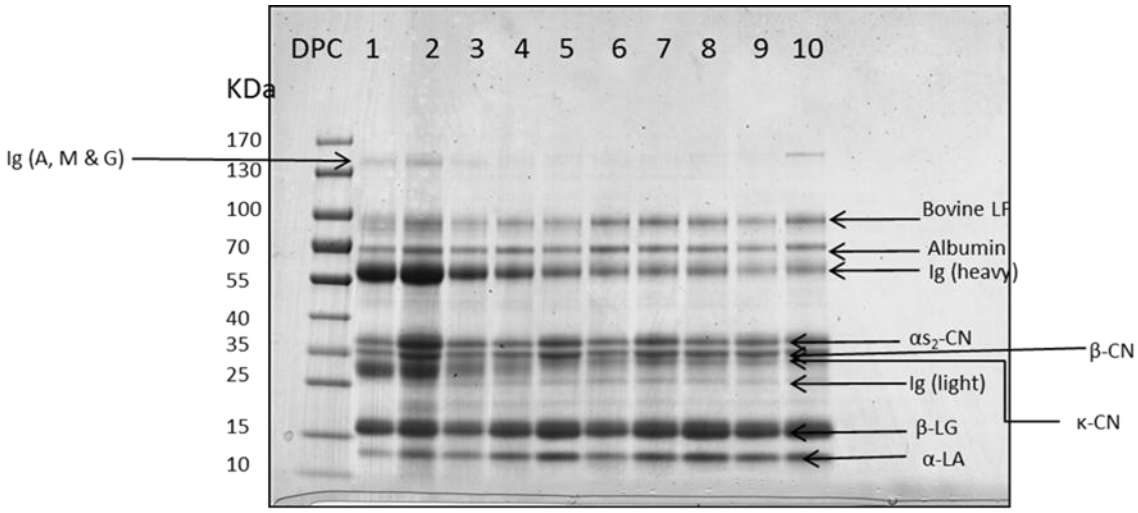

Figure 3-6: 1DE reducing gel electrophoretogram of immediate post-partum milk samples (day 1-10) pooled from healthy udder of cow A.
Ig (immunoglobulin), Bovine Lf (bovine lactoferrin), αS_2-CN (alpha S_2 casein), β-CN (beta casein), κ-CN (kappa casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin), DPC (days post-calving), kDa (kilo Dalton). Labelled by inference using data obtained from MS identification of similar samples by Mansor (2012) (PhD thesis, University of Glasgow) and Henderson (2013) (MRes dissertation, University of Glasgow); Appendix Chapter 3.

2DE SDS-PAGE

Figure 3-7 shows the 2DE gel image of colostrum sample (pooled from 4 quarters) of a healthy calving cow from Cochno Dairy farm, while Figure 3-8 shows the day 10 pooled milk 2DE of the same cow (pH 3-10). Spots on the gel were labelled by inference using extrapolations from reference 2DE gels described in Boehmer *et al*. (2008) and Mansor (2012) (PhD thesis, University of Glasgow). Comparing these 2DE gels, there is a reduction in the IgG heavy and light chains between day 1 and day 10 while the caseins, beta-lactoglobulin (ß-LG) and α-LA are more prominent at day 10.

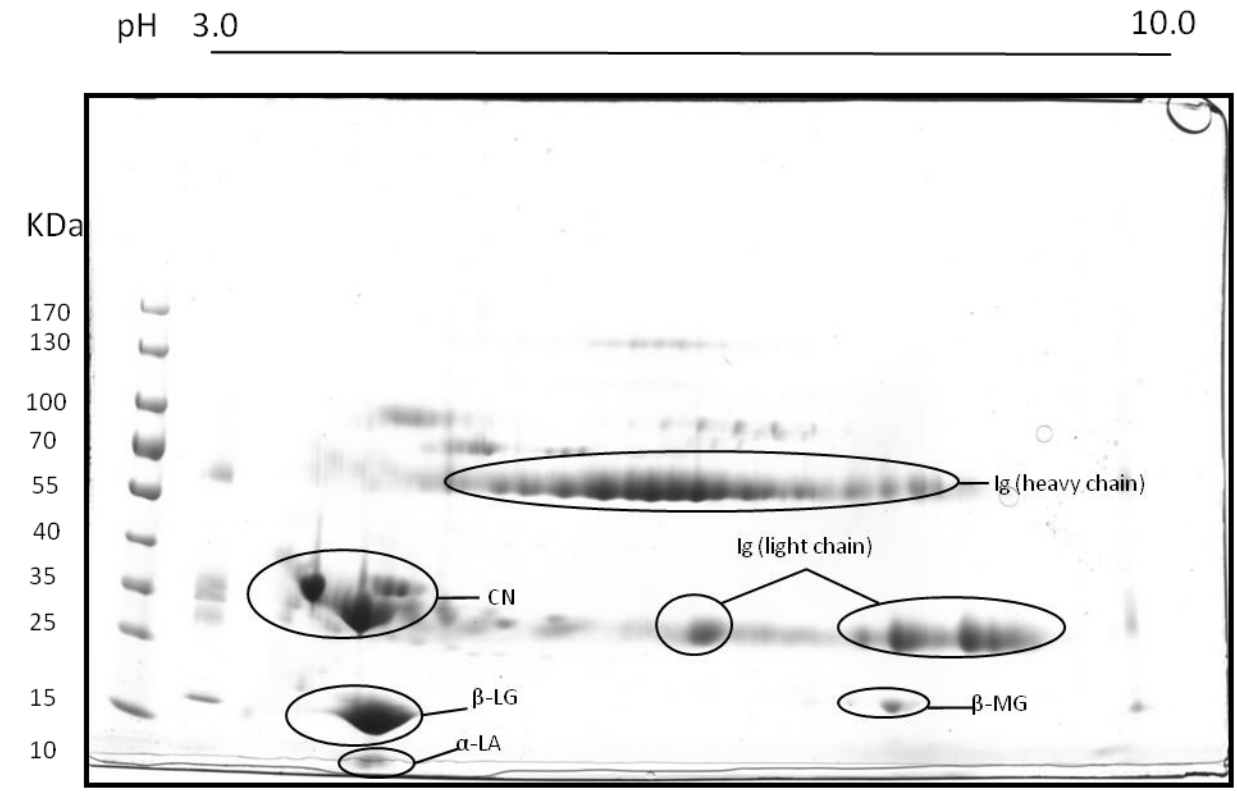

Figure 3-7: 2DE reducing gel of pooled (quarters) colostrum (day 1 post-calving) sample. Isoelectric range pH 3-10, from one representative calving cow of Cochno Dairy farm. Abundant spots of Ig (heavy and light chain) are seen which is characteristic of colostrum. Ig (immunoglobulin), CN (caseins), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin), β-MG (beta-2 microglobulin)

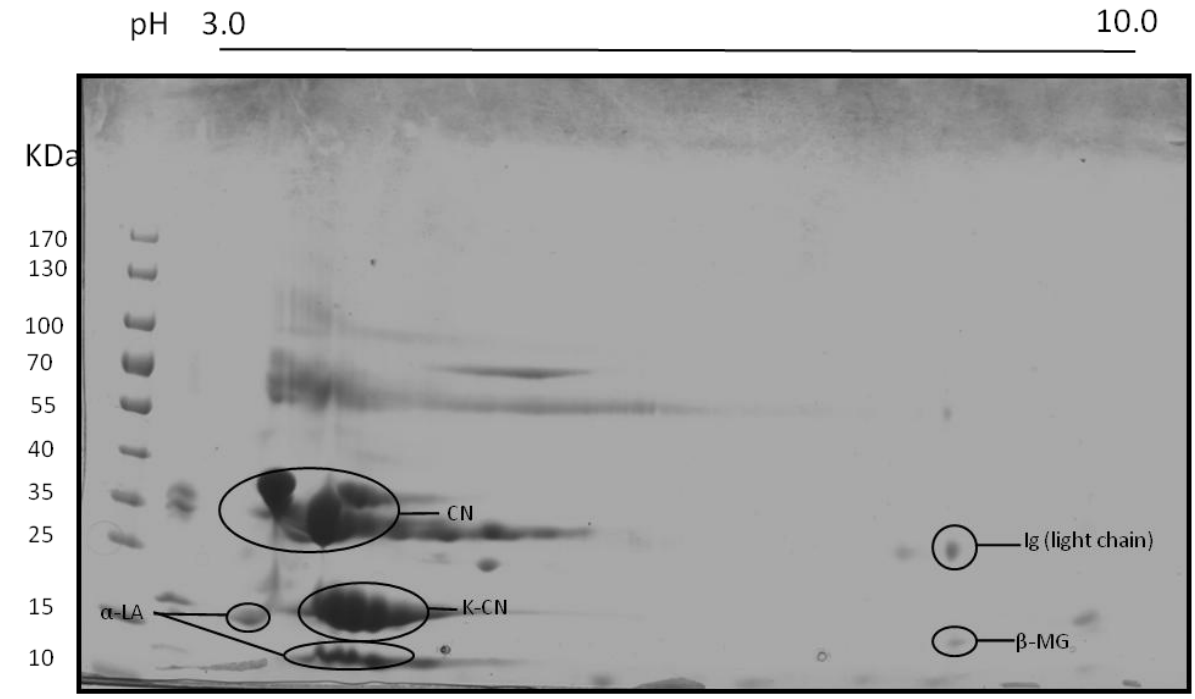

Figure 3-8: 2DE reducing gel of pooled (quarters) day 10 post-calving milk samples.
On a pH 3-10 range strip. Less Ig spots are seen here compared to the colostrum samples 2DE. Ig (immunoglobulin), CN (caseins), κ-CN (kappa caseins), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin), β-MG (beta-2 microglobulin).

3.4.1.5 Immunohistochemical localization of Hp in bovine mammary gland

Gross images of both mammary glands used for this study are shown in Figure 3-9. Figure 3-10 shows a healthy mammary gland stained with H & E showing the normal architecture of bovine mammary gland, while Figure 3-11 depicts an H&E stained section of a mastitis mammary gland (x100). Overall, the mastitis gland showed a high infiltration of neutrophils in most areas of the gland while Hp staining was strong in the neutrophils and alveolar duct epithelium.

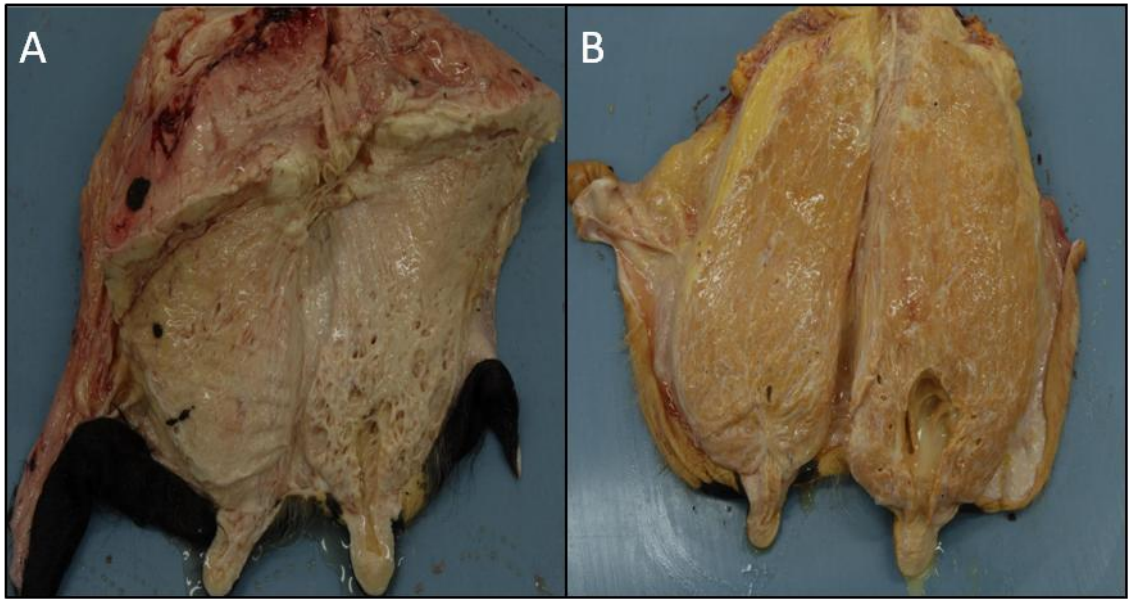

Figure 3-9: Gross images of the healthy involuted (A) and mastitic (B) mammary glands.
These were used for the immunohistochemistry analysis of Haptoglobin.

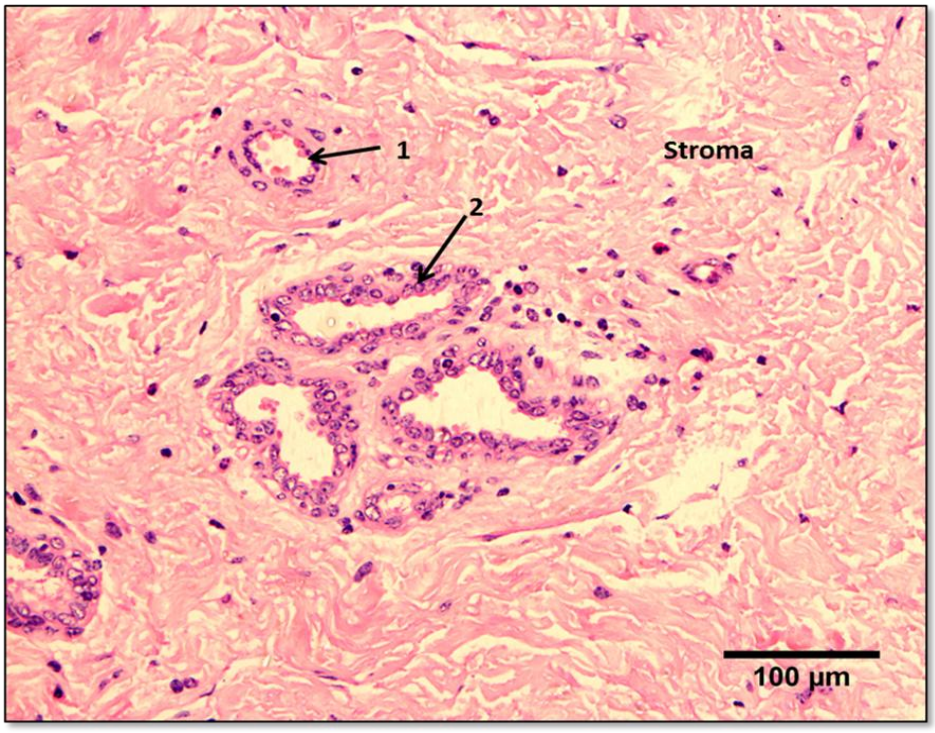

Figure 3-10: Healthy (involuted) bovine mammary gland section, H&E, x200.
Showing the normal mammary gland architecture, with occasional mononuclear cells infiltrating the periductular and perivascular stroma. Arrows indicate a blood vessel (1) and alveolar ducts (2).

Figure 3-11: Mastitis bovine mammary gland section, H&E, x100.
Arrows show duct epithelium (1), lamina propria (2), blood vessel (3), alveolar epithelium (4) and neutrophils within the alveolar duct (5). Moderate numbers of neutrophils as well as lower numbers of lymphocytes and plasma cells, macrophages infiltrate the lamina propria and extend into the mucosal lining of the duct cistern and alveoli in the mastitic glands. Moderate numbers of neutrophils are seen within the alveolar lumens.

Figure 3-12 displays the Hp IHC staining of healthy glands (1:800), x200 with occasional cytoplasmic activity of the ductal epithelial cells for Hp. Figure 3-13 is the Hp IHC stained (1:800) sections showing strong positivity in Hp in the duct, alveolar epithelial cells and neutrophils at x100.

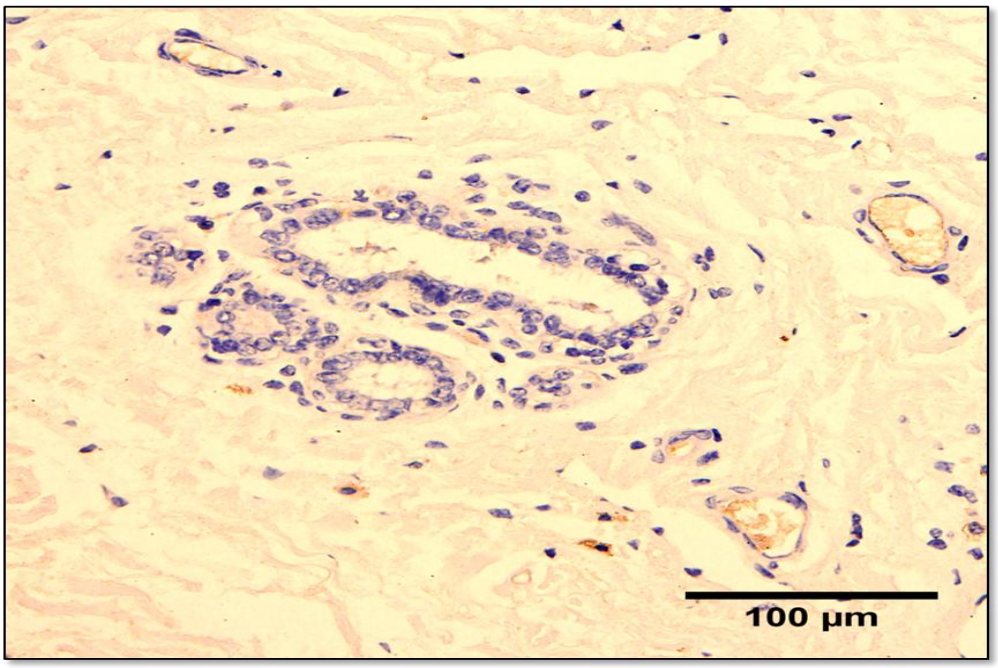

Figure 3-12: Haptoglobin immunohistochemistry (titre 1:800) staining of a healthy (involuted) bovine mammary gland, x200:
Very occasional ductal epithelial cells demonstrate minimal cytoplasmic positivity for Haptoglobin (brown staining).

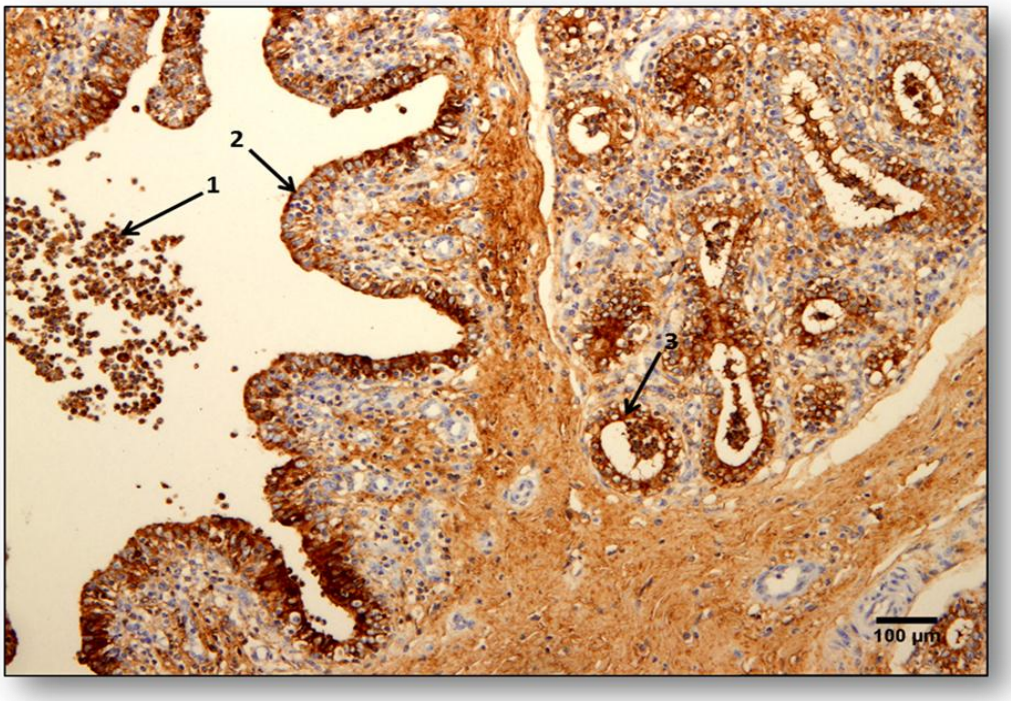

Figure 3-13: Haptoglobin immunohistochemistry (titre 1:800) staining of mastitic bovine mammary gland section, x100.
Arrow showing intensely staining neutrophils in the duct lumen (1), duct epithelium (2), alveolar ducts with Hp stained neutrophils (3). The densely staining region comprising the neutrophils and mammary epithelial cells are indicative of Hp producing sites. Mammary stromal staining (lighter brown) is considered a non-specific staining.

3.5 Discussion

3.5.1 Acute phase proteins in post-calving milk

3.5.1.1 Haptoglobin in post-calving milk

In the study on milk samples obtained from calving cows, milk Hp was determined from the first milking after calving, and daily milking thereafter to the tenth day post calving. Cows were milked twice daily, mornings and afternoons and samples were collected for the Hp analysis during the morning milking. It is well accepted that the periparturient period is one of the most critical periods, health wise, in the productive life of a dairy cow (Trevisi *et al.*, 2010) and this has been attributed to the negative energy balance and its associated immune suppression at the puerperal period. The levels of serum Hp at this period can give a picture of the physiologic stress the cow is undergoing. Variations in milk Hp in this early post-partum period can help to assess the potential of milk Hp concentrations as a method of distinguishing between physiological increases and identifying new post-calving IMI. Previous studies have reported increases in APP in serum during the first week(s) post-calving (Humblet *et al.*, 2006; Uchida *et al.*, 1993; Alsemgeest *et al.*, 1995), but few studies have demonstrated the effect of parturition on milk APP (McDonald *et al.*, 2001; Ceciliani *et al.*, 2005).

It has previously been determined that very minimal variations exist (below 10 %) in the APP (SAA and Hp) of healthy mid lactation (27 ± 9 weeks in lactation) cows over a course of 42 consecutive milkings (Åkerstedt *et al.*, 2011). The results of this study indicate that Hp is moderately high in the first few days post calving milk (colostrum) and gradually drops back to basal levels within 3 to 5 days after parturition. High individual variations exists, however by the fifth day, milk Hp had dropped to basal or non-detectable levels in over 80 % of quarter milk samples assayed in this study, which were considered healthy. In a few quarters, (~12 %), however, milk Hp increased beyond moderate or basal levels of the first few days of post-calving as days progressed. These quarters, can be suspected to be quarters developing new IMI or undergoing other forms of inflammatory stimulus that can influence the occurrence of an APR.

The major pattern of Hp in milk after parturition, followed a similar trend to those observed for SCC in the studies of Barkema (1999) and Sargeant *et al.*, (2001). The high level of milk Hp in the first few days post-calving may suggest a role for Hp in colostrum of conferring maternal protection to the new-born. On the other hand, it may be purely due to the stress induced by parturition, and its effect extending to the mammary gland.

Other APP that have been identified as being high in colostrum and shown to decrease in milk with days post calving are SAA (McDonald *et al.*, 2001), alpha-1 acid glycoprotein (Ceciliani *et al.*, 2005), lactoferrin and transferrin (Sánchez *et al.*, 1988). To the best of our knowledge this is the first report of daily variation in the levels of Hp from colostrum and milk over the first 10 days after parturition.

That there was no correlation found between the concentrations of first day Hp with the position of the quarter or parity indicates that neither of these factors has an effect on Hp in milk.

With the knowledge that Hp and other major APP are moderately high in colostrum in the first few days' milk post calving, caution should be exercised in using them for confirming the presence of IMI at this period. Compared to Hp concentration in peak stages of mastitis, milk Hp concentrations in colostrum are moderately raised. However, since most dairy farms measure SCC on a monthly basis by samples submitted to a central laboratory, APP assay can still come in useful for detecting new IMI in the periparturient period, especially after the first few days when a drop in Hp would be expected in the absence of IMI and provided they can be adapted to a rapid measurement format. Indeed a rapid test for Hp may be a better test for IMI than CMT which has been evaluated for their usefulness in detecting major pathogen caused mastitis in the immediate post-partum period (Dingwell *et al.*, 2003b).

3.5.1.2 M-SAA3 in post-calving milk

High levels of M-SAA3 observed in almost all composite colostrum samples are consistent with the first reports by McDonald *et al.* (2001) where high levels of MAA in bovine colostrum significantly dropped by day 4 post calving.

In this study it was observed that milk concentrations of M-SAA3 dropped faster than Hp levels post-partum, such that by the third day after calving values were almost all undetectable whereas milk Hp values were sustained in some animals to the 4-5th day. This suggests that M-SAA3 may have a critical role in conferment of innate immunity to the new born. Thus an M-SAA3 test may introduce spurious results if used for assessing for IMI at the first 4 to 5 days or first 6 milkings after calving, but it is therefore important that the natural levels of M-SAA3 in colostrum and early milk is recognized.

3.5.1.3 CRP in post-calving milk

Milk CRP was found to follow a similar trend as Hp and M-SAA3 in post calving milk, by being moderately high on the first 1 to 3 days and then gradually falling in concentration in healthy cows. This study confirms the reports of Schroedl *et al*. (2003) of the presence of CRP in bovine colostrum. In 2 cows, where CRP levels were low to moderate from day 1 to 8, but spiked in on day 9, and then dropped again on day 10, the spike in CRP on day 9 is not conclusively suggestive of a new IMI.

According to Lee *et al*. (2003) serum CRP levels correlated with lactation status, being highest during peak lactation period (2-4 months of pregnancy). Furthermore in the study of Zimmermann *et al*. (1998) plasma CRP levels in cows were increased post-partum with the increase higher in the group of cows that had undergone a normal puerperium than in groups with delayed involution or puerperal endometritis. There has been no previous report of the daily variation of CRP in bovine milk from the day of parturition up to the 10^{th} day post-partum. This pilot study should be extended in order to verify the observed pattern in milk CRP in the post-partum period.

3.5.2 Proteomic analysis of post-calving Milk

One dimensional gel electrophoresis is a robust and reproducible technique for protein separation. However, it has low resolution. From this study, 1DE of the daily milk samples displayed sufficient resolution to distinguish minor quantitative and qualitative variation of samples from first to tenth day post calving. The major milk proteins (caseins and whey proteins) as well as other high abundance proteins (for example immunoglobulins) have been identified

from similar studies by Henderson (MRes Dissertation, University of Glasgow, 2013), such that protein identification by MS was redundant.

As samples move from colostrum to milk, there is a decrease in immunological proteins such as Igs, bovine lactoferrin and serum albumin but the levels of major milk whey proteins; ß-lactoglobulin and α-lactalbumin remain virtually the same. No bands of IgA and IgM were observed in milk samples from day 4 post calving although they were seen from day 0 to 3. These findings agree with the reports of Stelwagen *et al*. (2009) of colostrum containing higher amounts of Igs especially IgG and other immune related proteins than milk. It should be noted that bands on a reducing 1DE gel, such as examined in the present study, have a high probability of containing multiple proteins (of similar mass) since only molecular weight separation was achieved using a 1DE. Hence to obtain better protein resolution and additional separation dimension (isoelectric point) can be employed as obtainable in 2DE.

Two dimensional gel electrophoresis of the milk samples from the first and tenth day post-calving had the additional advantage of being able to resolve proteins by both the isoelectric points and molecular mass. Various spots corresponding to milk proteins as well as immunological proteins were observed upon staining of 2DE gels. Although no mass spectrometry of gels spots were carried out in this study, inferences on the constituents of each spot was made by comparing gels spots analyses of similar samples carried out previously (Henderson, MRes, dissertation, University of Glasgow, 2013) and in comparison to published 2DE gels of milk protein (Hogarth *et al*., 2004; Smolenski *et al*., 2007; Boehmer *et al*., 2008).

Comparatively, visual examination of the 2DE gels of the day 1 and day 10 post-calving milk showed clear distinctions in relation to the relative abundance of milk proteins and immunoglobulin spots, with Day 1 milk having greater abundance of both classes of proteins, consistent with the documented composition of colostrum of being rich in immunoglobulins and other immune proteins. The 2DE gel analysis gave increased separation of the high abundance proteins of milk which were largely the same as those on the 1DE apart from the ß1-microglobulin, which with a high pi was well separated from other low Mw proteins such as α-LA.

Thus it can be concluded that 2DE provided a reasonable separation of high abundance proteins in milk and visual inspection of gels with comparison to previous results can identify protein for interpretation of the change in high abundance proteins of milk post calving. However 2DE does have limitations and more advanced proteomic technologies with greater sensitivity need to be applied to examine changes in lower abundant proteins and these issues are addressed in chapter 5 of this thesis.

3.5.3 Immunohistochemical localization of Hp in the bovine mammary gland

Immunohistochemistry is a technique that enables the localization of antigens or proteins in histological tissue sections by the use of labelled antibodies against the antigen of interest, which can then be visualized by staining with dyes.

In this IHC study of normal and mastitic bovine mammary glands, it was found that Hp stains highly within the neutrophils and MEC during mastitis. This agrees with previous reports of the neutrophils and MECs as one of the major sources of milk Hp (Lai *et al.*, 2009; Thielen *et al.*, 2007). However in addition to the alveolar epithelia cells, strong Hp staining was also observed for the ductal epithelial cells in this study. Similar findings on IHC localization of Hp have been observed for porcine lungs (Hiss *et al.*, 2007) and bovine female reproductive tract (Lavery *et al.*, 2003).

Furthermore, because MEC of both alveoli and ducts of the gland were found to stain highly for Hp, it can be inferred that the synthesis of Hp occurs in these sites of frequent communication between the body and the exterior lending further support to its possible role in the innate immune response.

It has been demonstrated that Hp is stored in granules within neutrophils, and undergoes exocytosis at sites of infection or injury (Theilgaard-Mönch *et al.*, 2006). However there is no documented evidence of its storage in this form, within MEC, as no granules have been associated with the MEC.

3.6 Conclusions

The APP profile in milk in the immediate post calving period was evaluated and with the conclusion that moderate increases occur in the concentration of Hp,

M-SAA3 and CRP during this period. These increases in APP can be distinguished from mastitis related increases in APP from the 4th day post calving, beyond which any sustained high value of APP would be suggestive of a new or on-going intra mammary infection. The advantage of proteomic studies was shown in distinguishing changes in colostrum and milk during the periparturient period.

Immunohistochemical studies have demonstrated the presence of Hp in neutrophil as well as the MEC, confirming these as possible origins of the source of milk Hp.

4 Proteins in milk from dairy cows with naturally occurring mastitis: effect of pathogen

4.1 Overview

This chapter discusses the analyses of milk samples from cows naturally affected by mastitis, which were submitted for diagnostic evaluation to a veterinary diagnostic laboratory in Scotland. Several analyses were carried out on these samples, ranging from quantitative assays, characterization of APP, as well as proteomic evaluations. The overall objective in this chapter was the identification of likely biomarkers of bovine mastitis from a panel of samples with a heterogeneous distribution of factors that relate to the occurrence of mastitis on dairy farms in the UK. In addition, milk collected, in these studies were subjected to a variant 2DE, to determine if milk isoforms of Hp correspond to the forms found in bovine serum during acute phase reaction. The milk protein was separated by a liquid phase isoelectric focusing and then 1DE SDS-PAGE in order to identify change in low abundance proteins.

4.2 Introduction

4.2.1 Natural mastitis caused by different pathogens

The incidence, types, aetiology, risk factors and distribution of mastitis has already been discussed in the introductory chapter of this thesis. A major determinant of the severity and course of a mastitis episode is the causative organism of the mastitis in a quarter at any given time. This can be further impacted by the presence of multiple species within the affected gland (Pyorälä *et al.*, 2011). Other factors that can also create risks for bovine mastitis include farm hygiene or management system, seasonal variation and breeds of cows.

In a number of studies, it has already been determined that different pathogens vary in the onset and level of release of inflammatory markers such as APP into milk during mastitis (Smolenski *et al.*, 2007; Pyorälä *et al.*, 2011; Kalmus *et al.*, 2013). Therefore the causative pathogen in any given mastitis condition could significantly affect biochemical composition of the milk and thus the findings during biomarker searches. Several studies have shown the variation of the APP

Hp and M-SAA3 in milk from mastitis caused by different pathogens. Furthermore Kruger and Neumann, (1999) have shown that significant variations exist in CRP levels of milk from mastitis caused by different pathogens. Having an in-house assay for milk Hp, as well as the hope of exploring the variation of APP from a wider spectrum of bacterial causes and comparing the dynamics of each APP with the other in the same samples, instigated further studies of APP dynamics in milk from mastitis caused by different pathogens.

The traditional method for determination of causative pathogen of mastitis has been the bacterial culture and isolation and this method has been recommended by the NMC (NMC, 2004). This method has disadvantages particularly of the length of time it takes for testing as well as incidence of frequent 'no growth' cases in mastitis milk cultures. Nonetheless this method continues to be a popular method for confirming the aetiologies of IMI.

In the search for markers for bovine mastitis, the use of samples from natural infections offer an added advantage over experimentally induced mastitis samples in that the disease takes its natural course as obtained in field or farm conditions. Indeed it would be part of validation of any commercial diagnostic test to ensure it can operate with samples from dairy farms with mastitis. Bovine mastitis is a 'natural infection' problem with high economic impact in most dairy farms across the world. Therefore, it was expected that exploring samples from natural infections from commercial dairy farms would give a better understanding of biomarker repertoires in naturally occurring mastitis affected milk. In addition, studying milk samples submitted from various dairy farms and locations in Scotland would help to understand the intricate contributions that other cow, farm or management factors have in the incidence, type and severity of mastitis.

4.2.2 Objectives

The objectives of this study were therefore to;

Determine the profile of APP (Hp, M-SAA3 and CRP) in samples of milk from cows with natural mastitis submitted for bacteriological examination and assess how these APP vary by causative pathogens and other factors.

Investigate major differences in the proteome of a model of gram positive and gram negative bovine mastitis milk, compared to a healthy bovine milk proteome using a liquid phase isoelectric focusing sample fractionation step.

4.3 Materials and methods

4.3.1 Reagents

General chemicals were obtained from Sigma-Aldrich, Poole, UK, except where otherwise stated. Reagents for polyacrylamide gel electrophoreses (PAGE) were obtained from Bio-Rad laboratories Ltd (Hemel Hempstead, UK). Buffers and stock solutions were prepared according to manufacturer's instructions. A 4-15 % Criterion™ precast 18-well comb and 11 cm IPG+1 well comb gels (Bio-Rad Lab, *Inc*. USA) were used for the 1DE and 2DE respectively. Milli Q water was used throughout.

4.3.2 Samples

The milk samples (n=63) used in this study were obtained from the Veterinary Diagnostic Services (VDS) (School of Veterinary Medicine, University of Glasgow), these were samples which were submitted from 7 dairy farms across Scotland, to the Laboratory between August 2012 to December 2013, in order to undergo bacteriological culture for the causative pathogen of mastitis. All samples were aliquoted (5 ml) and stored at -20^{o}C until analysed. The samples were obtained as part of collaborative study with Patricia Belinda Alves Simoes and Timothy Geraghty of the School of Veterinary Medicine, University of Glasgow. The results of bacterial isolation analyses performed by the VDS using bacteriological culture are presented along with APP results. Comparison of APP in milk caused by different mastitis pathogens was performed.

4.3.3 Milk APP concentration in natural mastitis caused by different pathogens

The APP, Hp, M-SAA3 and CRP were measured in the samples obtained from VDS (n=63, whose causative pathogens were determined by microbiology), using the in-house ELISA for Hp, the commercial ELISA kit from Tridelta development *Company* (Kildare, Ireland) for M-SAA3 and the commercial ELISA kit from Life

diagnostics (West Chester, USA) for CRP as described in Sections 2.3.2, 2.3.3 and 2.3.4 respectively, with modifications carried out in the dilutions used for samples with high APP levels. A maximum dilution of 1:1600 was used for Hp, 1:5000 for M-SAA3 and 1:2000 for CRP. An additional 12 samples with low SCC from Cochno dairy farm were analysed to provide a baseline for APP in milk from healthy cows.

4.3.4 Proteomic analysis of a model of gram positive and gram negative bovine mastitis whey

4.3.4.1 Sample preparation

Milk samples submitted to the VDS for bacteriological diagnosis (section 4.3.2) were used for this study. Pools of all *E. coli* (n=9), *S. aureus* (n=9) and *S. uberis* (n=13) mastitis milk were made by adding equal volume of each sample (500 µl) together and mixing thoroughly. A pool of healthy (non-mastitic) milk samples was also made from 12 samples having low SCC obtained from the Cochno dairy farm (as described in section 2.3.5.2 of this thesis).

Centrifugation was carried out in two steps to yield skimmed milk samples of each pool. First whole milk was centrifuged for 30-60 min at speed of 3,500 x*g* and 4°C temperature. Top layer (milk fat) was carefully removed and the supernatant (whey) decanted carefully into a new tube, while the bottom sediment (caseins) were discarded. The centrifugation step was repeated as above for the whey samples, and all residual fats and caseins removed. Once clear whey samples were obtained, they were dialyzed in milli Q water overnight at 4°C to remove salts. Samples were then filtered through a 0.45 µm Minisart® syringe filter (Sartorius, Epsom Surrey, UK).

4.3.4.2 Total protein concentration

Protein concentration in pooled whey samples for liquid phase preparative isoelectric focusing on the mini Rotofor™ cell was determined using spectrophotometric method (based on absorbance spectroscopy of proteins at 280 nm and concentration calculated from the equation 4-1 (Grimsley and Pace, 2004).

Equation 4-1; $A = \varepsilon \times \ell \times C$

Solving for *c,* where A=absorbance; ε= extinction coefficient of bovine serum albumin (6.6); *l*=path length (1 cm) and *c*=concentration (allowing for the molecular weight of albumin).

4.3.4.3 1DE SDS-PAGE

The pools of *E. coli*, *S. aureus, S. uberis* mastitis and healthy whey samples were each resolved by 1DE SDS-PAGE in different wells on a precast gel as described in section 3.3.1.6 as an initial study.

4.3.4.4 2DE: Rotofor™ and SDS-PAGE

Isoelectric focusing

Prior to the preparative isoelectric focusing on a Rotofor system (Bio-Rad Laboratories, Hemel Hempstead, UK), ampholyte concentration used in running the sample was calculated based on final protein concentration of each sample (>2 mg=2 %, 1 mg = 1.5 % and so on) and the final volume (18 ml) to be applied in the focusing chamber using the equation 4-2.

Equation 4-2; $C_1V_1 = C_2V_2$

Solving for V_1 where C_1=starting Bio-Lyte® concentration; V_1=unknown volume of Bio-Lyte®; C_2= Desired concentration of Bio-Lyte®; V_2=final volume for Rotofor (18 ml).

A mini Rotofor® system (220/240 V, 18 ml sample volume; Bio-Rad Laboratories, Hemel Hempstead, UK) was used to fractionate whey samples from *E. coli* mastitis, *S. aureus* mastitis, *S. uberis* mastitis and healthy milk according to various pi of protein molecules. The preparative isoelectric focusing was carried out according to the manufacturer's protocol;

The mini Rotofor cell electrodes were using 0.1 M phosphoric acid (H_3PO_4) as the anode (+) electrode and 0.1 M sodium hydroxide (NaOH) as the cathode (-) electrode. The focusing chamber was assembled by first sliding the anode electrode through the ceramic cooling finger and membrane core followed by the focusing chamber over the membrane core and ensuring that the membrane

core ports were not blocked by the focusing chamber screen. Next the cathode electrode was inserted through the cooling finger.

After covering the ports with sealing tape and cell-cover blocks, the focusing chamber was prepared by pre-running the cell with water at 5 watts constant power for 5 min. Next, a pre-sample solution of 1.5-2 % ampholyte (Bio-Lyte®; pH 3-10, 40 %; Bio-Rad laboratories, Hemel Hempstead, UK) in a solution of 5 M urea buffer (total volume 18 ml) was run at 12 watts constant power for 60 min. After discarding the pre-sample, 3 ml of whey sample in an ampholyte (1.5-2 %); 5 M urea solution (to make a final volume of 18 ml) was loaded using a syringe and needle into the focusing chamber and run at 12 watts constant power for 3 h. Fractions (n=20) were then collected by operating a vacuum pump through the harvest box, into pre-labelled culture tubes, and the pH of each fraction determined using a pH meter (Hanna instruments, Bedfordshire, UK).

Gel electrophoresis

Each whey fraction was then dialyzed in de-ionized water overnight to remove excess urea and concentrated using 0.5 ml centrifugal filter units (Merck Millipore, Germany), after which protein concentration of each fraction was determined and fractions were subjected to 1DE on SDS-PAGE to be resolved by molecular weight, as described in Section 3.3.1.6.

Bands of interest (selected based on visual quantitative or qualitative differences between the different whey fractions' 1DE) were carefully excised and placed in labelled 1.5 ml Eppendorf tube and taken for Trypsin digestion and further protein identification by liquid chromatography and mass spectrometry.

Trypsin digestion

The excised bands were placed in 1.5 ml Eppendorf tubes, cut into several pieces using pipette tips and washed for 30 min to 1 h by adding 500 µl of 100 mM ammonium bicarbonate (NH_4HCO_3) into the tubes and placing on a shaker. The wash was discarded and the gel pieces were further washed in 50 % (v/v) acetonitrile (ACN)/100 mM NH_4HCO_3 for another 30 min to 1 h. The wash was again discarded. For reduction, 150 µl of 100 mM NH_4HCO_3 and 10 µl of 45 mM DTT were added and incubated at 37°C for 30 min. For alkylation, 10 µl of 100 mM iodoacetamide was added and incubated in the dark for 30 min. Solvent was

discarded and gel pieces were washed in 500 µl of 50 % (v/v) ACN/100 mM NH_4HCO_3 with shaking for 30 min to 1 h. The wash was discarded and 50 µl of 100 % (v/v) ACN was added to shrink the gel pieces. After 10 min, the solvent was removed and gel pieces were dried completely in a vacuum centrifuge for 30 min. Sequencing grade modified Porcine Trypsin (Promega #V111, Southampton UK) in 25 mM NH_4HCO_3, was then added to the dried gel pieces enough to rehydrate each gel piece. Additional Trypsin solution was added if Trypsin was completely absorbed so that the gel band became fully rehydrated. The gel pieces were then incubated overnight to allow protein digestion at 37ºC. 30 µl of 100 % ACN was then added to the gel pieces and incubated for 20 min. Each solution in each tube was transferred to separate wells in a 96-well plate. 20 µl of 1 % (v/v) formic acid was added into each Eppendorf tube containing gel pieces and incubated for another 20 min. 40 µl of ACN (100 %) was added and further incubated for 20 min, after which the mixture was centrifuged briefly to sediment the gel and the solution was aspirated and transferred to the corresponding wells. Samples in the 96-well plate were then dried in a vacuum centrifuge which was stored at -20°C prior to LC-MS/MS.

LC-MS/MS

A nanoflow HPLC electrospray tandem mass spectrometry (nLC-ESI-MS/MS) was employed. Peptides samples (obtained after Trypsin digest) were solubilised in 2 % (v/v) acetonitrile with 0.1 % (v/v) trifluoroacetic acid and fractionated on a Thermo Scientific RSLCnano nanoflow uHPLC system (Thermo Scientific, Hemel Hempstead, UK). Peptide separation was performed on a Pepmap C18 reversed phase column (Thermo Scientific). Peptides are desalted and concentrated for 4 min on trap column followed by an acetonitrile gradient (in 0.1 % v/v formic acid) (3.2 – 32 % v/v 4 - 27 min, 32 % to 80 % v/v 27 - 36 min, held at 80 % v/v 36- 41 min and re-equilibrium at 3.2 %) for a total time of 45 min. A fixed solvent flow rate of 0.3 µl /min is used for the analytical column. The trap column solvent flows at a fixed rate at 25 µl/min using 2 % (v/v) ACN with 0.1 % (v/v) trifluoroacetic acid.

Online analyses were then carried out by electrospray ionisation (ESI) mass spectrometry on an Amazon Speed ion trap MS/MS (Bruker Daltonics, Coventry, UK). MS analyses were performed using a continuous duty cycle of survey MS

scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120 s. Mass spectra data were processed using Data Analysis software (Bruker, Coventry, UK) and the automated Matrix Science Mascot Daemon server (v2.4.1). Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI GenBank database, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses (Burchmore, pers. comm., 2014). LC-MS/MS analysis as well as data processing was carried out with the assistance of Ms Lorraine King under the supervision of Dr. Richard Burchmore (Glasgow Polyomics, University of Glasgow).

4.3.5 Statistical analyses

Tests for normal distribution were carried out on all data sets. Comparison of concentrations of the different APP in mastitis milk caused by different pathogens was carried out using an independent sample Kruskal-Wallis test. An independent Mann-Whitney's test was employed to compare APP of pathogen groups, pair wise in order to determine which pathogen had significantly different APP values from the other. Among the samples from the VDS, APP values from clinical and subclinical mastitis and healthy samples (drawn from Cochno composite samples with SCC < 200,000 cells/ml) as well as from different farms were also compared. Cross tabulation analysis for significant associations between factors was also carried out between farms, pathogen type and clinical status levels using Pearson Chi-square test and non-parametric correlations (Spearman's rho). P-value was considered significant at <0.05.

4.4 Results

4.4.1 Milk APP in natural mastitis caused by different pathogens

The distribution of the APP; Hp, M-SAA3 and CRP in milk analysed by microbiological culture and isolation at the VDS, across the different pathogen groups are shown in Figures 4-1, 4-2 and 4-3 respectively. The distribution of Hp, M-SAA3 and CRP for the different pathogens groups were significantly different (P=0.000). Similar and different pathogen groups are shown in Table 4-1. It was observed that *E. coli* had the greatest levels of each of the 3 APP among all the

pathogens. Next were the environmental pathogen *S. uberis* and *S. dysgalactiae* which also had APP significantly higher than that of other pathogens.

The type of mastitis (clinical or subclinical) was determined by the Veterinarian on the farm by presence or absence of clots in milk and history of signs of udder inflammation and therefore it was possible to classify the milk samples assayed in this study into CM and SM along with healthy samples. Clinical condition of the cows had a significant effect on the concentrations of each of the 3 APP assayed (P=0.000). Figures 4-4, 4-5 and 4-6 show the distribution of each APP across the various clinical conditions of the quarter/udder (clinical mastitis, subclinical mastitis, healthy or unknown). When subclinical mastitis samples were compared specifically with healthy samples (using independent samples Mann-Whitney U test), the 3 APP distributions were significantly different between the two categories (P=000). Clinical mastitis samples were also significantly different from subclinical (Hp- P=0.004; M-SAA3-P=0.041 and CRP-P=0.039) as well as healthy (Hp, M-SAA3 and CRP-P=0.000)

Farm of sample origin was another criteria used to categorise and compare the distribution of the APP in the milk samples. Figures 4-7, 4-8 and 4-9 displays the box plots showing median concentrations of Hp, M-SAA3 and CRP respectively, across the various farms of sample origin, which were also found to be significantly different across farms (P=0.000).

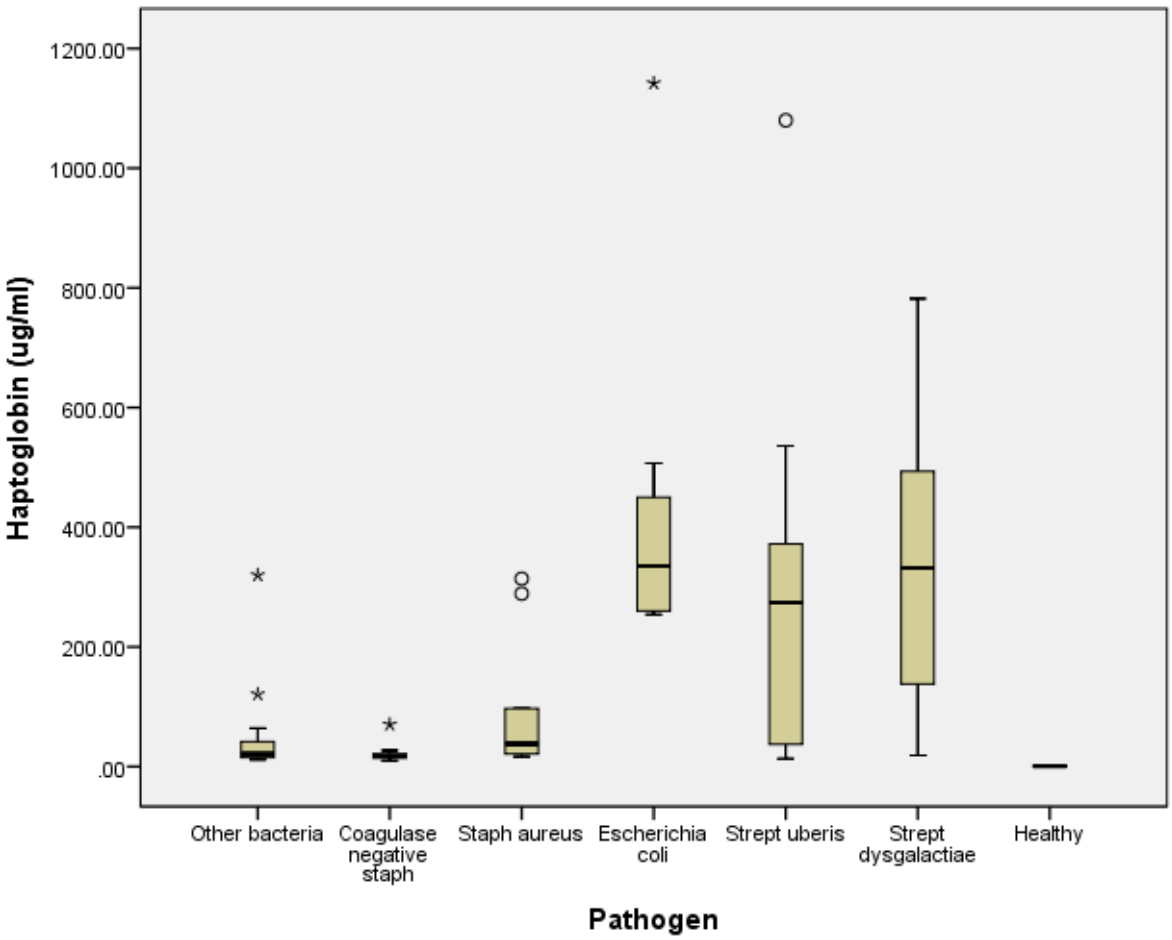

Figure 4-1: Box plot showing the levels of Hp in milk samples, across specific pathogens groups
* indicate extreme values, while º indicates outliers.

Table 4-1: P-values showing significant differences of the Hp values between the different pathogen groups
Significantly different P-values highlighted in light blue

HAPTOGLOBIN							
Pathogen	**Other bacteria**	**CNS**	***Staph aureus***	***Escherichia coli***	***Strept uberis***	***Strept dysgalactiae***	**Healthy**
Other bacteria	1	0.431	0.186	0.000	0.013	0.002	0.000
Coagulase negative	0.431	1	0.014	0.000	0.009	0.001	0.000
Staph aureus	0.186	0.014	1	0.002	0.238	0.036	0.000
Escherichia coli	0.000	0.000	0.002	1	0.155	0.541	0.000
Strept uberis	0.013	0.009	0.238	0.155	1	0.357	0.000
Strept dysgalactiae	0.002	0.001	0.036	0.541	0.357	1	0.000
Healthy	0.000	0.000	0.000	0.000	0.000	0.000	1

Other pathogens include:

-*Staph warneri* (n=2), *Staph. scuiri* (n=1*), Staph. pseudintermedius* (n=1), Staph*. chromogenes* (n=1), *Enterococcus faecalis* (n=2), *Corynebacterium spp* (n=3), *Lactococcus lactis* (n=1), *Enterococcus faecium* (n=1) and *Aerococcus. viridians* (n=1).

CNS-coagulase negative *Staphylococcus*

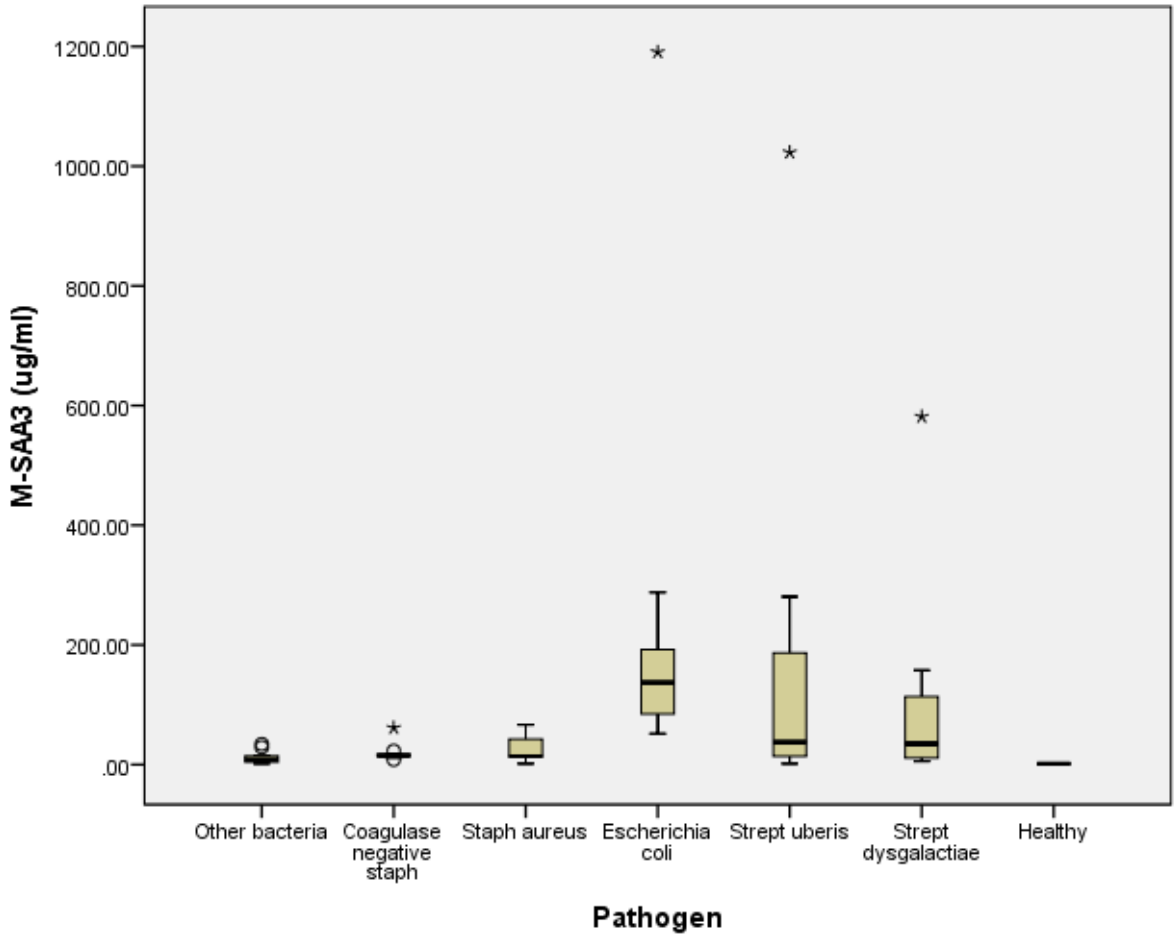

Figure 4-2: Box plot showing the levels of M-SAA3 in milk samples across specific pathogens groups
* indicate extreme values, while º indicates outliers.

Table 4-2: P-values showing significant differences of the M-SAA3 values between the different pathogen groups
Significantly different P-values highlighted in light blue

M-SAA3							
Pathogen	**Other bacteria**	**CNS**	***Staph aureus***	***Escherichia coli***	***Strept uberis***	***Strept dysgalactiae***	**Healthy**
Other bacteria	1	0.023	0.193	0.000	0.021	Sig.-0.025	0.000
Coagulase negative	0.023	1	0.863	0.000	0.238	0.481	0.000
5 Staph aureus	0.193	0.863	1	0.000	0.174	0.200	0.002
6 Escherichia	0.000	0.000	0.000	1	0.108	0.046	0.000
7 Strept uberis	0.021	0.238	0.174	0.108	1	0.776	0.000
8 Strept	0.025	0.481	0.200	0.046	0.776	1	0.000
Healthy	0.000	0.000	0.002	0.000	0.000	0.000	1

Other pathogens include:

-*Staph warneri* (n=2), *Staph. scuiri* (n=1*), Staph. pseudintermedius* (n=1), Staph*. chromogenes* (n=1), *Enterococcus faecalis* (n=2), *Corynebacterium spp* (n=3), *Lactococcus lactis* (n=1), *Enterococcus faecium* (n=1) and *Aerococcus. viridians* (n=1).

CNS-coagulase negative *Staphylococcus*

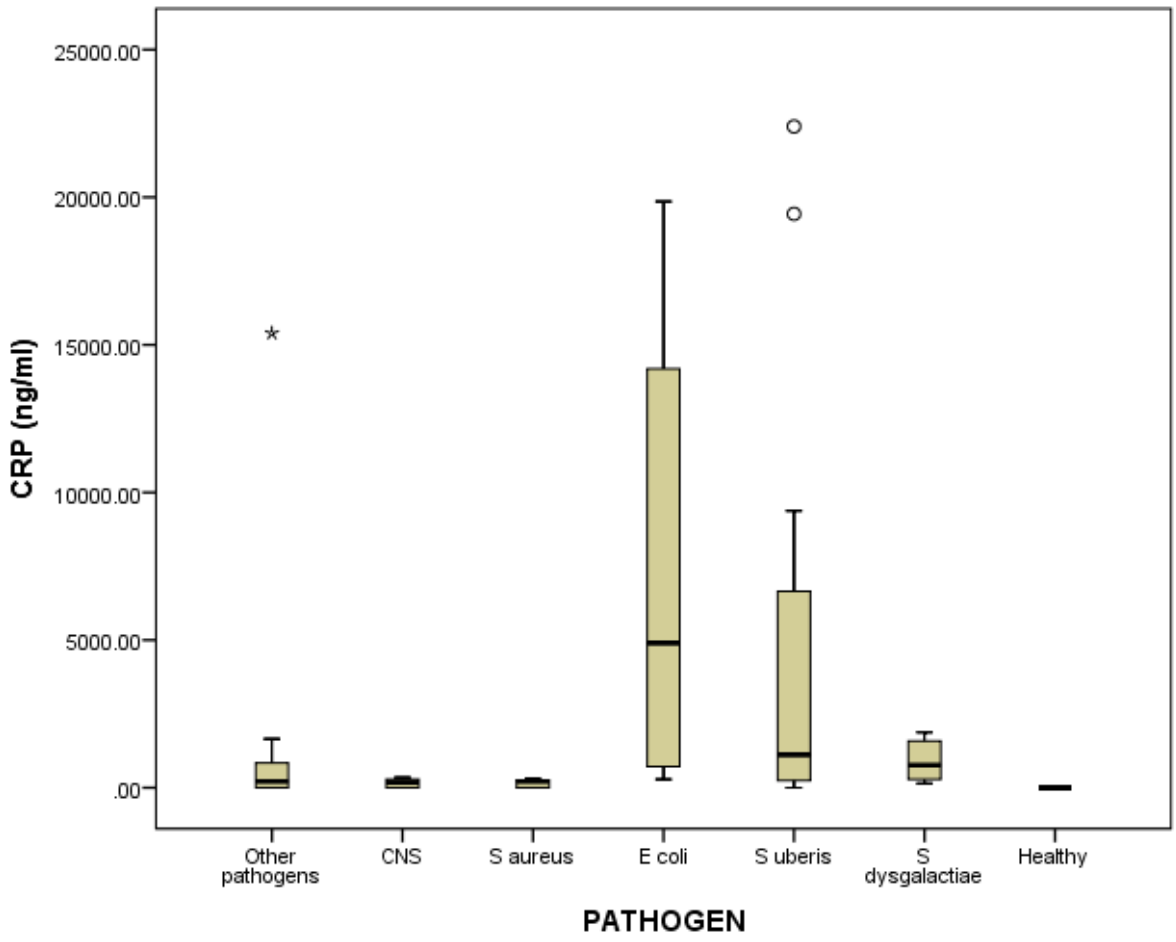

Figure 4-3: Box plot showing the levels of CRP in milk samples, across specific pathogens groups

* indicate extreme values, while º indicates outliers.

Table 4-3: P-values showing significant differences of the CRP values between the different pathogen groups
Significantly different P-values highlighted in light blue

CRP							
Pathogen	**Other bacteria**	**Coagulase negative**	**Staph aureus**	**Escherichia**	**Strept**	**Strept dysgalactiae**	**Healthy**
Other bacteria	1	0.556	0.750	0.002	0.413	0.185	0.000
Coagulasenegative staph	0.556	1		0.000	0.413	0.059	0.034
9 Staph aureus	0.750	1.000	1	0.000	0.169	0.038	0.004
10 Escherichia coli	0.002	0.000	0.000	1	0.035	0.015	0.000
11 Strept uberis	0.413	0.413	0.169	0.035	1	0.875	0.001
12 Strept dysgalactiae	0.185	0.059	0.038	0.015	0.875	1	0.000
Healthy	0.000	0.034	0.004	0.000	0.001	0.000	1

Other pathogens include:

-*Staph warneri* (n=2), *Staph. scuiri* (n=1*), Staph. pseudintermedius* (n=1), Staph*. chromogenes* (n=1), *Enterococcus faecalis* (n=2), *Corynebacterium spp* (n=3), *Lactococcus lactis* (n=1), *Enterococcus faecium* (n=1) and *Aerococcus. viridians* (n=1).

CNS-coagulase negative *Staphylococcus*

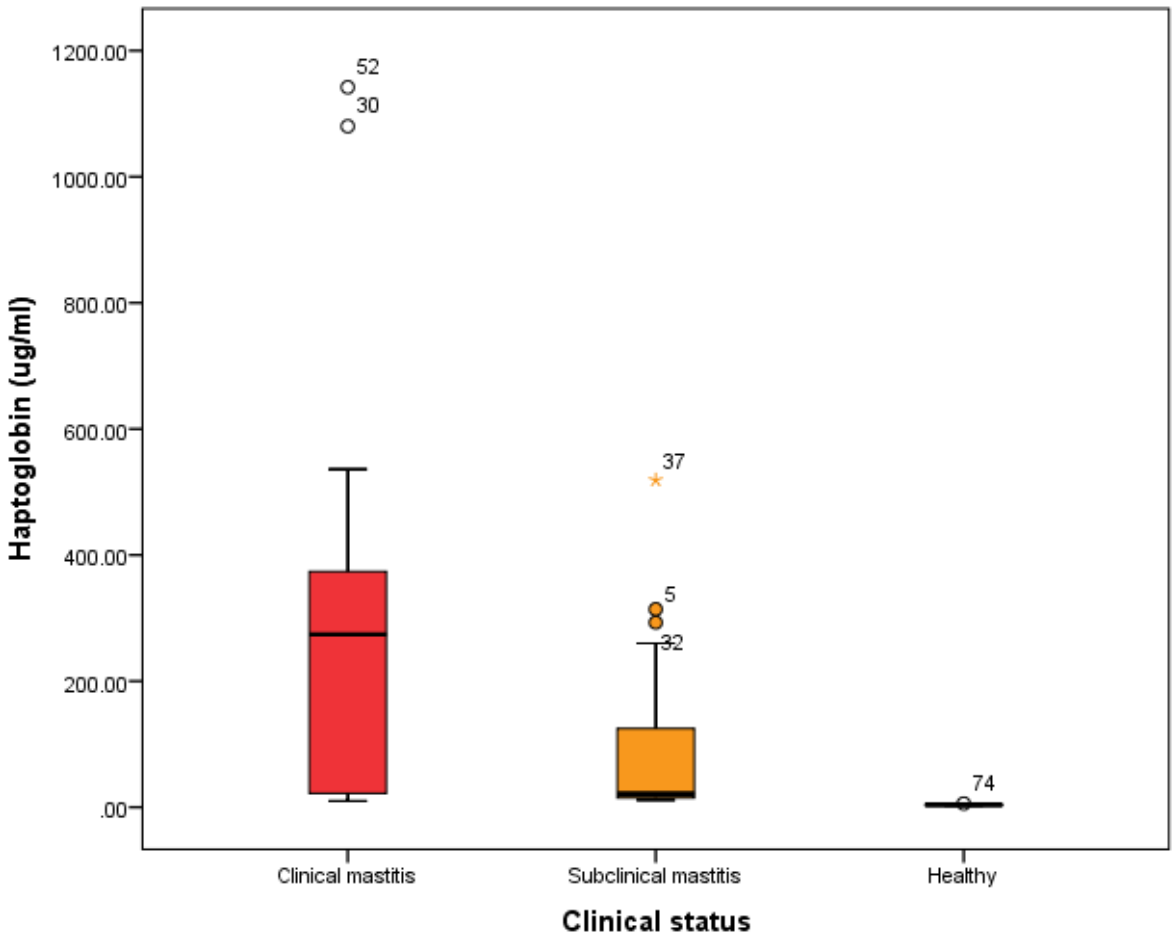

Figure 4-4: Box plot showing Hp concentration across the various clinical conditions of sample-source quarter/udder
* indicate extreme values, while º indicates outliers.

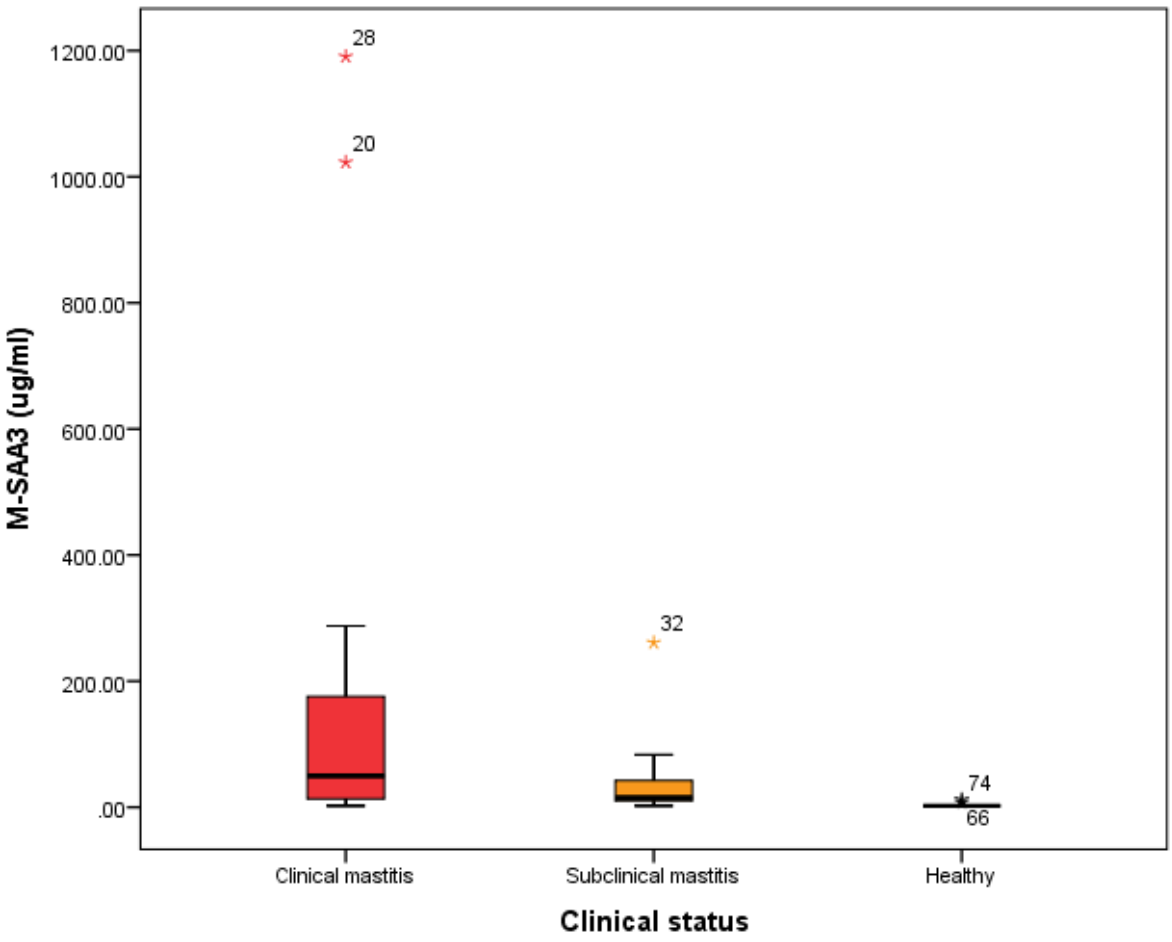

Figure 4-5: Box plot showing M-SAA3 concentration across the various clinical conditions of sample source quarter/udder
* indicate extreme values, while º indicates outliers.

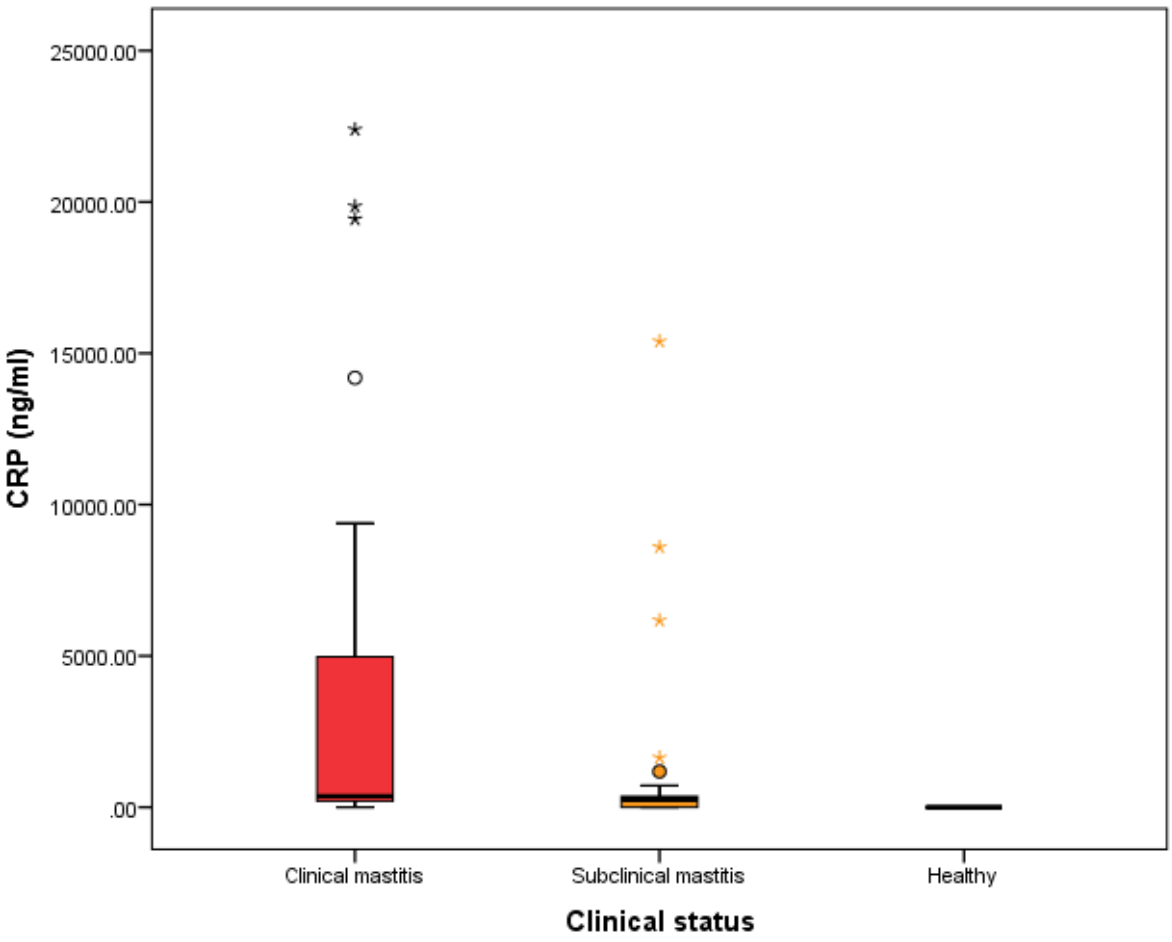

Figure 4-6: Box plot showing CRP concentration across the various clinical conditions of sample-source quarter/udder
* indicate extreme values, while º indicates outliers.

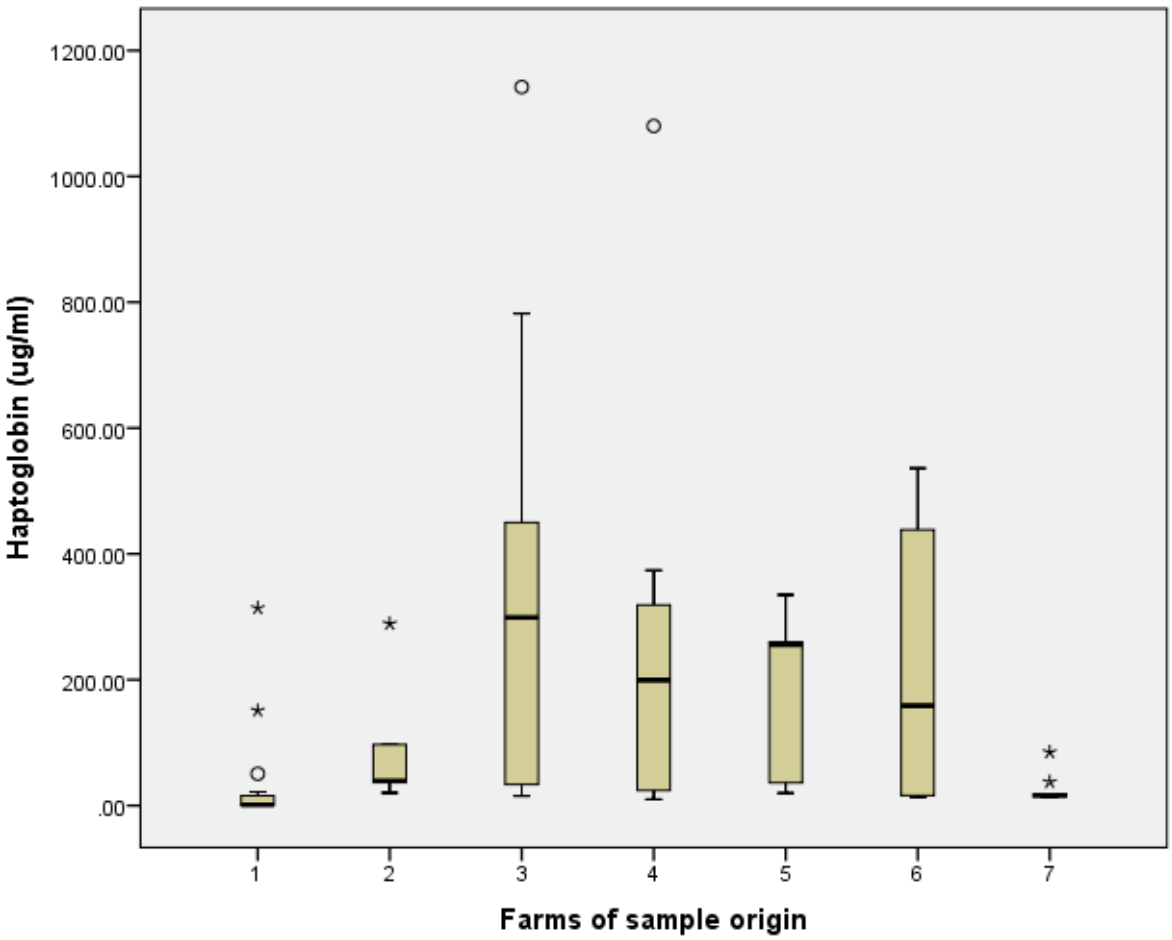

Figure 4-7: Box plot showing Hp concentration across the various farms from where the samples were obtained (Farm 1-7).
* indicate extreme values, while º indicates outliers.

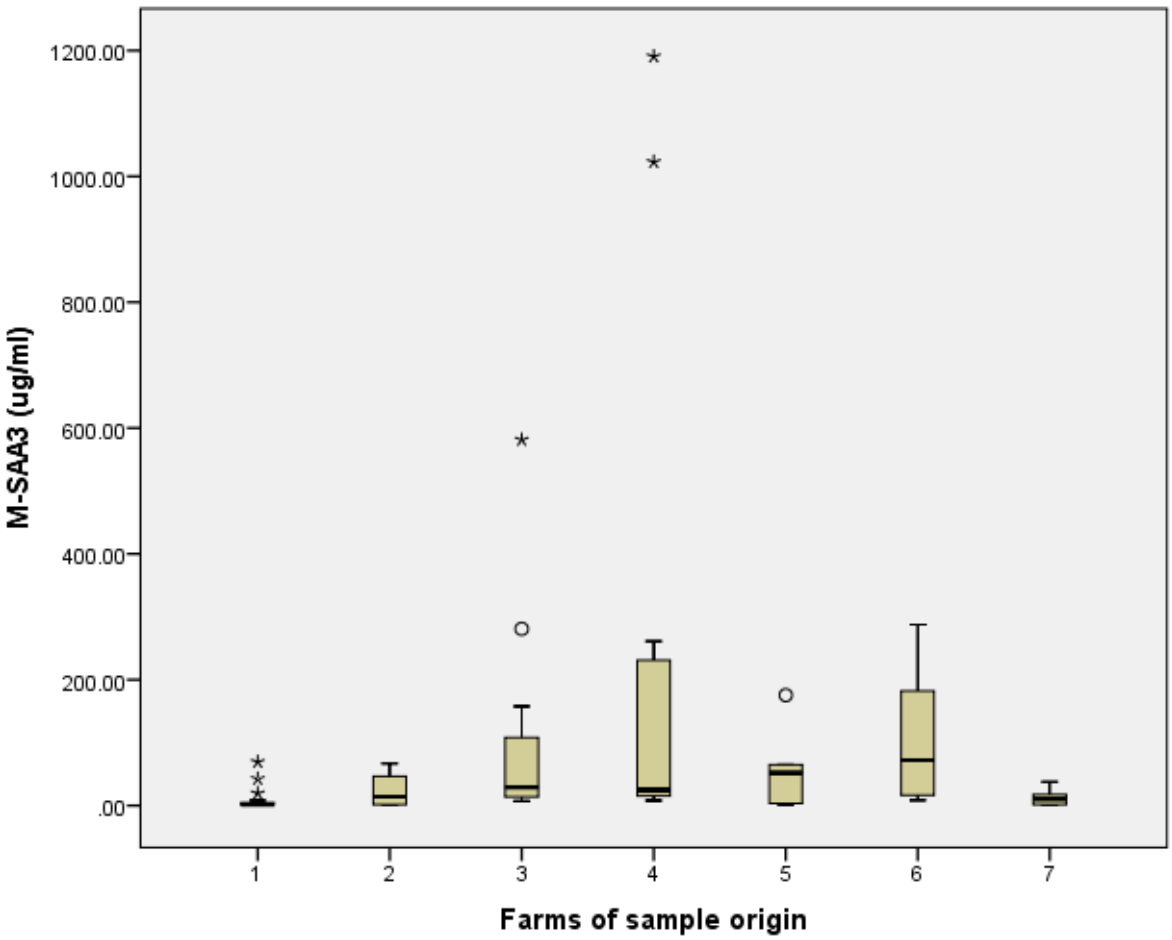

Figure 4-8: Box plot showing M-SAA3 concentration across the various farms from where the samples were obtained (Farm 1-7)
* indicate extreme values, while º indicates outliers.

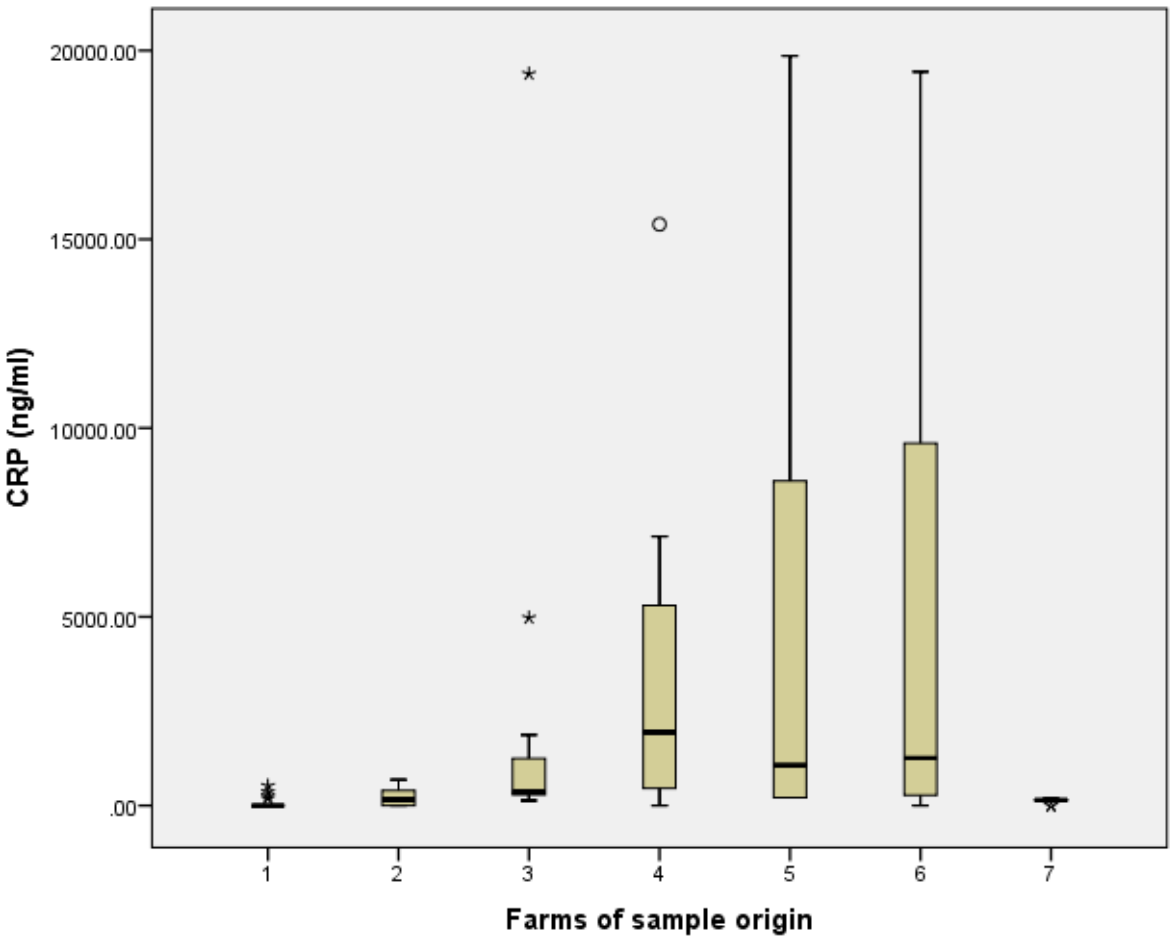

Figure 4-9: Box plot showing CRP concentration across the various farms from where the samples were obtained (Farm 1-7).
* indicate extreme values, while º indicates outliers.

There was a significant association between the clinical status of quarter/udder sampled and the pathogen detected in such samples (P=0.000). Figure 4-10 shows clustered bar charts of number of cases with different clinical statuses among the pathogens. There was also a significant association between pathogen-type detected and the farm of sample origin (P=0.013, shown in clustered bar charts in Figure 4-11). There was also significant associations between the clinical status and the farms (P=0.000, shown in clustered bar charts in Figure 4-12).

A significant positive correlation was found between Hp and M-SAA3 (P=0.01, r=0.72), between Hp and CRP (P=0.01, r=0.76) and between M-SAA3 and CRP (P=0.01, r=0.66) in this study.

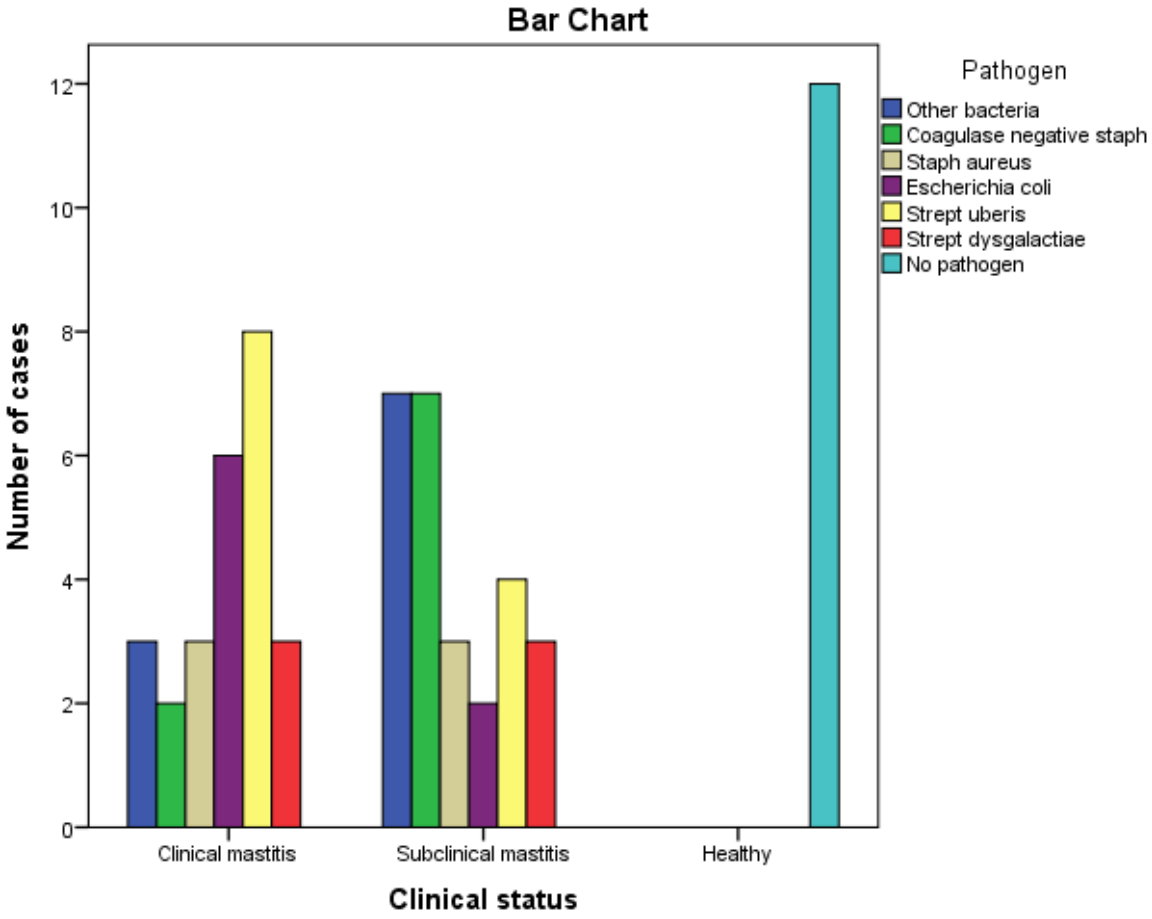

Figure 4-10: Clustered bar chart showing number of pathogen-type (cases) found between each clinical status of quarters/udders of samples origin
Pearson Chi-square test showed significant association between pathogens and clinical status (P=0.000), correlation between pathogen and clinical status (Spearman's rho) was also significant (P=0.003).

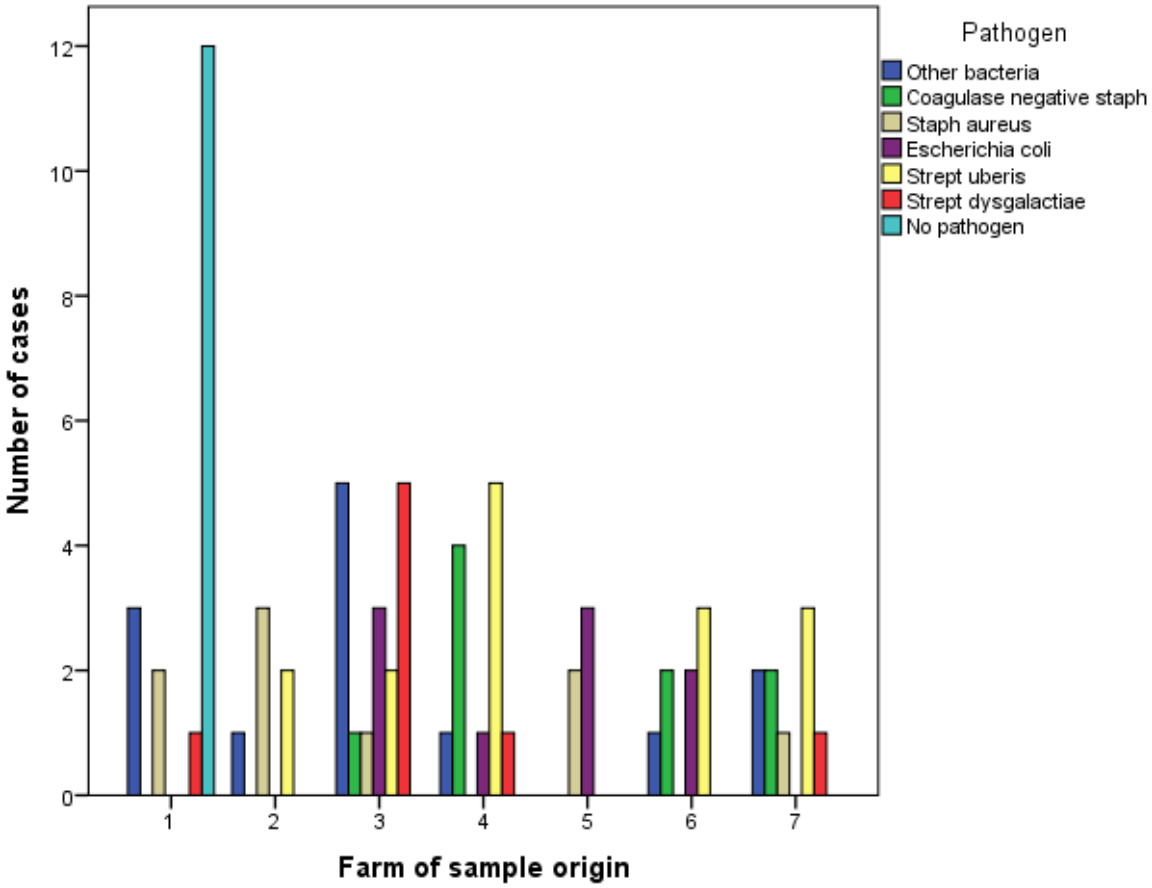

Figure 4-11: Clustered bar chart displaying the number of pathogen-type (cases) found in the different farms of sample origin
Pearson Chi-square test showed significant association of pathogens with farm (P=0.013).

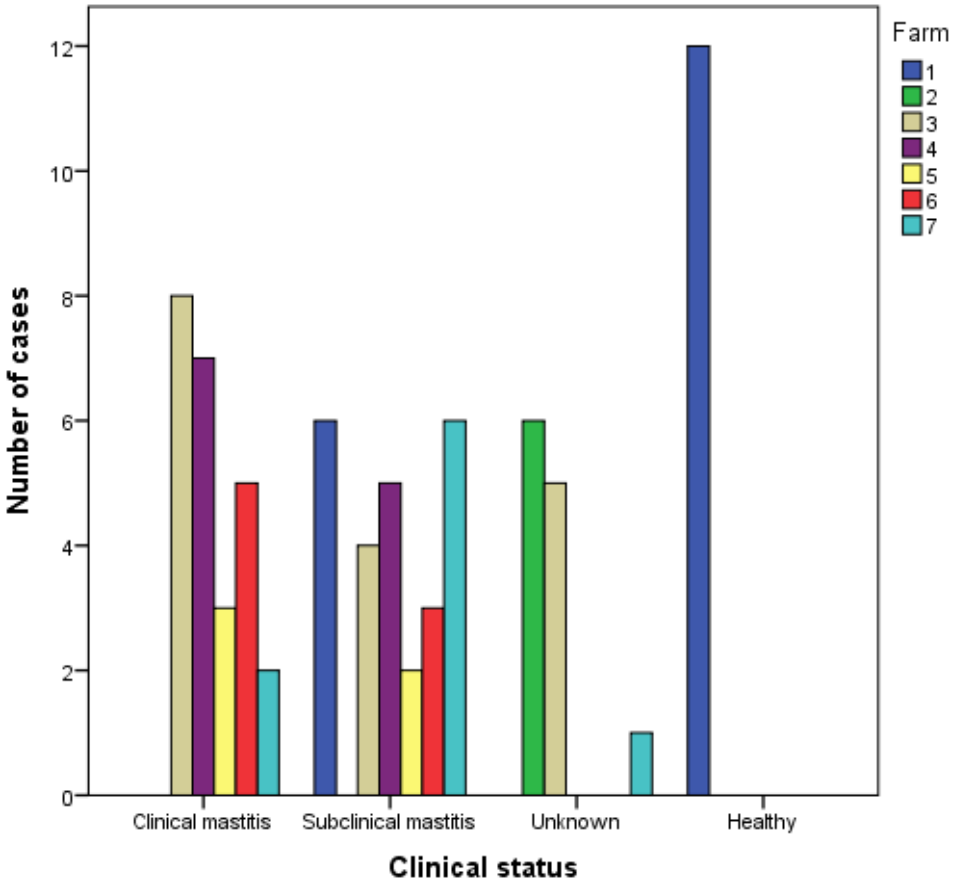

Figure 4-12: Clustered bar charts displaying number of cases in the different clinical statuses categories found per farm of sample origin.
Pearson Chi-square test showed significant association between clinical status and the farm of samples origin (P=0.000), correlation between farm and clinical status (Spearman's rho) was also significant (P=0.000).

12.1.1 Proteomic analysis of a model of gram positive and gram negative bovine bacterial mastitis

12.1.1.1 1DE SDS-PAGE

Figure 4-13 shows the 1DE gel image of the 3 pools (whey from *E. coli* (n=9), *S. aureus* (n=9) mastitis and healthy milk samples (n=9) loaded at a concentration of 2-3 mg/ml) per well.

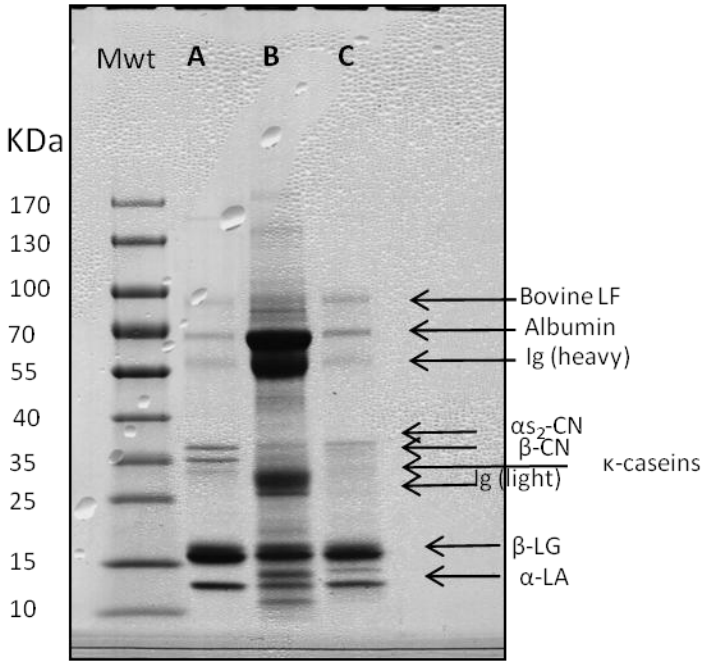

Figure 4-13: Skimmed samples from a pool of healthy (A), *E. coli* (B) and *S. aureus* (C) mastitis at concentration of ~2-3 mg/ml.
Ig (immunoglobulin), Bovine Lf (bovine lactoferrin), αS_2-CN (alpha S_2 casein), β-CN (beta casein), κ-CN (kappa casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin

12.1.1.2 Two dimensional electrophoresis: Rotofor® and SDS-PAGE

12.1.1.3 Rotofor® isoelectric focusing

Each pooled sample was subjected to isoelectric focusing to yield 20 fractions with pH ranging from 3.0-10.0 on the mini Rotofor® cell system. Figure 4-14 shows 1DE SDS-PAGE of 20 fractions with pH ranging from 2.71 to 9.92 for the healthy whey pool; Figure 4-15 shows *E. coli* pool fractions (pH 2.79 to 10.28) and the *S. uberis* fractions (pH 3.10 to 9.42) is shown in Figure 4-16. Bands excised for MS protein identification are indicated in circles. Table 4-4 gives a list of the bands and their corresponding proteins identified using the Mascot database search after LC-MS/MS analysis.

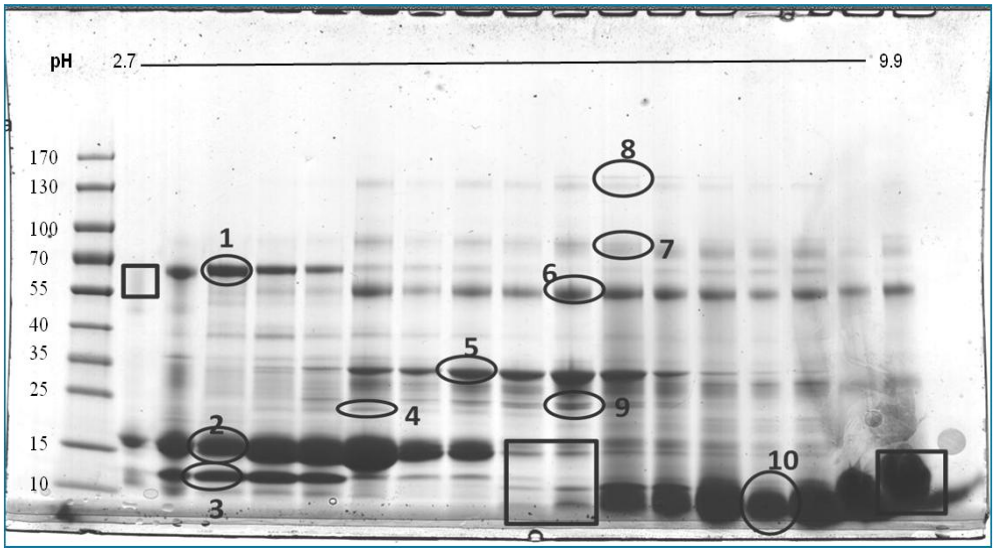

Figure 4-14: 1DE reducing gel of fraction of healthy milk samples with different pIs *(*following isoelectric focusing on Rotofor®)
Circled bands indicate bands excised for LC-MS/MS protein identification with the numbering corresponing to identifications listed on Table 4-4. Squared areas show bands representative of proteins not observable in conventional 2DE-SDS PAGE gels of the healthy milk sample.

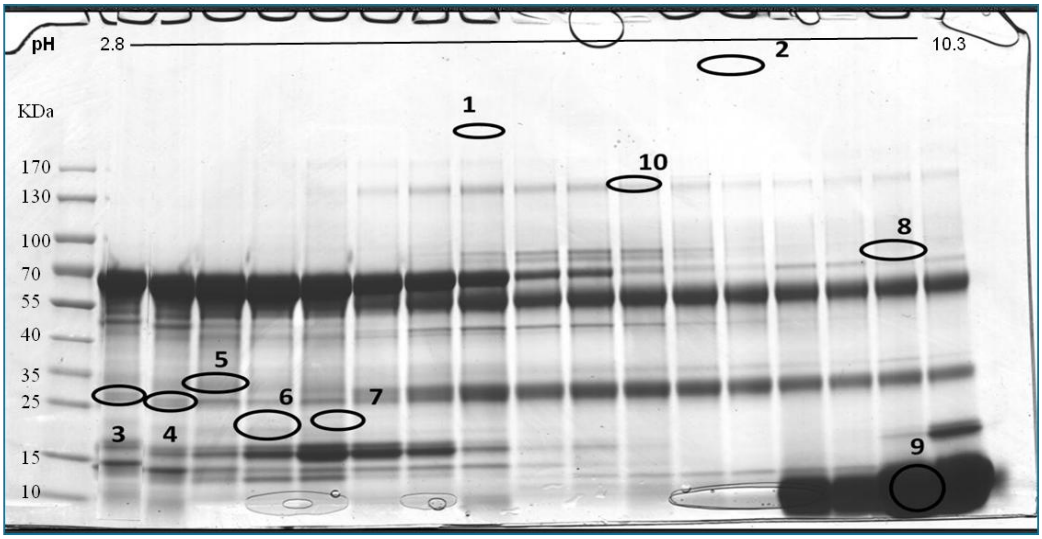

Figure 4-15: 1DE reducing gel of fractions of *E. coli* milk samples with different pIs (following isoelectric focusing using Rotofor®)
Circled bands indicate bands excised for LC-MS/MS protein identification with the numbering corresponing to identifications listed on *E. coli* section of Table 4-5.

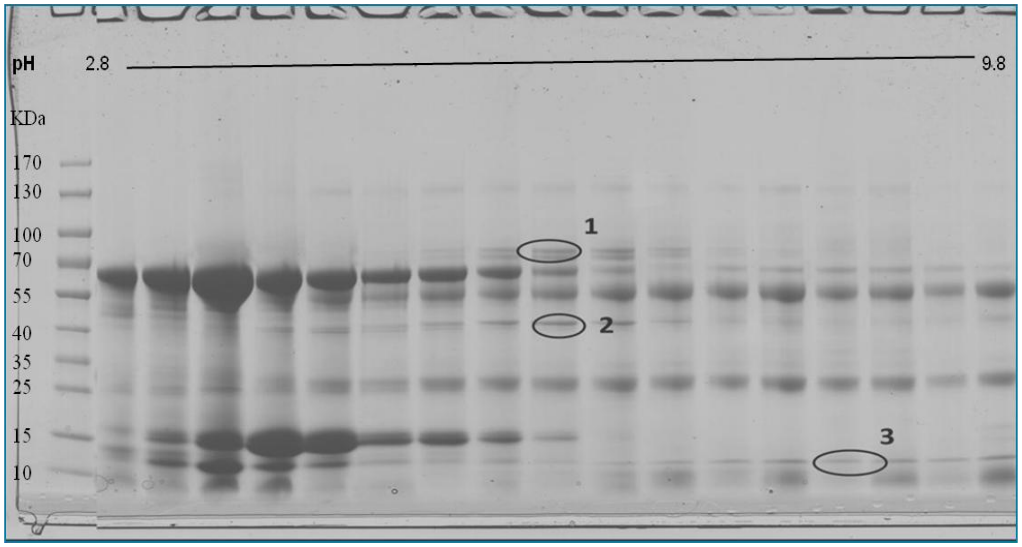

Figure 4-16: 1DE reducing gel of fractions of *S. uberis* whey samples with different pI (following isoelectric focusing on Rotofor®
Circled bands indicate bands excised for LC-MS/MS protein identification with the numbering corresponing to identifications listed on *S. uberis* section of Table 4-5.

Table 4-4: Excised bands from 1DE of Rotofor® fractions of healthy whey pool (shown in Figure 4-14) and the protein(s) identification.
The number of band corresponds to the Band ID.This was following Mascot searches after Trypsin digestion and LC=MS/MS analysis

Healthy pool							
Band ID	**Protein**	**Ac. Number**	**Calculated pi**	**Mass (Da)**	**MOWSE Score**	**Matches**	**% coverage**
1	Serum albumin	P02769	5.82	71244	4423	259(163)	68
2	Bovine Beta-Lactoglobulin	1BSO_A	4.76	18641	2588		97
3	Alpha lactalbumin	CAA44927	4.8	14603	1123	47(32)	40
4	Bovine Beta-Lactoglobulin	1BEB_A	4.83	18583	121	14(6)	50
5	Beta-casein A2 variant Lactoglobulin beta Chain L, Crystal Structure of Bovine Ab	AAB21950 732164A AAI02190	4.75 4.76 5.84	3798 18641 24910	42 72 86	7(1) - -	25 26 27
6	Serum albumin	P02769	5.82	71244	242	16(8)	11
	Ig heavy chain	AEY68824	6.1	51391	312	20(11)	10
7	Lactoferrin	AAA30610	8.73	80113	852	68(28)	46
8	Immunoglobulin heavy chain variable region	AEY68824	8.94	10127	39	3(1)	8
9	Ig lambda light chain constant region	AEM05851	8.49	11464	309	11(7)	47
10	Beta-lactoglobulin	AAA30412	4.6	6725	35	4(1)	56

Table 4-5: Excised bands from 1DE of Rotofor® fractions of the *E. coli* and *S. uberis* mastitis whey pools (gels shown in Figures 4-15 and 4-16) and the protein(s) identification.

The number of band corresponds to the Band ID. This was following Mascot searches after Trypsin digestion and LC=MS/MS analysis

***E. coli* whey pool**							
Band ID	Protein	AC. Number	calculated pi	Mass (Da)	MOWSE Score	Matches	% coverage
1	Serum albumin α-2 macroglobulin precursor	P02769 NP_001103265	5.82 5.71	71244 168953	727 599	67/30, 48/23	55 22
2	IgG1 heavy chain constant region	AAB37381	6.09	36510	452	33(16)	41
3	Neutrophil gelatinase associated lipocalin (NGAL)	XP_605012	9.35	22982	1174	75(36)	58
4	Neutrophil gelatinase-associated lipocalin isoformX2	XP_605012	9.35	22982	2191	97(72	65
5	Neutrophil gelatinase-associated lipocalin isoformX2 (NGAL)	XP_605012	9.35	22982	2191	97(72	65
6	Plasma retinol-binding protein Bovine Beta-Lactoglobulin Haptoglobin	P18902 1BEB_A HPBO	5.44 4.83 4.4	21397 18583 4734	350 259 95	16(10), 7(5), 2(2)	32 35 31
7	Apolipoprotein A-I preproprotein Bovine Beta-Lactoglobulin Neutrophil gelatinase-associated lipocalin isoformX2 precursor	P18902 1BEB_A XP_605012 P02769	5.71 4.83 7.08	30258 18583 11151	724 259 596	34(21), 7(5), 38(19)	55 35 58
8	Serum albumin Complement C3	P02769 Q2UVX4	5.82 6.41	71244 188675	973 1020	76(38), 56(36)	52 19
9	Beta 2-microglobulin	BAC56416	7.08	11151	270	22(12)	35
10	Serum albumin	P02769	5.82	71244	1502	115 (63)	61
***S. uberis* pool**							
Band ID	Protein	Ac. Number	Calculated pi	Mass (Da)	MOWSE Score	Matches	% coverage
1a,b	Serotransferrin precursor	DAA33056	7.13	79783	2621	137(87)	57
2	Serpin B3-like protein	XP_001254421	6.45	44318	1541	96(56)	41
3	ß-2-microglobulin	BAC56416	7.08	11151	284	19(14)	30

12.2 Discussion

12.2.1 Milk APP in mastitis caused by different pathogens

12.2.1.1 Haptoglobin

Since the extent of inflammatory signs and damage of udder tissue seen in any mastitis case depends on the pathogenicity of the mastitis causing bacteria (Pyörälä and Syväjärvi, 1987; Schukken *et al.*, 2012), the degree of acute phase response in the mammary gland also depends on the causative bacterial pathogen (Kalmus *et al.*, 2013). Wellnitz *et al.* (2013) also showed from their study that different bacterial endotoxins stimulated a variable panel of inflammatory cytokines.

In the present study, *E. coli*, *S. uberis* and *S. dysgalactiae* mastitis milk had significantly higher values of Hp (P=0.00) than other bacterial mastitis. This is in agreement with the findings from the study of Kalmus *et al.* (2013) and findings from Pyörälä *et al.* (2011) as well as Wenz *et al.* (2010) and the experimental models of Suojala *et al.* (2008).

Significantly lower milk Hp concentrations were observed for the CNS group of pathogens which are known to cause a mild form of mastitis (Pyörälä and Taponen, 2009; Simojoki *et al.*, 2011). Similarly, *S. aureus* mastitis in this study, showed relatively low milk Hp on average. However 2 out of 9 samples assayed from this group had high levels of Hp (289 and 314 μg/ml) compared to a range of 16.8-96.9 μg/ml (mean= 39.94 μg/ml) for the remainder of the samples. It has been suggested that the clinical course of an *S. aureus* mastitis depends on the genotype isolated (Pyörälä *et al.*, 2011) and consequently this could affect the level of APR detected in each case.

In the study of Pyörälä *et al.* (2011), Hp was undetectable in a sizable proportion of mastitis milk samples, this may be due to a high detection limit of the assay used (7.8 mg/l), and this was anticipated as being a problem with the use of Hp in the diagnoses of mastitis. However in the present study, all samples from clinical mastitis cases assayed fell within the working range of the assay.

Milk Hp concentration were found to be highest in samples from *Arcanobacterium pyogenes* mastitis compared with milk from other mastitis-

causing bacteria, whereas SAA concentration in the same samples were very low (Pyörälä *et al.*, 2011). *A. pyogenes* causes severe clinical mastitis (summer mastitis) characterized by purulent discharge and poor prognosis (NMC, 2001), therefore a high Hp value in *A. pyogenes* mastitic milk is more reflective of the inflammatory state of the gland than the low SAA values seen in milk isolated from this mastitis milk in the study of Pyörälä *et al*. (2011).

In addition, Hp showed more association with inflammation than did MAA in the study by Kalmus *et al*. (2013), as it changed more to reflect infections by a more diverse range of pathogens than did MAA (for example as in *A. pyogenes* mastitis). This suggests that different pathogenic or host response mechanisms underlie the secretion of different APP in response to inflammatory stimuli, and Hp may be more useful for indication of inflammation to support the identification of different bacteria in milk (higher sensitivity for broader spectrum of mastitis-causing pathogens) than M-SAA3.

In the study of Kim *et al*. (2011), different strains of *S. aureus* were used to induce mastitis and differences in serum cytokines (IL-8, IFN-γ, and TGF-β1) and milk cytokine (IFN-γ) were observed for the different strains. Strain typing of the various pathogens detected in the present study was not determined; however it would be interesting to determine the APP variation in milk from mastitis caused by different strains of the same pathogen.

Significant variations seen in the clinical conditions of sampled udders, show that levels of APP can be computed to reference ranges for clinical mastitis, healthy and subclinical mastitis milk samples, which would further improve the value of APP for mastitis diagnosis.

Significant associations were found between specific farms, clinical conditions of sampled udder/quarter and specific pathogens, with the environmental pathogens (*E. coli* and *S. uberis*) as well as *S. dysgalactiae* being more associated with CM and certain farms. This would suggest the presence of environment-related risk factors (e.g. poor milking hygiene, dirty pens etc.) in such farms that need to be addressed. Variations in Hp across farms may also be indicative of presence of different strains of similar pathogens in different farms which may stimulate APR to different degrees (e.g. some *S. aureus* infected milk having very high Hp concentrations relative to the remaining majority with low

Hp). Although the sample-size per different-bacterial-mastitis milk in this study was small, conclusions can be drawn that different organisms are able to stimulate the APP response in the mammary gland during mastitis to varying degrees.

12.2.1.2 M-SAA3

Gram negative coliform, *E. coli,* and gram positive *S. uberis* and *S. dysgalactiae* mastitis milk samples had higher average M-SAA3 levels than any other bacteria-mastitis samples assayed. *E. coli* and *S. uberis* have been described as environmental pathogens that have the ability to cause severe forms of mastitis; it was therefore not surprising that the APP response to mastitis by these pathogens were higher than for other pathogens. For contagious pathogens such as *S. aureus* and CNS, a milder M-SAA3 response was observed with low to moderate concentrations in samples. These findings also agree with the findings of Kalmus *et al.* (2013) and Pyorälä *et al.* (2011). Farm variations in M-SAA3 levels from samples with the same pathogens may indicate differences in strains of the pathogens (on different farms) which could stimulate APP secretion to different extents.

12.2.1.3 CRP

C-reactive protein concentration was found to also vary significantly with the causative pathogen of mastitis indicating variable level of stimulation of its secretion by the virulence factors of different pathogens. The results from this study agrees with the findings of Kruger and Neumann, (1999), who observed variable levels of milk CRP in milk from mastitis caused by different pathogens, however, common mastitis causing pathogens such as *E. coli*, *S. aureus* and *S. dysgalactiae* were not examined in that study. The present results therefore represents the first report of the comparison of CRP levels in mastitis milk from *E. coli*, *S. aureus, S. dysgalactiae* and CNS organisms. A similar pattern with the various pathogens of mastitis as observed for Hp and M-SAA3 was seen in the CRP analysis. This suggests that similar pathways of stimulation may be elicited for the secretion of the different APP secretion by the pathogens. This is further confirmed by the high positive correlation observed between the 3 APP.

All three APP examined in this first set of milk samples showed divergent levels that were able to distinguish clinical from subclinical mastitic samples. In addition, the values of the three APP were able to distinguish subclinical mastitis samples from healthy samples (SCC below 200,000 cells/ml) (P=0.00 for each APP). Hence it can be concluded that Hp, M-SAA3 or CRP can be applied to differentiate samples from healthy quarters from those with mastitis which are not showing any clinical signs of IMI (SM).

Overall, from this study on milk of naturally occurring mastitis from commercial dairy farms, it can be seen that several factors can affect the APP variation, notably the causative pathogen of mastitis. However, this does not undermine the usefulness and sensitivity of APP in indicating on-going inflammatory processes in the sampled quarter/udder. Indeed the assay of APP can be used as a confirmatory test for active (on-going) mastitis infections, where a technique such as PCR has been employed and has detected several pathogens, in other to discriminate the presence of milk contaminants or mammary gland commensals (in samples with one or more detected pathogens but with low APP) from actual mastitis-causing pathogens (samples with high APP), and also indicate false negative PCR or microbiological culture results (where no pathogens have been detected by PCR or microbiology but the same samples have high APP).

The level of the APP measured in milk samples could also give an indication as to the type or class of pathogen(s) responsible for the IMI; for example, as shown in this study and previous studies, environmental pathogens generally elicit a more pronounced APR with higher levels of APP than contagious pathogens.

12.2.2 Proteomic analysis of a model of gram positive and gram negative bovine bacterial mastitis

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis following Rotofor preparative isoelectric focusing was able to resolve proteins into their isoelectric points with numerous bands being visible. Compared with using a gel based isoelectric focusing of proteins, it can be concluded that a liquid phase isoelectric focusing could offer an advantage of concentrating the low abundance proteins away from the high abundance proteins (caseins and major whey proteins) at their pi of 4-5, thus making these lower abundance proteins

easier to identify, for example apolipoprotein A precursor, serpin and α-2 macroglobulin.

Some of the proteins which were differentially expressed in the mastitis (*E. coli*) whey pool included NGAL, complement C3, α-2 macroglobulin and apolipoprotein A. Some of these proteins have already been reported in experimental *E. coli* mastitis milk proteomics (Boehmer *et al.*, 2008; Boehmer, 2011) and play a role in the innate immunity against mastitis. Neutrophil gelatinase associated lipocalin has been described in several human studies as a biomarker for kidney disease (Magnusson *et al.*, 2012; Devarajan, 2008; Hinze *et al.*, 2008; Wheeler *et al.*, 2008; Suzuki *et al.*, 2008).

In bovine, NGAL was first isolated and purified from colostrum and an ELISA was developed for its measurement (van Veen *et al.*, 2006). There have been no reports of its presence in normal milk or even its presence in mastitis milk from previous studies of milk proteomics in relation to bovine mastitis. Lipocalins are a group of proteins produced in several organs of the body notably the liver, kidney, uterus and the mammary gland. They are involved in transport of small hydrophobic molecules and play crucial role in inflammation; examples include alpha-1-microglobulin, alpha-1-acid glycoprotein, apolipoprotein D; beta-lactoglobulin, complement component C8 and NGAL. Neutrophil gelatinase-associated lipocalin are specifically localized in some granules of neutrophils. They have been suggested to function in defence against bacterial infections by limiting bacterial assess to iron as well as transport of iron, retinol and fatty acids (van Veen *et al.*, 2006). It can be speculated that the isolation of this protein from bovine colostrum by van Veen *et al*. (2006) may be related to its requirement for conferment of maternal protection to the new-born while its observation in *E. coli* mastitis milk in the present study may also relate to its functions in host defence against bacteria. A similar protein; Lipocalin-type prostaglandin D synthase has been reported in mastitis milk by Baeker *et al*. (2002).

In the natural-infection *S. uberis* mastitis whey pool, fewer differential bands were seen from the healthy and *E. coli* pools thus only 4 bands were excised from the *S. uberis* gel. Three out of the identified bands were also proteins not normally detected in healthy whey samples namely serotransferrin precursor and

serpin B3, which were also identified in mastitis milk (Boehmer, 2011). These two proteins are more commonly seen in the blood where serotransferrin acts in the binding and transportation of iron (Chung, 1984) and serpin B3, acts as a serine protease inhibitor (Villano *et al.*, 2013). Therefore the presence of these proteins in the *S. uberis* infected whey samples is likely to be due to the seepage of blood constituents into milk from the damaged blood-milk barrier, which occurs during mastitis. In a recent study by Smolenski *et al.* (2014), a model of experimental *S. uberis* mastitis was subjected to a 2DE, MALDI-TOF MS and also a Gel LC-MS/MS proteomic analysis, and a large number of proteins (68) were identified in milk including a number of proteins related to host immune responses which were also identified for an *E. coli* challenge (Boehmer, 2011), and also serpins and serotransferrin as identified in the present study. In addition, four new proteins were identified including azurocidin 1, pancreatic adenocarcinoma up regulated factor (PAUF), common salivary protein BSP30b and serum amyloid P-component. Due to the fact that limited number of bands from the *S. uberis* pool gel was selected for protein identification in this study, no new proteins other than those in similar bands on the *E. coli* gel were detected.

12.3 Conclusions

The conclusions that can be drawn from this chapter include the fact that different pathogens have the ability to stimulate the synthesis and secretion of milk APP to varying degrees, and these levels of milk APP are also related to the severity of mastitis caused by each pathogen.

Furthermore, the use of a liquid phase first dimension fractionation of milk whey was observed to have an advantage of concentrating the high abundance milk proteins at their isoelectric points and therefore enabling the isolation of other les abundant milk proteins away from similar pi. Proteins of potential biomarker significance were observed in whey samples using this technique.

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13 Investigations of experimental *Streptococcus uberis* mastitis

13.1 Overview

In this chapter, the acute phase proteins profile and general proteomic studies of milk following an experimental mastitis challenge with a host adapted strain of *Streptococcus uberis* (*S. uberis*) is discussed. Proteomics technologies including 1DE, 2DE and difference gel electrophoresis (DiGE) as well as peptidomics analysis were employed in these studies.

13.2 Introduction

13.2.1 *Streptococcus uberis* mastitis

Streptococcus uberis is one of the most prevalent causes of bovine mastitis in the UK (Zadoks, 2007) and other countries (Verbeke *et al.*, 2014; Wang *et al*, 2013; Katholm *et al.*, 2012; Shima *et al.*, 2004). Generally classified as an environmental pathogen, *S. uberis* has been determined to have a wide genetic diversity with the occurrence of several of strains having varying levels of virulence (Zadoks *et al*. 2013; Tassi *et al.*, 2013; Wang *et al.*, 2013). There have also been reports of strains capable of cow to cow transmission within herds (contagious pathogens) (Tassi *et al.*, 2013).

In addition to the high prevalence rates of *S. uberis* mastitis, mastitis caused by this pathogen has been known to have a high tendency to reoccur in the same cow (Abureema *et al.*, 2014). This has been attributed mainly to reinfection of quarters with a new strain of the bacteria. In the study of Tassi *et al*. (2013), the immunological response of Holstein-Friesian cows to mastitis challenge with two separate strains of *S. uberis*; one, a host adapted one and the other non-host adapted one, were studied. Clear differences were observed between the host responses to the strains.

Numerous studies have attempted to elucidate the molecular properties of *S. uberis* in relation to bovine mastitis, several other studies have also been dedicated to understanding the host-pathogen responses to *S. uberis*, for

example Pedersen *et al*. (2003) studied the early inflammatory responses of the host to an experimental *S. uberis* infection showing the rise in milk APP. Bannerman *et al*. (2004) also explored the innate immune response to *S. uberis* mastitis in terms of the cytokines (IL-1 beta, IL-8, IL-10, IL-12, IFN-gamma and TNF-alpha) as well as CD14 and lipopolysaccharide binding protein (LBP).

Since strain specific clinical, cellular and immunological responses have been shown to occur during *S. uberis* mastitis, it follows that earlier reports of the APP profile during *S. uberis* may not represent the general pattern for *S. uberis* mastitis due to particular strain characteristics. Adaptation to host has been shown to develop in strains that persist for long period in herds (Tassi *et al.*, 2013). It would therefore be useful to further explore the APR to a different strain than previously reported.

In another study for by Smolenski *et al*. (2014), identification of suitable biomarkers for *S. uberis* mastitis in milk has been targeted. Utilizing a 2DE and gel electrophoresis liquid chromatography tandem mass spectrometry (GeLC-MS/MS) proteomics approach, new proteins in milk were found, having specific biomarker potentials for *S. uberis* mastitis (strain O140J). It would also be useful to identify changes in the proteome with course of *S. uberis* mastitis challenge that may vary from strain to strain.

13.2.2 Milk proteomics

General aspects of proteomics have been discussed in Section 1.1.7.14. After, genomics and transcriptomics, proteomics was the next major technological approach to the study of biological systems. However proteomics is more complicated as proteins within a system are more dynamic and subject to post translational modifications.

Milk is a major complex biological extracellular fluid that has been studied using proteomics. Caseins are the predominant milk proteins and the less abundant milk proteins are usually difficult to detect in proteomic analyses due to the masking effect of the high abundance milk proteins (Roncada *et al.*, 2012).

In the proteomic study of Hogarth *et al*. (2004), milk whey from mastitis cows was seen to have a composition nearing that of serum as a result of the compromised blood milk barrier, and subsequent leakage of serum proteins into

milk. Smolenski *et al.*, (2007) observed from their study the complexity of the milk proteome especially in relation to mastitis by using two different proteomic approaches 2DE-MALDI-TOF MS and LC-MS/MS. Several chaperonins which play a role in pathogen recognition were discovered, along with many other immune related proteins that had not been previously identified in milk, from that study.

Boehmer *et al*. in a series of studies (2008, 2010 and 2011) used a coliform mastitis model to study several features of changes in the milk proteome during mastitis. Firstly, new proteins not previously reported were identified in both normal and mastitic whey and multiple isotypes of serum albumin in mastitis whey samples (Boehmer *et al.*, 2008) and then a label free approach, specifically the number of peptides per identified protein, was employed for quantification of differentially expressed proteins (Boehmer *et al.*, 2010).

A similar study by Hinz *et al*. (2012) also identified several inflammatory markers and proteolytic products of casein hydrolysis in milk following an LPS challenge using a 2DE-MALDI-TOF MS approach. Using 2DE coupled to MALDI-TOF- MS/MS on normal and mastitic whey and serum samples, Alonso-Fauste *et al*. (2012), detected a panel of APP, antimicrobial proteins and other immune related proteins that were differentially expressed or regulated which have the potential to be used in diagnosing mammary infections.

Bislev *et al*. (2012b) used a quantitative proteomic approach to differentially examine the expression of 20 selected host-response related proteins in healthy and *S. uberis* mastitis mammary glands, and was able to demonstrate multiple fold changes in some of the proteins. From the study of Smolenski *et al*. (2014), 68 host defence proteins including 4 new proteins not previously seen in milk, and as many as 43 and 16 others identified in previous *S. aureus* (Reinhardt *et al.*, 2013) and *E. coli* (Boehmer *et al.*, 2008) studies respectively, were identified. Upon further validation of some of the proteins using western blotting technique, variable levels of response (of the proteins) were seen between milk from different quarters of individual cows and between quarters of separate cows. It was also observed that cathelicidins and S100 proteins showed relatively higher increases in response to mastitis than other proteins examined and were thus suggested as potential biomarkers of mastitis. Most

secretory products in milk were found to be decreased in response to mastitis (Smolenski *et al.*, 2014).

In all, these proteomic studies have contributed to the recognition of close to a hundred new proteins related to host immune response in milk in relation to mastitis. The application of milk proteomics continues to grow, particularly in the area of search for new mastitis biomarkers (Ceciliani *et al.*, 2014) and in the characterization of milk from different species in order to detect possible inter species adulteration of milk or cheese (Hinz *et al.*, 2012), as well as gaining a better understanding of systems biology of the mammary gland (Ferreira *et al.*, 2013).

Typical limitation with proteomics studies involves the complexity of samples and the need for optimal preparation and the heterogeneity of protein molecules. In addition, a varying dynamic range of proteins is challenging with high abundance proteins that can mask the detection of low abundance proteins.

13.2.3 Peptidomics

13.2.3.1 General

Peptidomics, which was first used in literature in the early 2000s, is another of the newly emerging and rapidly growing 'omics' technologies, has been described as an integrated analysis of the peptide content of an organism, body fluid, tissue or cell (Baggerman *et al.,* 2004). It may be considered a subfield of proteomics. Peptidomics has proven successful and holds further promise in a number of areas including neuroendocrine research, biomarkers for diseases such as cancer (Eleftherios, 2006) and drug discovery. Peptides are chains of two or more amino acids linked by amide (peptide) bonds, having molecular weight of 20 kDa or less and which play crucial roles in many biological processes. Polypeptides comprise of longer continuous chains of peptides while proteins are distinguished from peptides by being composed of two or more polypeptides arranged in a functional way within the body. In a biological sample or system, peptides are either intact small peptide such as hormones, cytokines and growth factors, or peptides released during processing or proteolytic breakdown of protein molecules. The peptidome is therefore the collection of all peptide molecules within a given biologically system and these have the potential to

change with the physiological and pathological state of the system. Bioactive peptides include cytokines, hormones, neuropeptides involved in nervous system function such as neurotransmission.

Peptidomic analyses of some biological samples such as serum and urine has led to the discovery of panel of biomarkers for several disease conditions such as cancers (Berry *et al.*, 2013; Bauça *et al.,* 2014), metabolic disorders such as diabetes (Budde *et al.*, 2005), kidney disorders (Good *et al.*, 2010) amongst others.

13.2.3.2 Methodologies for peptidomics

Peptides are too small to be visualized on 2D gels as they either run off the gels or do not pick up even the most sensitive stains (Clynen *et al.*, 2008). Peptidomic analysis depends largely on mass spectrometry techniques such as MALDI-TOF or ESI combined with MS/MS, and usually requires identification of all available peptide molecule entities unlike in proteomics where not all digested peptide fragment needs be identified for protein recognition.

Peptide analysis in many studies also entails their relative quantification, especially if such content would be assessed in relation to biomarkers discovery; and this is carried out by isotope affinity labelling such a iTRAQ or secondary antibodies (TMAB) which allow quantification using either the MS or MS/MS spectra (Clynen *et al.*, 2008).

Peptidomics also requires identification of all available peptide molecule entities, unlike proteomics where not all digested peptide fragments need to be identified for protein recognition. Concentration methods to enhance analysis of low abundance peptides in a complex biological matrix or solution include combinatorial and affinity peptidomics, where low abundance peptides are enriched and high abundance proteins or peptides in the samples are reduced (Soloviev and Finch, 2006). Tandem mass spectrometry (MS/MS) is often employed for identification of peptides or *de novo* sequencing can be applied to identify peptides based on their amino acid sequence from mass peaks as well as comparison of observed mass spectra to theoretical peaks obtainable from a genome (Costa *et al.*, 2013).

A new identification tool for peptides after tandem mass spectrometry has been described by Costa *et al*. (2013), this analyses the 6 frame translation of a genome and is not limited to sets of *de novo* reconstruction sequences and search space and is specifically designed for peptidomics. Peptide analysis frequently also entails their relative quantification, especially if such data could be assessed in relation to biomarker discovery, and this can be carried out by isotope affinity labelling such iTRAQ, secondary antibodies (TMAB) and so on, which are then quantified using either the MS or MS/MS spectra(Clynen *et al*., 2008).

Following mass detections, peptides can be identified by several methods including database searches, spectral matching algorithms, *de novo* sequencing or hybrid approaches; many of these tools can be accessed from the internet. Databases such as SwePep, PeptideDB, Peptidome and Erop-Moscow are a few examples of bioactive peptide databases, while more general peptide databases including X! Hunter and SpectraST, PeptideAtlas are available for peptides searches. A bovine specific peptide atlas has now been produced at 'CowMilk2011-https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/buildDeta-lsatlas_build_id=320' (Bislev *et al*., 2012a). These databases can be searched based on peptide characteristics such as length, mass or amino acid sequence for data validation. Peptide identification is often validated by means of statistical tests including identification probabilities and false discovery rates (FDR) computation. Conventional separation technologies for peptides within complex mixtures comprise chromatographic techniques such as liquid chromatography, including reversed phase HPLC, and ion exchange chromatography (Schrader and Schulz-Knappe, 2001).

Combinatorial peptidomics is a modified method of peptidomic assay which has been described by Soloviev and Finch (2005). It is said to have the added advantage of being able to detect and quantify more proteins in an easier procedure which uses special functional groups contained within amino acids that make up the peptides, to affinity-purify the peptides in the complex protein mixtures (as obtainable in biological samples) (Soloviev and Finch, 2005). From the human diagnostics point of view, peptides within cerebrospinal fluid (CSF) and synovial fluid have been shown to have potential for use in diagnosis of Alzheimer's disease, and osteoarthritis respectively (Clynen *et al*., 2008). A

specific panel of peptides in saliva have also been linked to diseases of the teeth in a recent study (Trindade *et al*., 2014).

Peptidomics has also been used to elucidate proteolytic regulation of bioactive peptides as a key to understanding the physiology and identifying possible drug targets of these peptides (Kim YJ *et al*., 2013).

13.2.3.3 Milk peptidomics

Although several milk peptidomic studies have been carried out as reviewed in Section 1.1.7.15, most have been focussed on recognizing the patterns and contributors to proteolysis in milk during mastitis. Only a few studies have explored the possibility of the use of these proteolytic breakdown products in milk (peptides) as biomarkers that can differentiate mastitic from normal milk and even milk samples from mastitic quarters caused by one pathogen from those caused by a different pathogen.

One such study was carried out by Mansor *et al*. (2013). In that study, a panel of peptides (n=154) that could differentiate milk of mastitic quarters from milk of normal (healthy) quarters, and differentiate milk from *S. aureus* and *E. coli* mastitis (n=47) and serve as potential biomarker panel of peptides were recognized. CE-MS as well as LC-MS/MS methods were used to characterize the peptides in milk from healthy and naturally occurring *E. coli* and *S. aureus* mastitis. Thus, the potentials of a peptidomic approach to biomarker discovery for bovine mastitis were highlighted in that study. Suggestions for further studies, which would additionally exploit the peptidomic tool, to resolve the critical need for biomarkers of bovine mastitis, were underscored.

13.2.4 Objectives

The objectives of this study were:

- To explore the changing profile of milk APP following experimental challenge of six dairy cows' quarters with a host-adapted strain of *S. uberis*. This was done in order to evaluate the usefulness of the APP; Hp, M-SAA3 and CRP for identifying new infections and in reflecting the stage of disease comprising the early, peak and resolution stages of the

infection, as well as to gain a better understanding of host response to *S. uberis* mastitis as it relates to secretion of these APP.

- To utilize the different gel based proteomics approaches (1DE, 2DE and DiGE) as a pre-fractionation method prior to identification of qualitative and quantitative protein changes in the proteome of milk during the course of the experimental mastitis challenge.

- To elucidate the changing peptide composition of milk during key time points of the *S. uberis* mastitis challenge using a capillary electrophoresis mass spectrometry-based peptidomics analysis of milk samples.

These experiments, it is hoped, will offer insight into the course of proteolytic changes in milk and facilitate a better understanding in associating the changes caused by a specific pathogen, with time duration and host responses to mastitis. In addition it was anticipated that a valuable biomarker panel of peptides will be recognized in order to distinguish mastitic milk from healthy samples.

13.3 Materials and methods

13.3.1 Reagents

Reagents for polyacrylamide gel electrophoreses (PAGE) were obtained from Bio-Rad laboratories Ltd (Hemel Hempstead, UK). Buffers and stock solutions were prepared according to manufacturer's instructions. A 4-15 % Criterion™ precast 18-well comb and 11 cm IPG+1 well comb gels (Bio-Rad Lab, *Inc.* USA) were used for the 1DE and 2DE respectively. Milli Q water was used throughout.

13.3.2 Experimental challenge

For this investigation samples were provided by Dr Tom McNeilly and Professor Ruth Zadoks (Moredun Research Institute, Penicuik, Scotland) from a previous intramammary challenge study of six quarters from six cows using a putative host adapted strain of *S. uberis*, strain FSL Z1–048 carried out as described by Tassi *et al*. (2013). The intramammary challenge was carried out at the experimental unit of the Moredun Research Institute, Penicuik, Scotland. Samples were obtained at 19 time points from each challenge-positive quarter

(n=114) comprising 0, 6, 12, 18, 24, 30, 36, 42, 48, 57, 72, 81, 96, 120, 144, 168, 192, 240 and 312 h post challenge and 7 time points (n=42) including 0, 12, 36, 57, 96, 192 and 312 h post challenge, for the challenge control quarters which were infused with 2 ml of sterile phosphate buffered saline (PBS). Skimmed milk was prepared by centrifuging 50 ml of milk at 2,800 *x g* at 4°C for 20 min. The fat layer was discarded and the supernatant was transferred to a new 50 ml Falcon tube. Centrifugation was repeated and the supernatant was stored at −20°C until analysed. Somatic cell counts (SCC), milk bacterial counts, clinical scoring and the cytokine profile for these samples were determined at the Moredun Research Institute and reported in Tassi *et al*. (2013).

13.3.3 Acute phase proteins during experimental *S. uberis* mastitis

13.3.3.1 Haptoglobin

ELISA

All samples collected from positive challenge (19 time points from each of the 6 infected quarters, n=114) and challenge control quarters (7 time points from each of 6 quarters, n=42) during the course of the *S. uberis* mastitis challenge were analysed for Hp concentration using the in house Hp ELISA as earlier described (2.3.2.1.2), with modifications carried out in the dilutions used for samples with high Hp (maximum dilution of 1:1600 was used).

Western immunoblotting analysis of milk Hp

Samples from challenge positive quarters ranging from 0 to 192 h post challenge were subjected to western immunoblotting for Hp as described in Section 3.3.1.3.2.

13.3.3.2 M-SAA3

The M-SAA3 was also measured in (skimmed) milk samples of challenge positive quarters (19 time points) (n=114). Analysis was carried out using the commercial ELISA kit as described in Section 2.3.3.3; with modifications carried out in the dilutions used for samples with high M-SAA3 (highest dilution used was 1:5000).

13.3.3.3 CRP

Challenge positive samples from the infected quarters (n=114) were analysed for CRP concentration as described in details in Section 2.3.4, with modifications carried out in the dilutions used for samples with high CRP (maximum dilution of 1:2000 used).

13.3.4 Proteomics

13.3.4.1 Total protein concentration

The total protein concentration of whey samples used for the proteomics analyses were determined by Bradford method (Appendix Chapter 4) with bovine serum albumin (BSA) used as the standard.

The Bicinchoninic acid assay (BCA) Uptima from interchim (Montluçon, France) was used to determine protein concentration in samples for peptidomic analysis prior to CE-MS investigation. About 25 µl of each standard and milk extracted samples were pipetted into microplate wells in duplicates. The BCA assay reagent was then added (200 µl) and mixed. The sample mixtures were incubated at 37°C for 30 min or 2 h at RT. The microplate was cooled to RT and absorbance read at wavelength of 562 nm. The protein concentrations of the samples were interpolated from a standard calibration curve of protein concentration using bovine serum albumin (BSA) (Sigma Aldrich, Dorset, UK).

13.3.4.2 1DE SDS-PAGE

The samples from 19 time points of one representative challenge quarter and the 7 time points of another representative control quarter were subjected to 1DE SDS-PAGE. Protocol for this procedure has been described (3.3.1.5.1).

13.3.4.3 2DE SDS-PAGE

Whey samples from six time points selected across the duration of the intramammary challenge (including 0, 36, 42, 57, 81, 168 and 312 h post infection) were each subjected to a 2DE SDS-PAGE after pooling each time point together from all infected quarters. The 2DE SDS-PAGE procedure has been described in Section 3.3.1.5.2.

13.3.4.4 DiGE

Samples

Pooled samples of three selected time points; 0, 81 and 312 h post challenge following the *S. uberis* mastitis challenge were analysed using DiGE. These corresponded to samples from pre-infection, peak and resolution phase of the mastitis challenge, respectively.

Sample Preparation

Preliminary sample preparation was carried out by pooling 1 mg/ml of protein from each of the six samples from time points 0 h, 81 h and 312 h, to give a volume with a total protein content of 6 mg of each of the 3 time points. Acetone precipitation was then carried out on each of the pools by adding 4 times the sample volume of ice cold 100 % acetone to the samples; this was mixed thoroughly and kept at -20°C overnight. Precipitate was separated from supernatant by centrifugation at 1400 x*g* for 30 min at 4°C. The pellets formed were then washed by mixing thoroughly with ice cold 80 % (v/v) acetone and then centrifuged at 1400 x*g* for 30 min once again. This step was repeated two more times. Finally all supernatant was separated from the pellet and the pellet allowed to air dry by exposing to air. Subsequently the pellet was re-suspended in 250 µl DiGE lysis buffer (Appendix Chapter 5). The total protein concentration of the re-suspended pellet was then determined by the Bradford method and adjusted to 5 mg/ml by addition of more DiGE lysis buffer.

CyDye Labelling

One µl of CyDye was added to 10 µl of sample and incubated in the dark at 4°C for 30 min. To stop the reaction, 1 µl of 10 mM lysine was added and further incubated in the dark for 10 min. The different dyes to sample mixtures that went into each DiGE gel are shown in Figure 5-1.

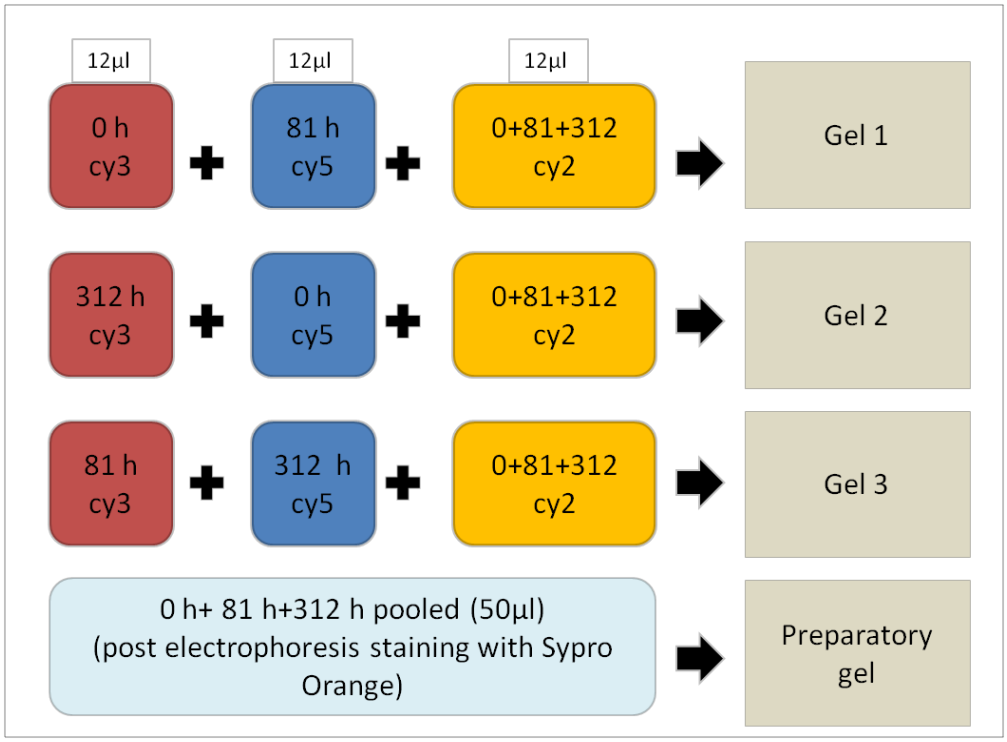

Figure 13-1: Protocol of samples and corresponding dye labels, and pools used for each gel in the DiGE experiment.
Red dye-cy3, blue dye-cy5, yellow dye-cy2 (pooled standard). 50µl of pooled samples were used for the preparatory gel.

Isoelectric focusing

The DiGE samples (labelled Cy3, Cy5 and pool standard labelled Cy2; ~12 µl of each) were mixed together and 424 µl of rehydration buffer (RHB) was added into the sample mixture and mixed thoroughly. 50 µl of the unlabelled pooled standard was added to 410 µl of the RHB. 450 µl of each DiGE pool and preparative gel pool was then pipetted along the length of each strip holder, avoiding any formation of bubbles. The IPG strip (24 cm pH 4-7, GE Healthcare, UK) was put onto the strip holder and covered and this was left for ~ 30 min for the gels on the strip to rehydrate passively. 1 ml of mineral oil was then applied over the top of the strip, just enough to cover it. The lid was placed back on and the strip holder was placed on the IPGphor system (GE Healthcare, UK). Isoelectric focussing was carried out on the IPGphor using the protocol in Table 5-1. All aspects of the DiGE experiment were carried out with the assistance of Mr Alan Scott (Institute of Infection Immunity and Inflammation, University of Glasgow) and under the supervision of Dr. Richard Burchmore (Glasgow Polyomics, University of Glasgow).

Table 13-1: Protocol for running isoelectric focusing of DiGE IPG strips

Step	Volts	Time (h-hours)
1	30 V step and hold	10 h
2	300 V step and hold	2 h
3	600 V gradient	2 h
4	1000 V gradient	2 h
5	8000 V gradient	1 h
6	8000 V step and hold	9 h
7	1000 V step and hold	1 h

After isoelectric focussing, the strips were taken out of the holders, washed briefly in milli Q water to remove excess mineral oil and put into the plastic tubes. 10 ml of SDS EB I (see Appendix Chapter 5) were placed onto the strips and the plastic tubes were put on a large flat rocker desk for 15 min. The buffer was poured off and SDS EB II (see Appendix Chapter 5) was added and was rocked gently for another 15 min. The buffer was poured off and the strip(s) were inserted horizontally between then DiGE gel (precast Ettan DALT® gels; 26 x 20 cm) cassettes and allowed to make contact with the gel. 1 ml of 0.5 % (w/v) agarose was added on top of the DiGE gel (before putting the gel in the electrophoresis running tank). Running buffer was added into the tank assembly and electrophoresis was carried overnight at 1W/gel.

DiGE Gel Scanning

DiGE gels were scanned on a 3 Laser Typhoon 9400 scanner (GE Healthcare life sciences, Buckinghamshire, UK). A preliminary scan was performed on each DiGE gel at a low resolution (1000 microns). Spot saturation was checked on each of the resulting images, and the photomultiplier tube voltage adjusted so that the most intense protein spots were not saturated (according to DiGE instruction manual, GE Healthcare). A high resolution (100 microns) scan of each DiGE gel was then done. Once satisfactory images were obtained, DiGE gels were stored in a wet tray and kept at 4°C for a maximum of one week. The preparative gel was fixed in 7 % (v/v) acetic acid + 10 % (v/v) methanol for 1-2 h and then rinsed in water and stained with Sypro orange (1/10, 000 dilution in 7 % (v/v) acetic

acid for 2 h. The prep gel was scanned using the green filter of the Typhoon scanner at high resolution (100 microns). The prep gel was wrapped and stored at 4°C until spot picking.

Image manipulation

DiGE images were cropped using ImageQuant software (GE Healthcare life Sciences, Buckinghamshire, UK) on the Typhoon scanner. The cropping removed all edges of the gel image (gel spacers, IPG strip and dye front). ImageJ software (National Institute of Health, Maryland, USA) was also used to obtain jpeg format images of each DiGE gel. Images were then loaded into DeCyder™ (GE Healthcare life sciences, Buckinghamshire, UK) 2-D differential analysis software for processing.

DeCyder Biological Variation Analysis

A DeCyder 2D (version 7.0) differential In-gel analysis (DIA) and biological variation analysis software (BVA) (GE Healthcare life sciences, Buckinghamshire, UK) were used to analyse the gels and create gel to gel matching of spots (qualitatively and quantitatively) within the three gels produced. The software produced statistical comparisons (one way ANOVA), after normalization of the ratio of spot volume between the spots of different dyes on a single gel using the internal standard (pool of all samples labelled with Cy2), between the 3 different gels across the 3 time points (designated: before, peak and after for 0, 81 ad 312 h post-infection times, respectively). It was therefore possible to identify spots which were quantitatively and qualitatively different across gels.

A few of the significantly varying spots once identified, were then excised from the preparative gel (gel 4) using the Ettan spot picker (GE Healthcare life sciences, Buckinghamshire, UK) and processed by trypsin digestion for protein identification using LC-MS/MS as described in Sections 4.2.4.4.3 and 4.2.4.4.4 respectively.

13.3.5 Peptidomics

13.3.5.1 Sample extraction for CE-MS

Milk samples used for peptidomic analysis were from six time points in the course of the *S. uberis* mastitis challenge including 0 h (pre-challenge), 36, 42,

57, 81 and 312 h post challenge (n = 6 at each time point). Selection of these time points was based on the results from APP analysis and the published results for cytokine and SCC to represent stages of mastitis; pre-infection (0 h), initial infection (36/42 h), peak infection (57/81 h) and resolution (312 h).

Skimmed milk samples were defrosted and 0.1 % phenylmethylsulfonyl fluoride (PMSF) was added to each milk sample to prevent further protease activity. An aliquot of 150 µl of sample was added to 150 µl of urea buffer (2 M urea, 100 mM sodium chloride (NaCl), 10 mM ammonium hydroxide (NH_4OH) and 0.02 % (w/v) SDS) in a Centrisart ultrafiltration tube (Sartorius, Gottingen, Germany), and centrifuged for 60 min at 3,400 rpm, at 4°C in order to remove higher molecular weight (HMW) proteins with peptides being in the ultrafiltrate for peptidomic analysis.

During this time, a NAP-5 column (GE Healthcare, Sweden) was placed onto a 50 ml collecting tube, and equilibrated by rinsing with 15 ml of ammonium (NH_4) buffer, in preparation for desalting, enrichment of the polypeptides and removal of urea (Mansor *et al.*, 2013). After centrifugation, 200 µl of the Centrisart filtrate was passed through the column. 300 µl of NH_4 buffer was further passed through the column. The columns were then placed in 2 ml Eppendorf tubes and eluted using 700 µl of the NH_4 buffer. The eluate was frozen and later freeze-dried.

The protein concentrations of milk polypeptides samples extracted were determined using the BCA method as described in Section 5.3.4.1. A final concentration of 2 µg/µl was then achieved by re-suspending the freeze-dried samples in an appropriate volume of Milli Q water prior to CE-MS.

13.3.5.2 CE-MS analysis

Buffers and Solutions

Buffers for CE-MS analysis are prepared freshly every week, these includes 1M sodium hydroxide (NaOH), NH_4OH (prepared by adding 3.76 ml of NH_4 solution (25%) to deionised water and the volume adjusted to 50 ml with deionised water), running buffer (prepared by adding 10 ml of ACN and 472 µl of formic acid to a final volume of 50 ml with deionised water),and the sheath flow liquid

(prepared by mixing 15 ml of 2-propanol and 200 µl of formic acid with the volume adjusted to 50 ml with deionised water).

The standard protein/peptide solution (0.5 pmol/µl) for the calibration of CE-MS analysis contains lysozyme (14,303 Da, L4919, Sigma-Aldrich, Dorset, UK), ribonuclease (13,681 Da, R5500, Sigma-Aldrich, Dorset, UK), aprotinin (6,513 Da, A1153, Sigma-Aldrich, Dorset, UK) and 4 synthetic peptides including; ELMTGELPYSHINNRDQIIFMVGR (2,832 Da), TGSLPYSHIGSRDQIIFMVGR (2,333 Da), GIVLYELMTGELPYSHIN (2,048 Da) and REVQSKIGYGRQIIS (1,733 Da) (Method described in Mansor *et al.*, 2013).

Sample Analysis

A Beckman Coulter P/ACE MDQ CE system (Fullerton, USA) was used for the CE-MS analysis. A 90 cm 360 µm OD, 50 µm ID uncoated capillary with a tapered tip (New Objective, Woburn, USA) was used on-line and coupled to a microdot MS (Bruker Daltonics, Bremen, Germany). This (capillary) was first conditioned with NaOH (pressure 50 psi) for 15 min and then washed with NH_4OH solution (50 psi) for 15 min and 20 min with running buffer (50 psi). The capillary was not connected to the MS until the conditioning procedure has been completed. The Beckman CE was set to run in reverse mode when connected to the MS system using the external detector adapter. Running buffer (pressure applied 50 psi) was used to rinse the capillary prior to each injection.

Re-suspended samples were centrifuged at 14,000 x*g* for 10 min at 4ºC shortly before analysis. The samples were then injected at a pressure of 2.0 psi for 99 s resulting in a loading volume of 290 nl of sample. Separation was done with +25 kV at the injection site for 30 min with a capillary set temperature of 35°C. Additional pressure to initial +25 kV was applied as follows; 0.1 psi for 1 min, 0.2 psi for 1 min, 0.3 psi for 1 min, 0.4 psi for 1 min and 0.5 psi for 30 min. The sheath liquid was applied coaxially at a running speed of 0.02 ml/h. The capillary was rinsed with deionised water (50 psi) for 1 min after each run followed by a washing step with NH_4OH solution (50 psi) for 3 min and lastly by a flushing step with deionised water (50 psi for 3 min). Electro-spray ionisation sprayer (Agilent Technologies, Palo Alto, CA, USA) was grounded to achieve electric potential of 0, and the electro-spray interface potential was set between -4 and -4.5 KV.

The mass calibration of the MS was performed on a weekly basis using the standard protein/peptide solution (0.5 pmol/µl) for CE-MS analysis. The acquisition of data and MS were automatically controlled by the CE via contact close-relays and MS spectra accumulated every 3 s, over a mass-to-charge range of 350 to 3000 for about 60 min depending on the analysis requirements. The CE-MS analysis was performed with the assistance of Dr Angelique Stalmach and under supervision of Dr. William Mullen of the Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK.

13.3.5.3 CE-MS data analysis

The Mosaiques Visu software (www.proteomiques.com) (Mosaiques Diagnostics, Hannover, Germany), was used to analyse the peptide mass spectra ion peaks, involving deisotoping and deconvolution along with spot picking.

The Mosaiques Visu software examines all mass spectra from a CE–MS investigation for signals above the threshold (SNR^4) and uses a probabilistic clustering algorithm and both isotopic distribution and conjugated masses for charge-state determination of the entities (Kolch *et al.*, 2005). This resulted into a list in which all signals that are interpretable were defined by mass/charge, charge, migration time and single intensity (ion counts). Only signals observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. Signals with a calculated charge of +1 were automatically excluded to minimize interference with matrix compounds or drugs.

Capillary electrophoresis migration time and ion signal intensities were normalized. Reference signals of over 380 milk entities or milk “housekeeping polypeptides” were used for CE-time calibration by local regression. The same peptides were used for ion signal intensity normalization by a applying a global linear regression. The resulting peak list contained the molecular mass (Da) and normalized CE migration time (min) for each feature. Normalized signal intensity can be used as a measure of relative abundance. Data sets were accepted only if the following quality control criteria were met: A minimum of 950 chromatographic features (mean number of features minus one standard deviation) must be detected with a minimal MS resolution of 8000 (required resolution to resolve ion signals with z = 6) in a minimal migration time interval (the time window, in which separated signals can be detected) of 10 min. After

calibration, the mean deviation of migration time (compared to reference standards) must be below 0.35 min. Control and disease-specific polypeptide patterns were generated using support vector machine (SVM)-based MosaCluster software.

13.3.5.4 LC-MS/MS peptide sequencing

In order to determine the sequences of significant biomarker polypeptides, an LC-MS/MS peptide sequencing was carried out using a Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberley UK). 5 µl of each sample were loaded onto a Dionex 100 µm x 2 cm 5 µm C18 nano trap column at a flow rate of 5 µl/min by an Ultimate 3000 RS auto sampler (Dionex, Camberley UK). This was then separated on a C18 reverse phase column (75 µm ID × 150 mm, 100 Å, Acclaim PepMap RSLC C18, Dionex). Eluting of peptides was carried out with gradient of 1-5-40 % B (80 % ACN, 0.2 % formic acid) in 0-5-60 min at a flow rate of 300 nl/min. The eluent from the column was directed to a Proxeon nano spray ESI source (Thermo Fisher, Hemel, UK) operating in positive ion mode then into an Orbitrap Velos Fourier transform mass spectrometer (FTMS). The ionisation voltage was 2.5 kV and the capillary temperature was 200°C. The mass spectrometer was operated in MS/MS mode scanning from 380 to 2000 atomic mass unit (amu). The top 20 multiply charged ions were selected from each full scan for MS/MS analysis, the fragmentation method was CID at 35 % collision energy. The ions selected for MS^2 using data dependent method with a repeat count of 1 and repeat and exclusion time of 15 s. Precursor ions with a charge state of 1 were rejected. The resolution of ions in MS^1 was 60,000 and 7,500 for HCD MS^2. LC-MS/MS analysis was performed by Dr. William Mullen of the Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK.

13.3.5.5 LC-MS/MS data analysis

Raw spectral data from LC-MS/MS analysis of the samples were uploaded to Thermo Proteome Discoverer 1.3. Peak picking was performed under default settings for Fourier transform mass analysers (FTMS) analysis i.e. only >+2 peptides were considered with signal to noise ratio higher than 1.5 and belonging to precursor peptides between 380 – 6000 Da. Peptide and protein identification was performed with SEQUEST algorithm. An in house compiled database containing proteins from the latest version UniProt SwissProt database was compiled to include only *Bos taurus* and *S. uberis* entries. No enzyme cleavage was selected and oxidation of methionine and proline were chosen as

variable modifications. Precursor mass tolerance was set at 5 parts per million (ppm) and 0.1 Da for MS/MS fragment ions. Resulting peptides and protein hits were further screened by excluding peptides with an error tolerance higher than 10 ppm and by accepting only those hits listed as high confidence by Proteome Discoverer software. Target FDR was 0.01 (strict) and 0.05 (relaxed).

13.3.6 Statistical analyses

Acute phase proteins

Test for normality of data were run on each APP set of data per time point. The concentration of each APP across time points were compared using related samples Friedman's two-way analysis by ranks test (non-parametric ANOVA). For pair wise (time point) comparison of APP values, a related sample Wilcoxon's signed rank test was utilized. P-value was considered significant at < 0.05.

DiGE

A one way ANOVA test was carried out by the BVA module of the DeCyder software following normalization and ratio matching of spots, to determine the spots that changed across the 3 time points in each of the gels. A student T-test was also used to determine differences in spot intensities at pre-infection (0 h) and peak infection time (81 h).

CE-MS

Only polypeptides that were found in more than 70 % of the samples in at least one of the groups were considered for biomarker definition. The pre-defined sets of polypeptides were further validated by randomly excluding 30 % of available samples. This was repeated (bootstrapping procedure) up to 10 times. For multiple testing corrections, P-values were corrected using the stringent maxT test or using false discovery rate adjustments of Benjamini-Hochberg (BH).

A model was generated for the classification of the samples depending on their CE-MS profile. In the model, 6 samples from time 0 (control) and 6 samples from time 81 (peak infection) were used as training cohort (n=12). For the validation of the model, samples from 36 h, 42 h, 57 h and 312 h (n=23) were used to discriminate the time points of progression and resolution of infection. A non-

parametric Wilcoxon test was carried out to analyse the significant peptides (significant adjusted BH p-value) in the training set. From the significant peptides, those with AUC of 1 were selected and a model based in these was used to score the validation set and box-plots to find visually possible changes per time were plotted.

13.4 Results

13.4.1 Milk acute phase proteins in an experimental *S. uberis* mastitis

13.4.1.1 Haptoglobin

ELISA

The profile of Hp over time during an *S. uberis* mastitis challenge is shown in Figure 5-2. The earliest significant rise in Hp levels was seen between 36 h post challenge (PC) (P=0.028). Peak values of Hp was achieved at 42 h in one cow, 57 h in another, 72 h in two others, 81 h and 96 h in last two respectively. In all the average time of peaking in Hp was at 57 h.

At the final time point sampled (312 h) which corresponded to the time of resolution of the infection, two cow-quarters still had high Hp concentration relative to basal values; one these two quarters was shown to still be positive for *S. uberis* colony forming units (CFU) at this time point whereas the others were negative. The remaining four cow-quarters' Hp had lowered significantly, however there was still a significant difference in the total median values of Hp at 312 h and at 0 h (possibly due to the two quarters still having high Hp at 312 h). Time 0 h milk Hp ranged from <0.4 - 3.21 μg/ml in the infected quarters whereas in milk from controls range was <0.4 - 2.25 μg/ml. In control quarters milk the overall range of Hp in milk from 0 h to 312 h was <0.4 - 6.38 μg/ml.

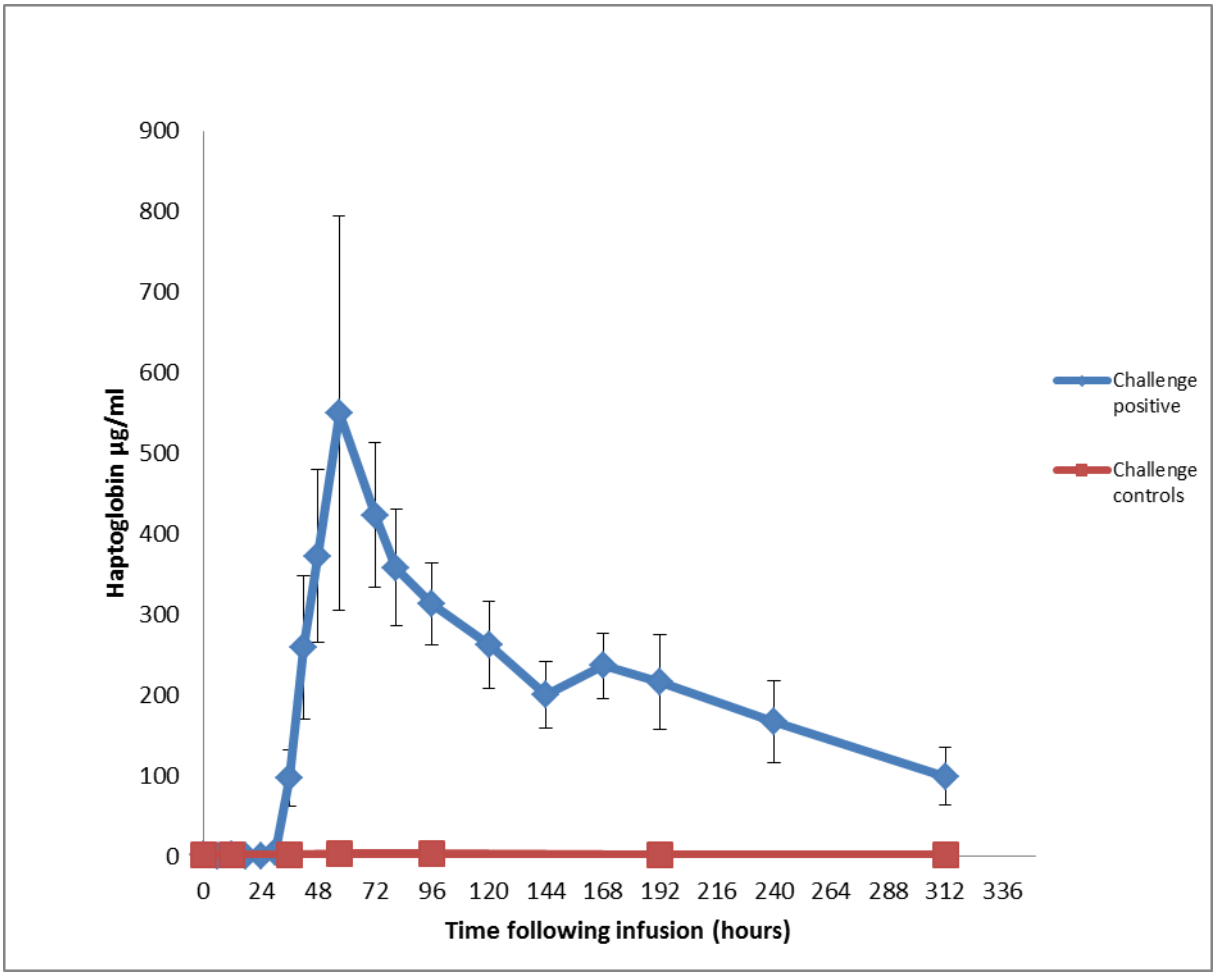

Figure 13-2: Concentrations of Haptoglobin (mean ± SEM) during the course of an experimental *S. uberis* infection.
Host adapted strain of *Streptococcus uberis* was used; challenge positive samples (blue line) and challenge controls (red line) over several time points from 0 to 312 h.

Hp western immunoblotting

Haptoglobin bands became visible on the western blotted NCM first at the 36 h post challenge (ß-chain) and the intensity of the bands increased to a peak at 57 and 72 h post challenge (Figure 5-3). Bands were once again absent by 120 h post challenge. This is in agreement with the results of ELISA on the milk samples that showed an average peak at 57 h post challenge.

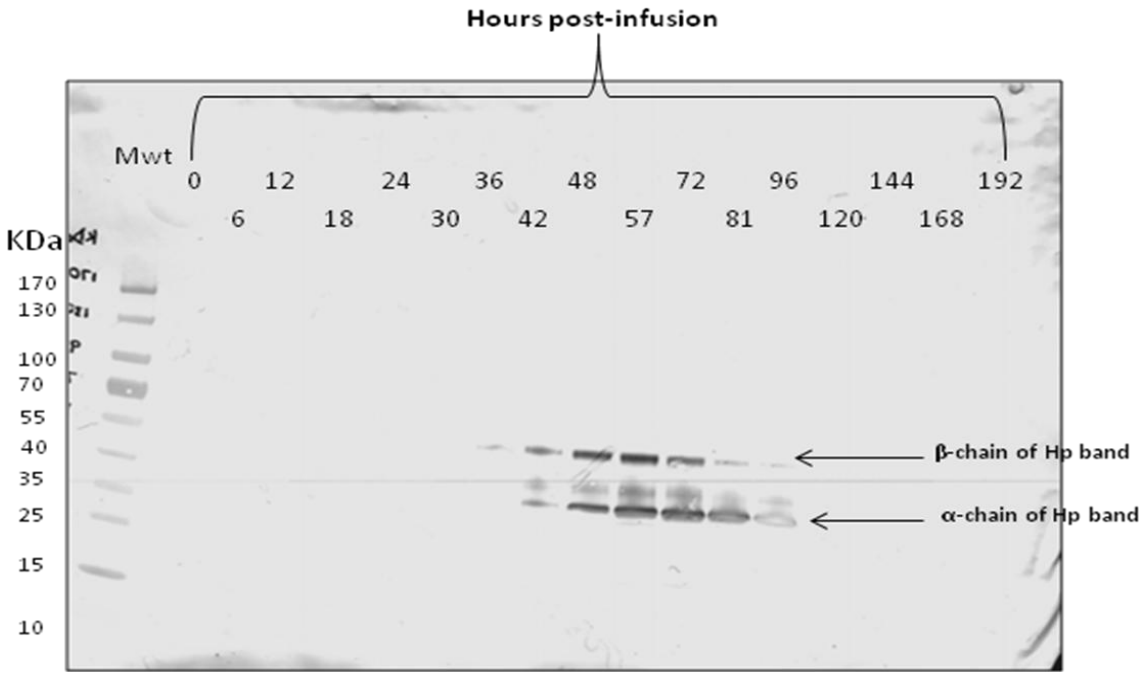

Figure 13-3: Western blot of pooled *S. uberis* challenge whey samples.
Lanes represent time 0 to 192 h post infusion samples, showing bands of the β and α chain of Hp from 36 h (faint, β-chain) to 96 h

13.4.1.2 M-SAA3

The profile of M-SAA3 over time during an *S. uberis* mastitis challenge is shown in Figure 5-4. The earliest significant rise in M-SAA3 levels was seen between 36-48h PC in 2 quarters, however in all quarters, 57 h PC was the time when a significant change was observed in M-SAA3 concentrations from the 0 h. Peak values of M-SAA3 were achieved at 96 h in 5 out of six cows and 81 h in the last cow. At the final time point sampled (312 h) which corresponded to the time of resolution, two cow-quarters still had high M-SAA3 concentrations relative to basal values; these two quarters were the ones which also showed high Hp concentrations at 312 h one of which had not cleared the bacteria from milk samples by 312 h.

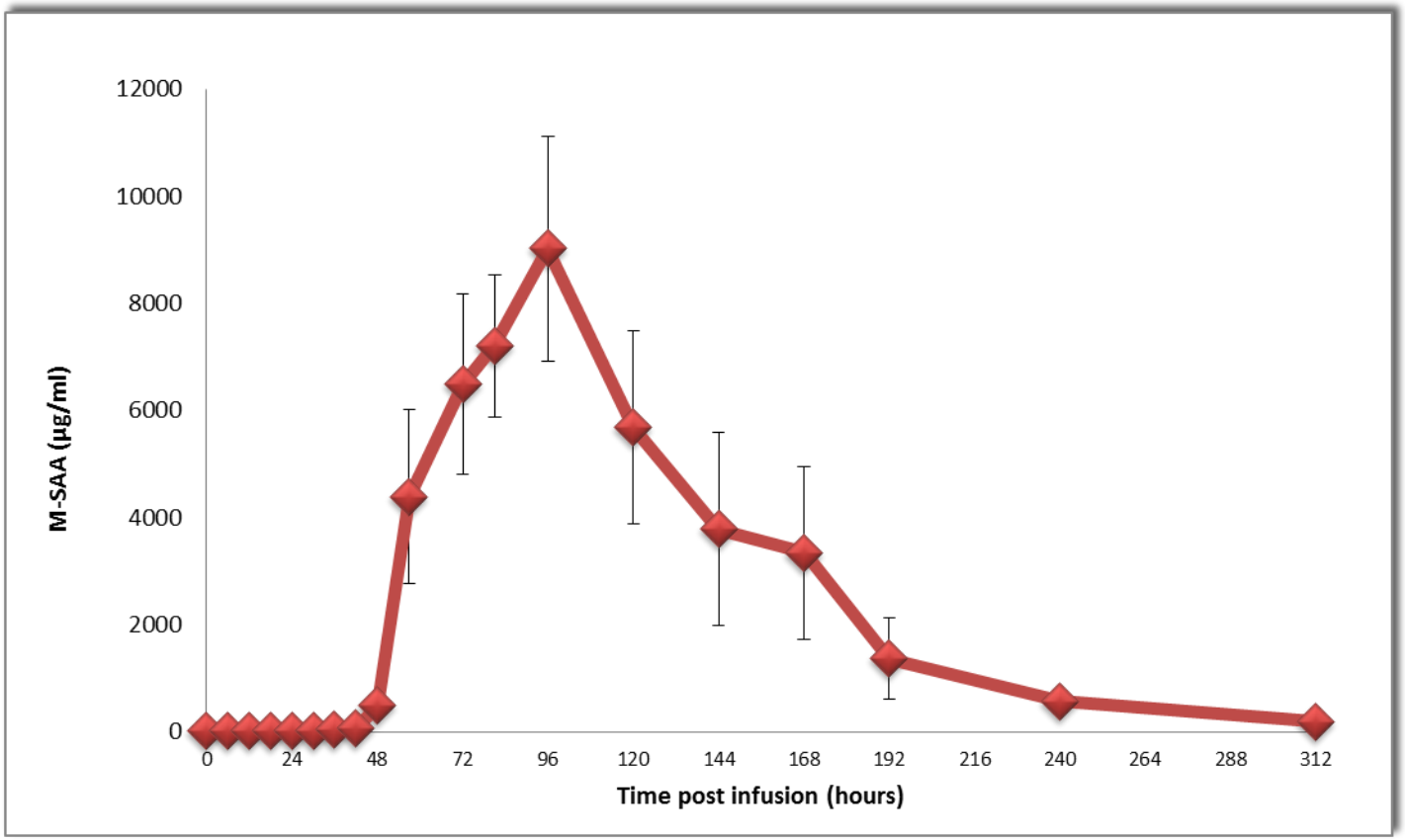

Figure 13-4: Concentrations of M-SAA3 (mean ± SEM) during the course of an experimental infection with host-adapted strain of *S. uberis*.

13.4.1.3 CRP

The changing profile of milk CRP through the course of the mastitis challenge is shown as a plot of mean ± SEM in Figure 5-5. CRP was first noticed to rise from baseline levels at 30 h PC two peaks of mean CRP concentration were observed during the course of the infection, one at 42 h and the other at 120 h PC. Concentrations of CRP at 240 h were still significantly higher than at 0 h, but at 312 h, the CRP levels had dropped back to similar values as at 0 h. A related samples Friedman's two way analysis of variance by ranks showed that distribution of CRP across the various time points were significantly different (P=000).

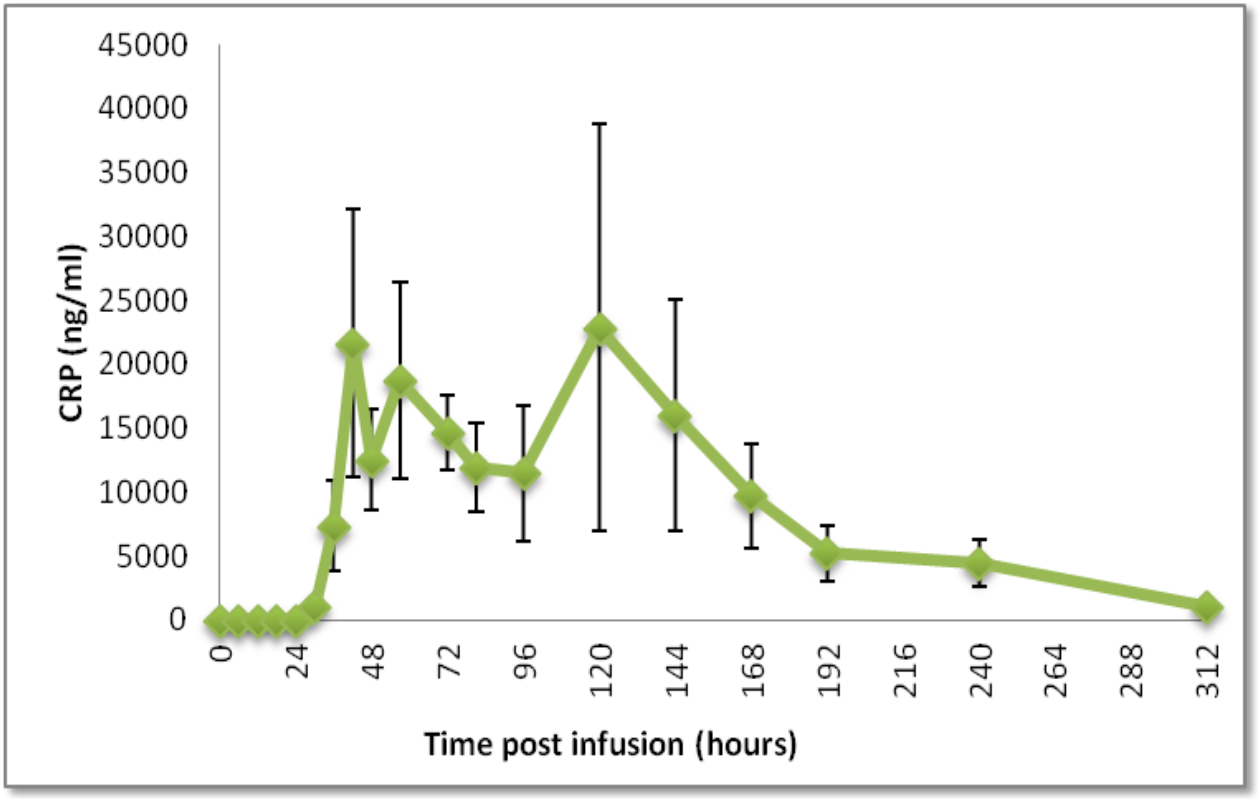

Figure 13-5: Concentrations of CRP (mean ± SEM) during the course of an experimental infection with host-adapted strain of *S. uberis*.

13.4.2 Proteomics

13.4.2.1 1DE-SDS PAGE

The 1DE electrophoretogram image of challenge positive samples is shown in Figure 5-6 while that from control quarters infused with PBS at seven of the time points is shown in Figure 5-7. Bands on the gels were identified by inference from results of MALDI-MS identification of 1DE bands of clinical mastitis milk samples carried out by Mansor, R. (PhD dissertation, University of Glasgow, 2012).

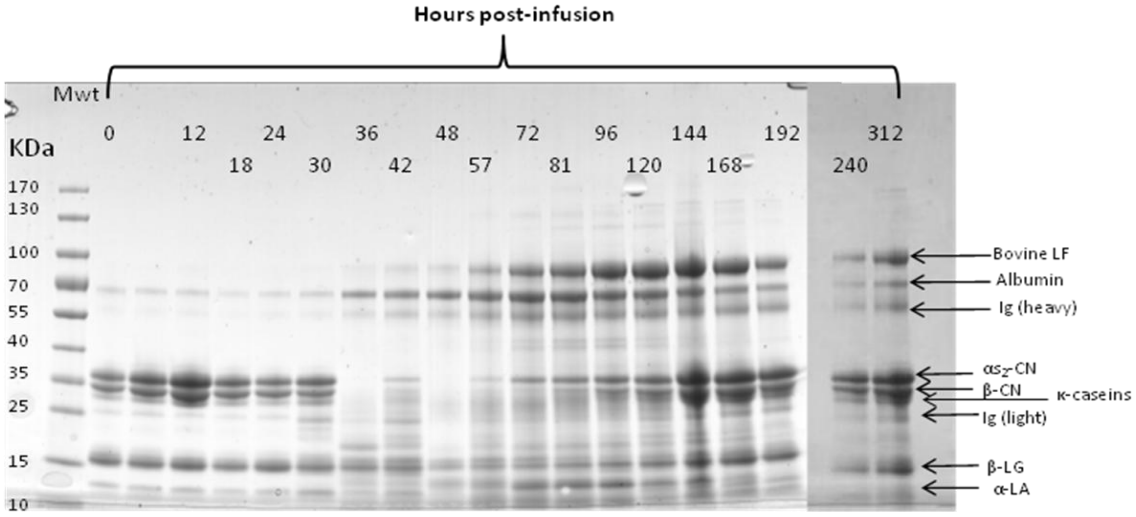

Figure 13-6: 1DE reducing gel image of *S. uberis* challenge positive skimmed samples at 19 time points during course of challenge.
Ig (immunoglobulin), Bovine Lf (bovine lactoferrin), αS_2-CN (alpha S_2 casein), β-CN (beta casein), κ-CN (kappa casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin

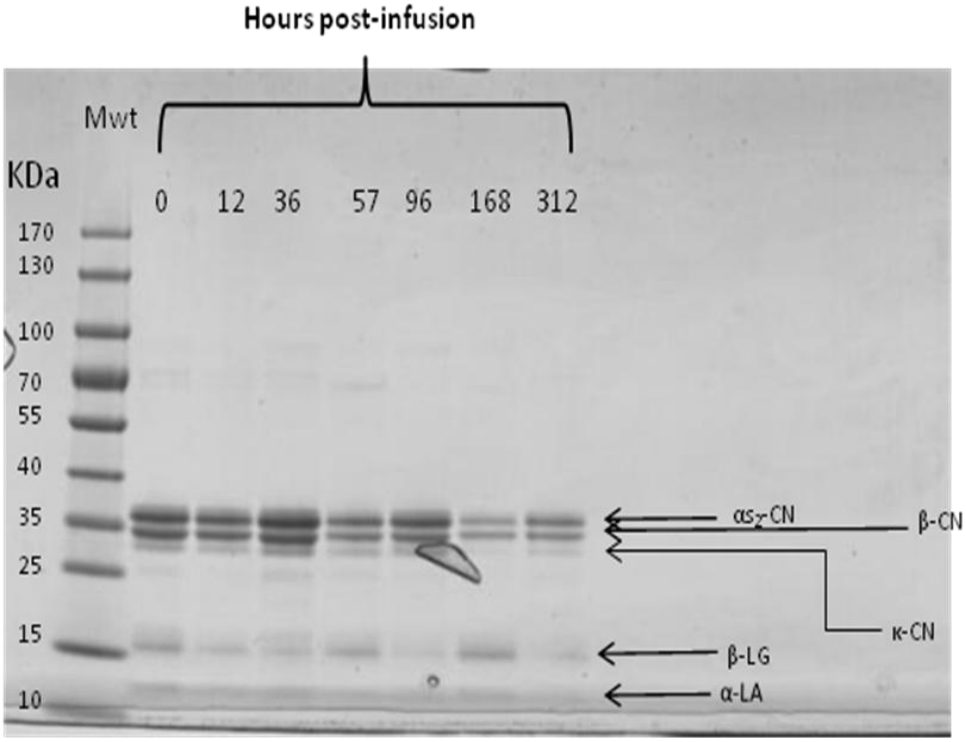

Figure 13-7: 1DE reducing gel image of pooled samples from *S. uberis* control-challenge quarters
Including the 7 time points sampled during the course of the control-challenge (PBS). αS_2-CN (alpha S_2 casein), β-CN (beta casein), κ-CN (kappa casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin)

13.4.2.2 2DE-SDS PAGE

Pooled samples from six time points of the *S. uberis* challenge positive quarters, were resolved with a 2DE SDS-PAGE (pH 4-7), and the gel images of the time points 0 h, 36 h, 57 h, 81 h, 168 h and 312 h are shown in Figures 5-8, 5-9, 5-10, 5-11, 5-12 and 5-13 respectively. Spots were identified by inference to spots analysed from 2DE of normal and mastitis milk whey as described in Boehmer *et al*. (2008), Smolenski *et al*.(2007) and Hogarth *et al*. (2004).

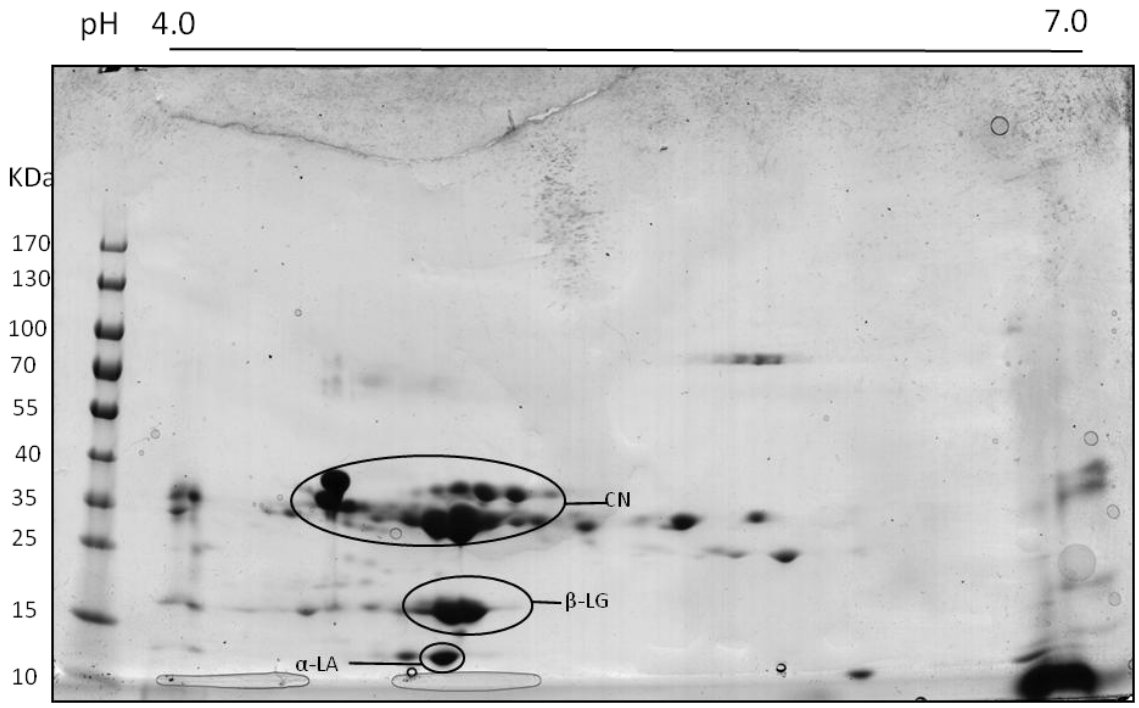

Figure 13-8: 2DE reducing gel image of pooled skimmed milk samples positive for *S. uberis* challenge at 0 h
Isoelectric range pH 4-7, CN (caseins),β-LG (beta lactoglobulin), α-LA (alpha lactalbumin.

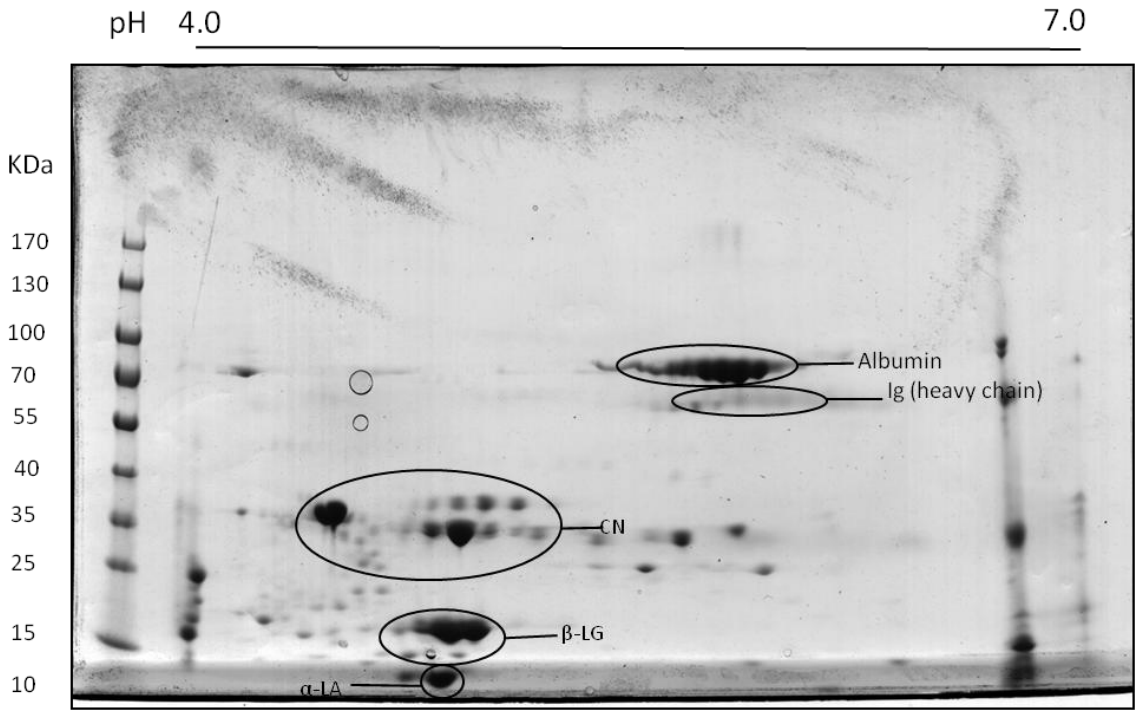

Figure 13-9: 2DE reducing gel image of pooled skimmed milk samples positive for *S. uberis* challenge at 36 h
Isoelectric range pH 4-7, Ig (immunoglobulin), CN (casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin).

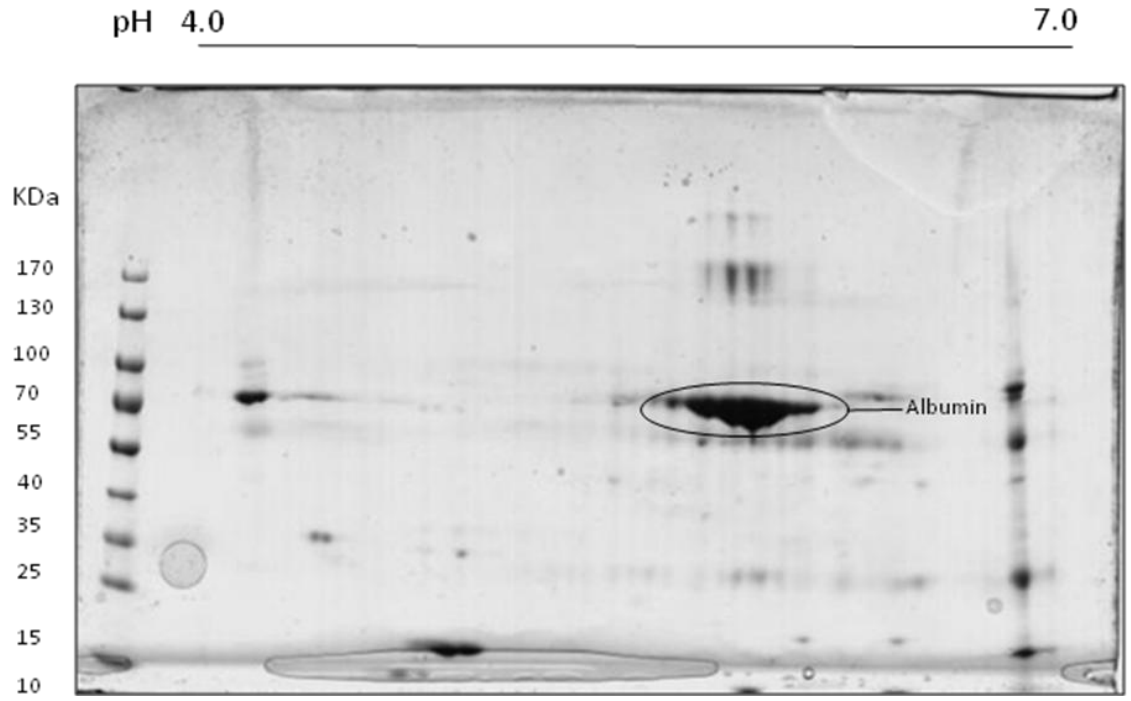

Figure 13-10: 2DE reducing gel image of pooled skimmed milk samples positive for *S. uberis* challenge at 57 h
Isoelectric range-pH 4-7

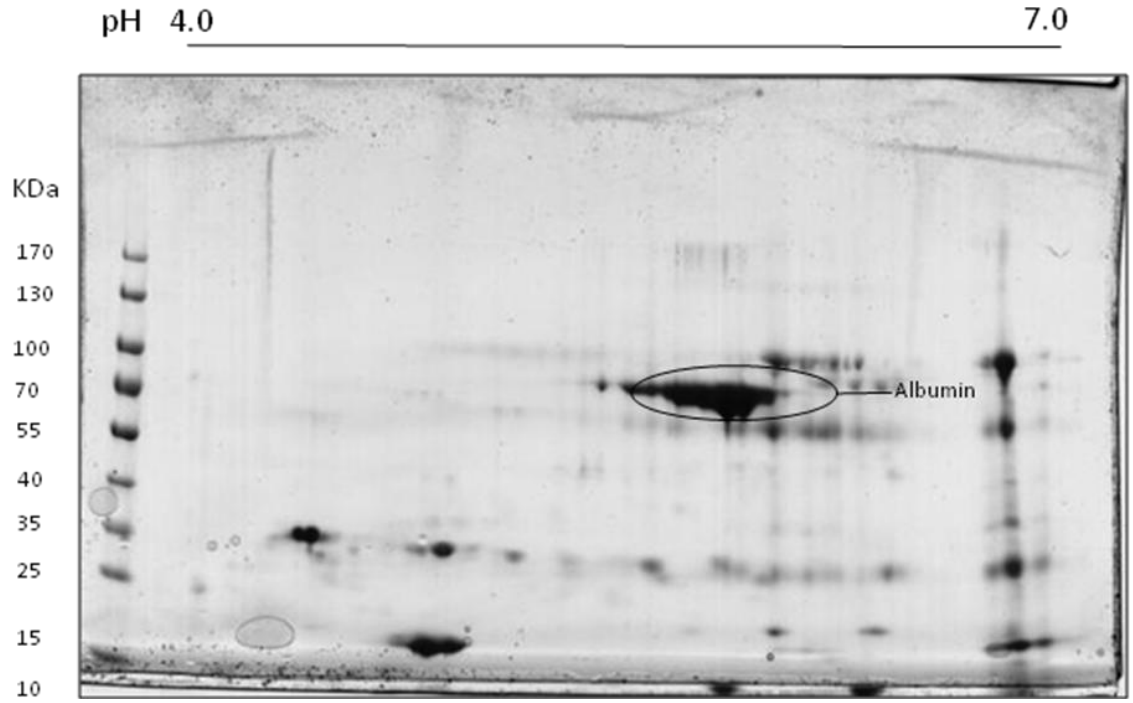

Figure 13-11: 2DE reducing gel of pooled skimmed milk samples positive for *S. uberis* challenge at 81 h.
Isoelectric range- pH 4-7

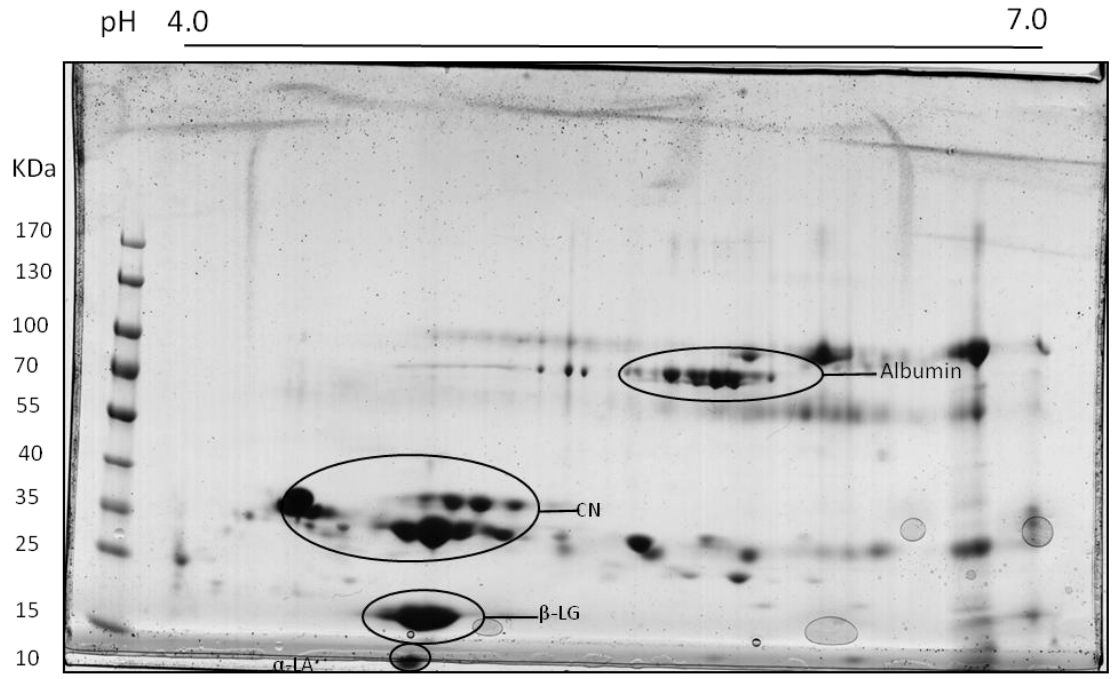

Figure 13-12: 2DE reducing gel image of pooled skimmed milk samples positive for *S. uberis* challenge at 168 h
Isoelectric range- pH 4-7, CN (casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin)

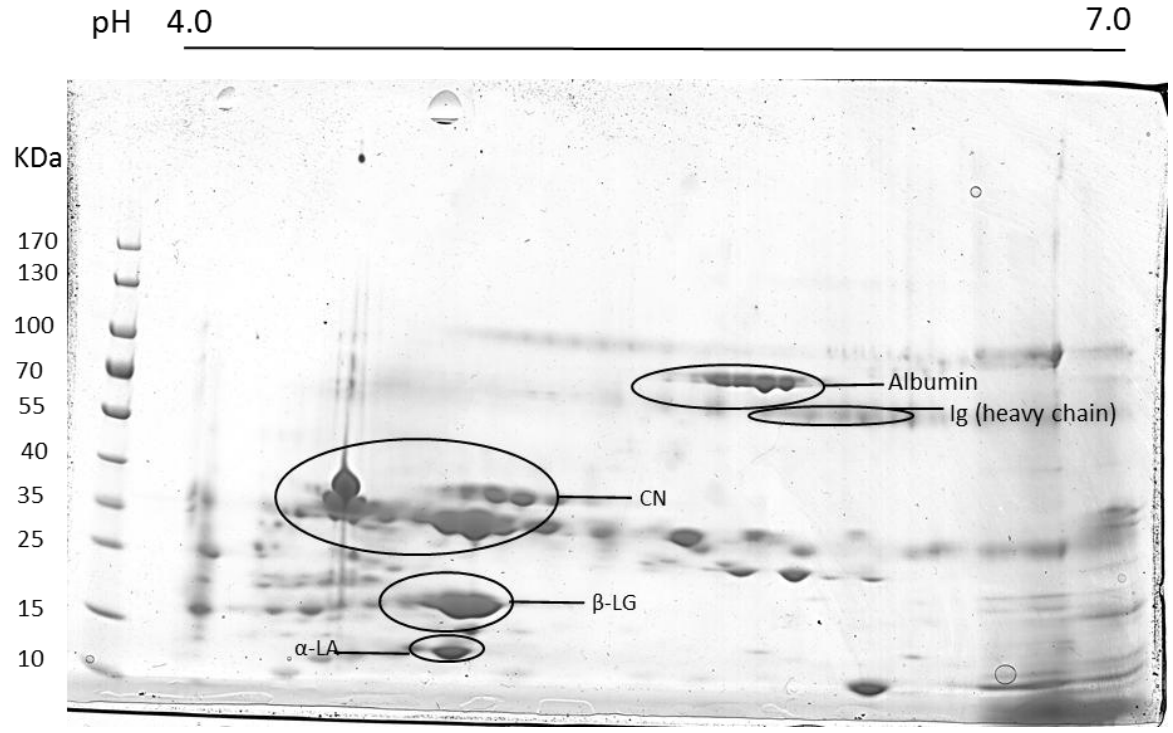

Figure 13-13: 2DE reducing gel image of pooled skimmed milk samples positive for *S. uberis* challenge at 312 h
Isoelectric range- pH 4-7. Ig (immunoglobulin), CN (casein), β-LG (beta lactoglobulin), α-LA (alpha lactalbumin)

13.4.2.3 DiGE

Different combinations of three time-points' samples from the *S. uberis* mastitis challenge (time 0, 81 and 312 h post infection) were run on 3 DiGE gels. The ImageQuant software images of the 3 different DiGE gels are shown in Figures 5-14 to 5-16. Numerous new protein spots were seen at 81 h which were not present at 0 h. Also, some major differences in the intensity of spots present at 0 h and 312 h were observed at 81 h. There were no appreciable differences between spots at 0 h and at 312 h (Figure 5-15). The Preparative (prep) gel image is shown in Figure 5-17. This gel comprised of a pool of the 3 time points considered for the DiGE experiment, from which spots of interest were excised (spots indicated as numbers on the image).

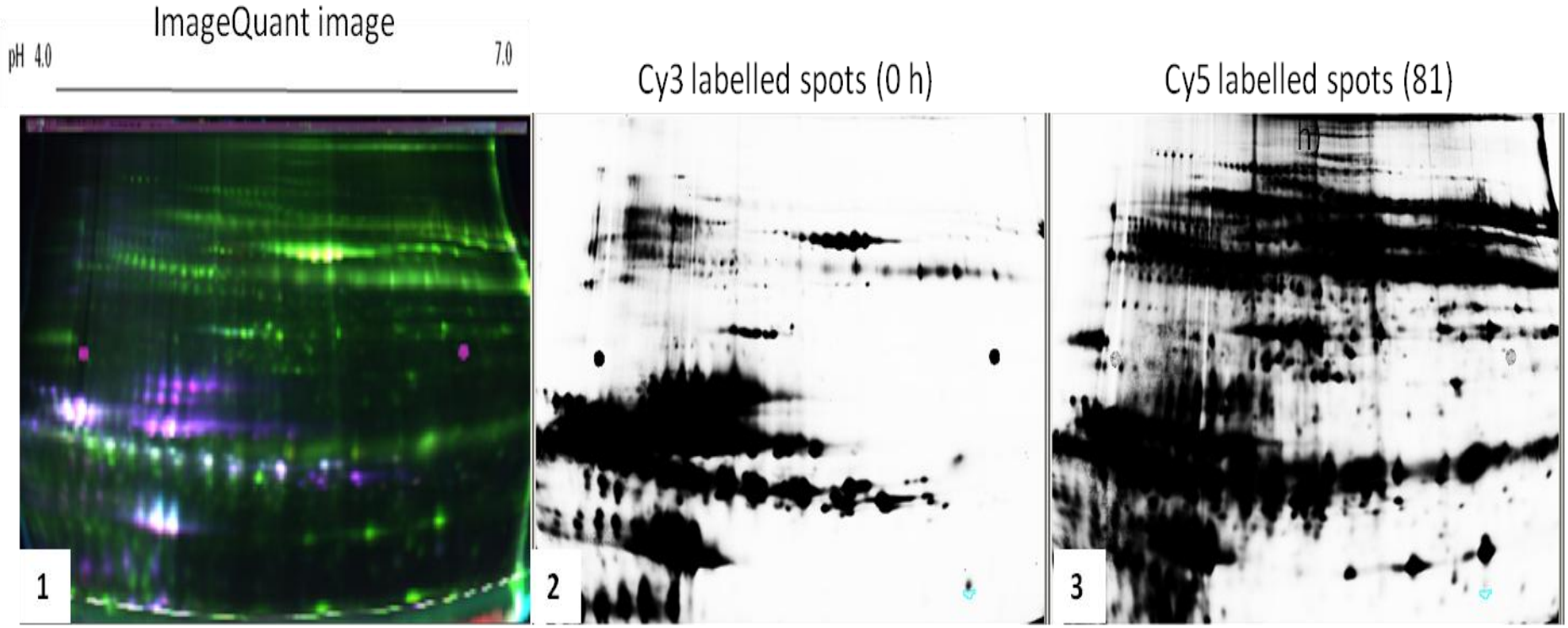

Figure 13-14: Difference gel electrophoresis (DiGE gel 1) on bovine whey from experimental *S. uberis* mastitis showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots.
Pool of time point 0 labelled with Cy3/red fluorescent dye (appearing purple) and pool of time point 81 labelled with Cy5/blue (appearing green) on ImageQuant.

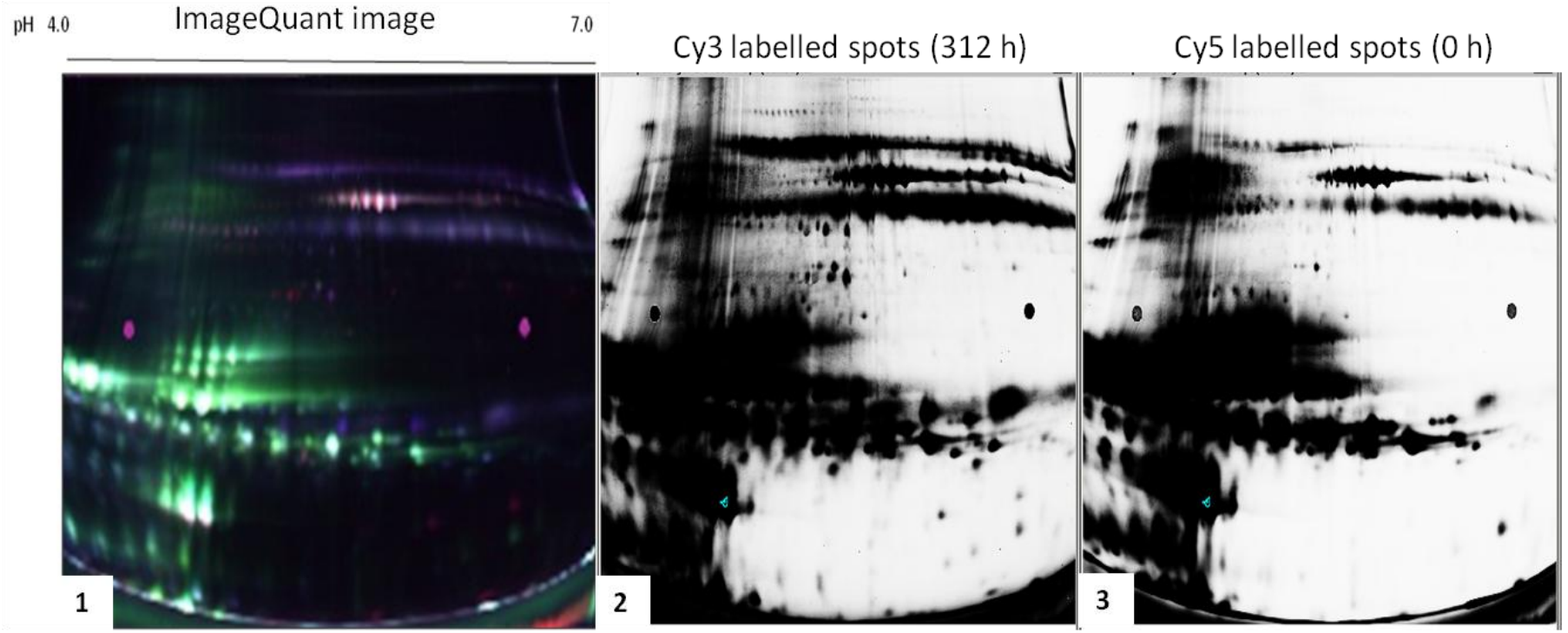

Figure 13-15: Difference gel electrophoresis (DiGE gel 2) on bovine whey from experimental *S uberis* mastitis, showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots.
Pool of time point 0 labelled with Cy5/blue (appearing green) fluorescent dye and pool of time point 312 labelled with Cy3/red (appearing purple) on ImageQuant.

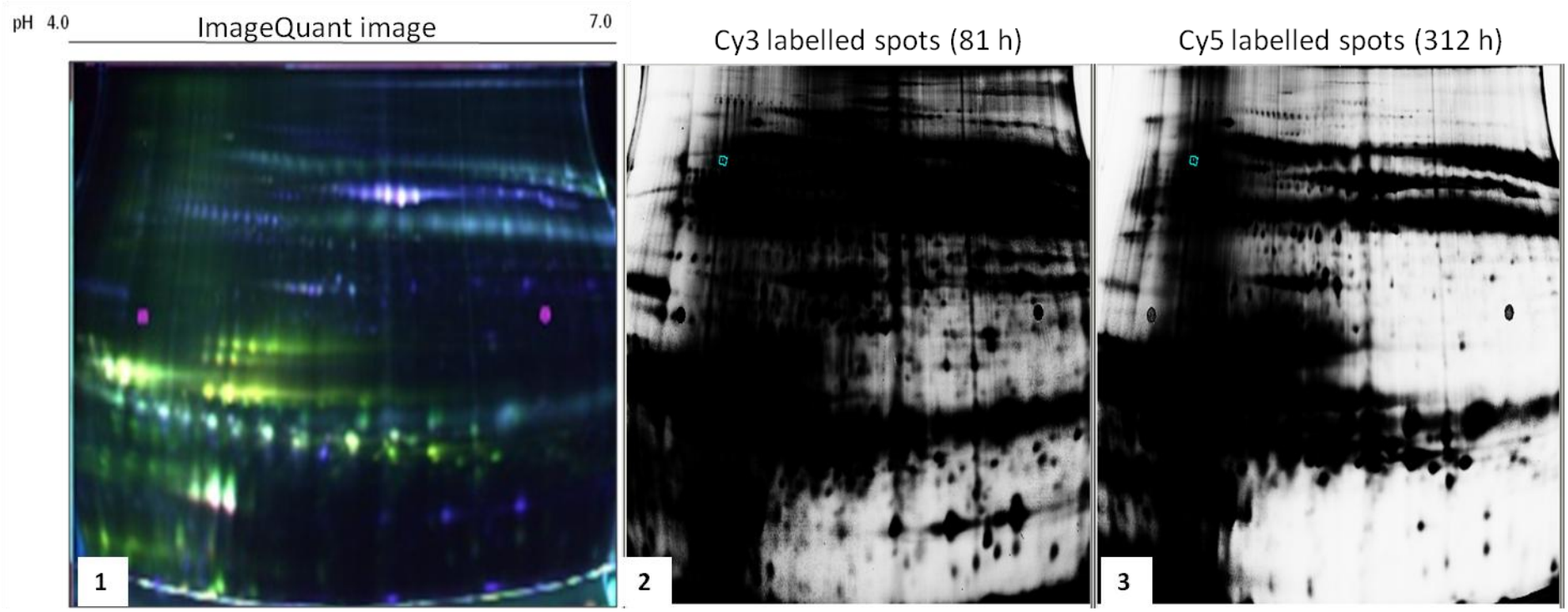

Figure 13-16: Difference gel electrophoresis (DiGE gel 3) on bovine whey from experimental *S uberis* mastitis, showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots.
Pool of time point 81 labelled with Cy3/red (appearing bluish/purple) fluorescent dye and pool of time point 312 labelled with Cy5/blue (appearing green) on ImageQuant.

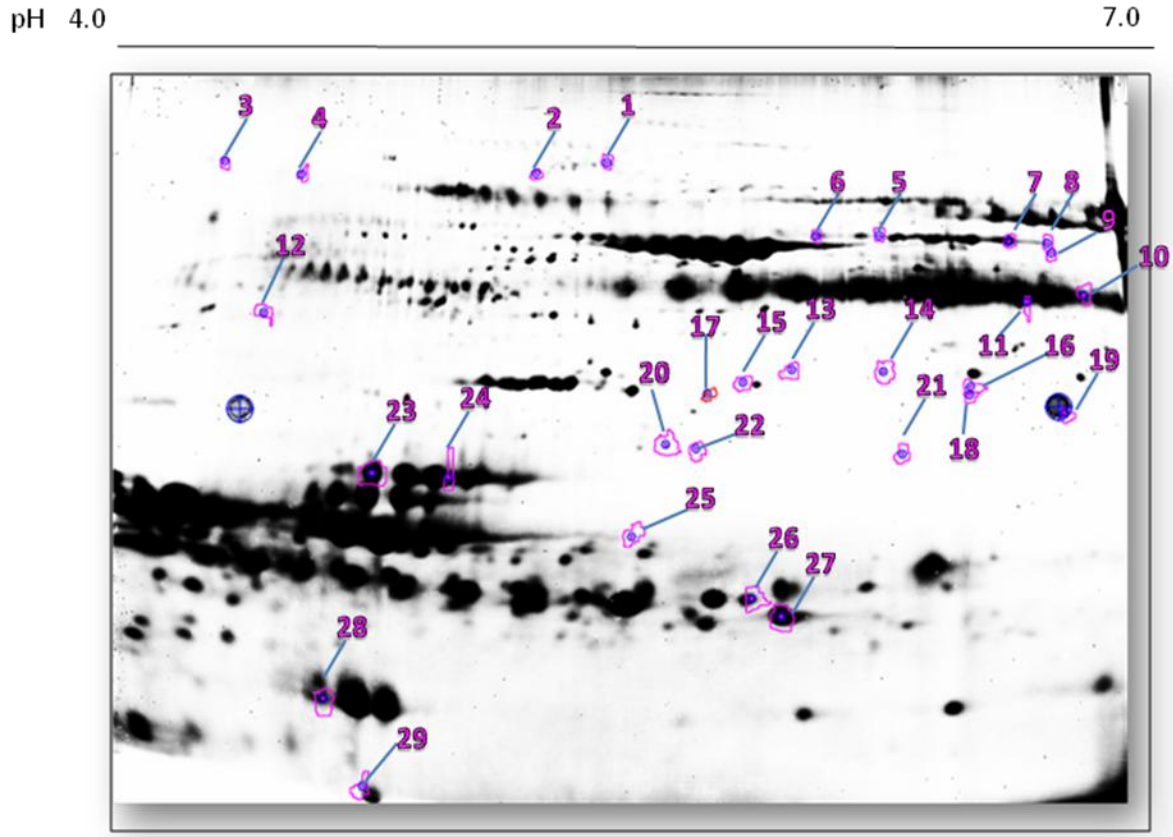

Figure 13-17: DiGE preparative gel comprising of the pool of 3 time points (0, 81 and 312 h). Spots of interest (selected based on changing profile during the course of the 3 time points) (n=29) excised for LC-MS/MS analysis are shown numbered and identified as listed in Table 5-2.

A total of 2154 spots were identified in the first DiGE gel (0 h + 81 h) by the DeCyder software and 1077 of these were matched on the prep gel. While 2577 and 2358 spots were seen in second and third DiGE gels respectively (0 h + 312 h and 81 h+312 h). A total of 2577 spots were matched in the preparatory gel in the second DiGE gel while 1077 matched to the prep gel on the third DiGE gel. Therefore because the second DiGE gel had the higher number of matches in the preparatory gel, it was selected for matched-spots picking to detect proteins of interest (spots varying significantly across the 3 time points and in the three gels). After the BVA test, 728 showed differences across the pre-infection, peak-infection and resolution phase of the challenge study. A total of 521 of these spots had a statistically significant variation (ANOVA, $P<0.05$) with time, while the variation in 207 spots were not statistically significant ($P=0.05$-0.97). Out of the significantly varying spots, 29 were selected based on level of change, for protein identification. Table 5-2 shows the 29 spots picked from the prep gel for LC-MS/MS analysis and their protein identifications.

Table 13-2: Protein identification of spots excised from the DiGE preparative gel (Figure 5-17).
Following matching of spots on preparatory gel with 3 DiGE gels, spots excision and protein identification using LC-MS/MS.

Spot No= gel image	Spot ID	Protein	Ac. Number	Calculated pi	Mass (Da)	MOWSE Score	Characteristics at peak infection
1	**78**	Poor protein quality hCG1811380-like	DAA33614	9.03	169671	23	up regulated
2	**132**	Benzodiazepine receptor (peripheral) associated protein 1 Poor protein quality	DAA19201	5.55	173983	22	up regulated
3	**141**	Ankyrin repeat and sterile alpha motif domain-containing protein 1B	XP_005198370	5.41	90486	18	Down regulated
4	**156**	ND					Down regulated
5	**495**	Albumin	754920A	5.76	68083	51	up regulated
6	**562**	Albumin	754920A	5.76	68083	51	up regulated
7	**607**	Serotransferrin	Q29443	6.75	79870	81	up regulated
8	**636**	IQ motif containing E-like	DAA15162	8.43	88179	21	up regulated
9	**689**	Albumin	754920A	5.76	68083	55	ND
10	**960**	Albumin	754920A	5.76	68083	74	up regulated
11	**962**	Albumin	754920A	5.76	68083	48	up regulated
12	**1042**	Refeldin A-inhibited guanine nucleotide-exchange protein 3	XP_871474	5.54	245336	16	Down regulated
13	**1117**	Factor XIIa inhibitor precursor	NP_777246	6.19	51919	16	Up regulated
14	**1137**	Centromere protein A-like	DAA25906	11.19	52963	16	Up regulated
15	**1140**	Leucine-rich repeat flightless-interacting protein 2	DAA17146	5.37	45494	28	ND
16	**1155**	RAC-beta serine/threonine protein kinase isoform x4		5.98	55905	20	Up regulated
17	**1170**	ND					Up regulated
18	**1209**	ND					Up regulated
19	**1236**	SH2 domain-containing adapter protein B	NP_001179863 XP_618475	9.07	55667	21	Up regulated

20	**1336**	RAC-beta serine/threonine-protein kinase	NP_001193075 XP_870006	5.98	56004	23	Up regulated
21	**1370**	ND					Up regulated
22	**1375**	Albumin	754920A	5.76	68083	76	Up regulated
2	**1429**	ND					Down regulated
24	**1457**	ND					Down regulated
25	**1701**	Immunoglobulin heavy chain variable region	CAA10182	8.62	12561	19	Down regulated
26	**1960**	ND					Down regulated
27	**2023**	ND					Down regulated
28	**2236**	Beta-lactoglobulin	CAA32835	4.85	20307	25	Down regulated
29	**2402**	Poor protein quality; zinc finger protein	XP_003587295	9.32	64407	17	Down regulated

*ND-not determined due to poor protein quality

13.4.3 Milk peptidomics

13.4.3.1 CE-MS analysis

Model for infected milk biomarkers

Using a non-parametric Wilcoxon test to compare peptides identified at 0 h and 81 h, 460 polypeptides were detected to have significant Benjamini-Hochberg (BH) p-value, and used to train the SVM algorithm. Out of these, 205 had an AUC of 1 (specificity-100 %, sensitivity-100 %, cut off ≤-0.915 and interval of confidence 0.805 to 1) and were thus selected as discriminatory polypeptides with prospective biomarker significance and assessed in a validation set of samples (n=23).

Model of pre-infection (n=6) versus peak-infection (n=6) polypeptides is shown in box-and-whisker plots used as training sets for validation are shown in Figures 5-18 and 5-19. The Y-axis in these box plots represents the biomarker score, the case (disease) is scored as 1 and the control is scored as -1. So on the Y-axis it is possible to determine how much or how little of the disease is present based on the scores. A cut off point between healthy and diseased is somewhere between -1.0 and 1.0 and for biological samples it is not always zero.

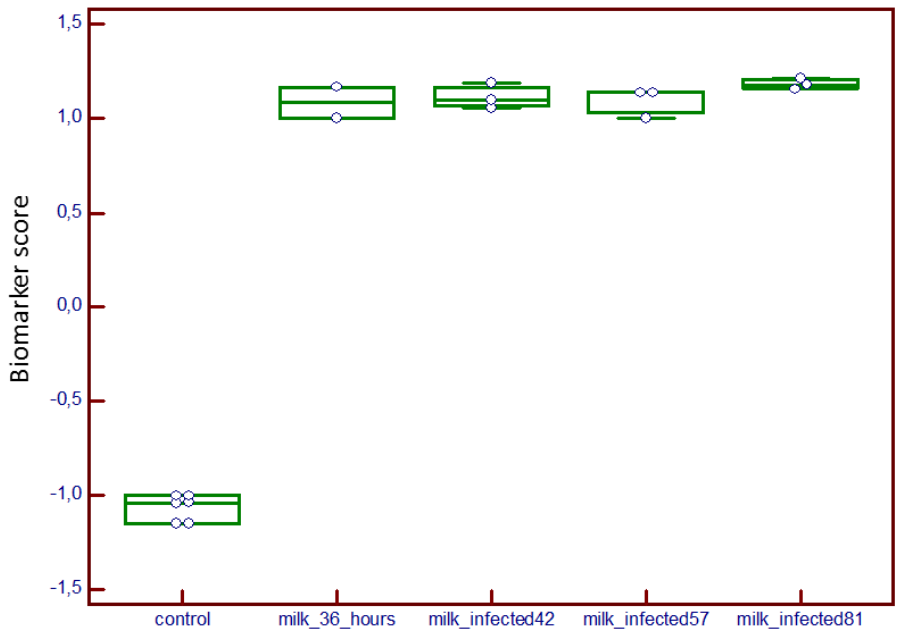

Figure 13-18: Box plot of non-total cross validation of the CE-MS polypeptides training set
Biomarker scores; Control= -1.0, Diseased= 1.0

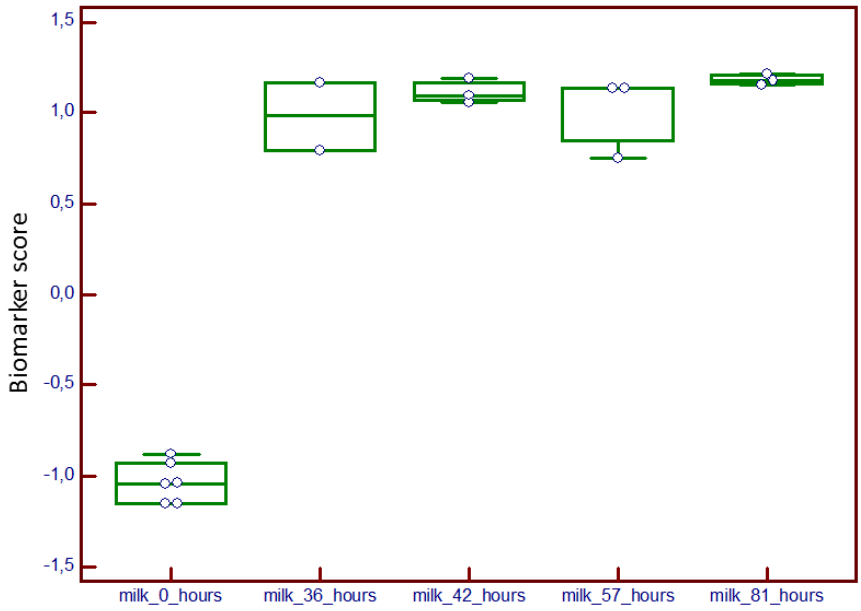

Figure 13-19: Box plot of total cross validation of the CE-MS polypeptides training set
Biomarker scores; Control= -1.0, Diseased= 1.0

Figure 5-20, displays the box plot of the validation using the test (training) set and shows control obtained from training set used for the validation.

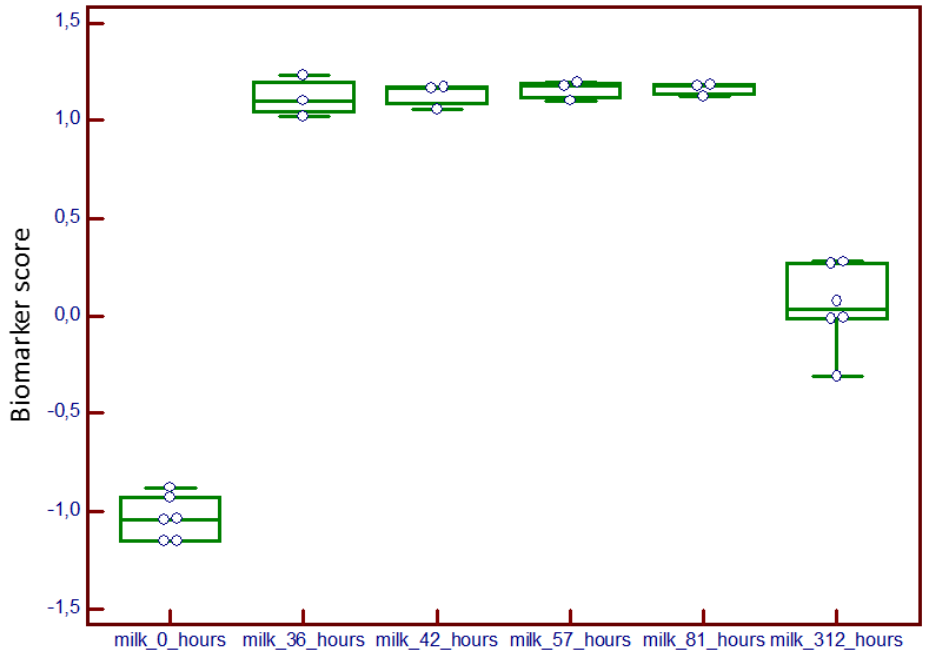

Figure 13-20: Box plot of test set validation of polypeptides using the training set model
Biomarker scores; Control= -1.0, Diseased= 1.0

There was little discrimination between the biomarker sets of 36 h and 81 h samples, therefore to gain a better biomarker model, CE-MS profile of the highest level of infection seen (81 h post infection) was used to establish a biomarker panel and this was tested against all the other time points. Figure 5-21, shows the box plot of the of test set used for validation. A total of 205 polypeptides were observed to fit into the biomarker panel using this model.

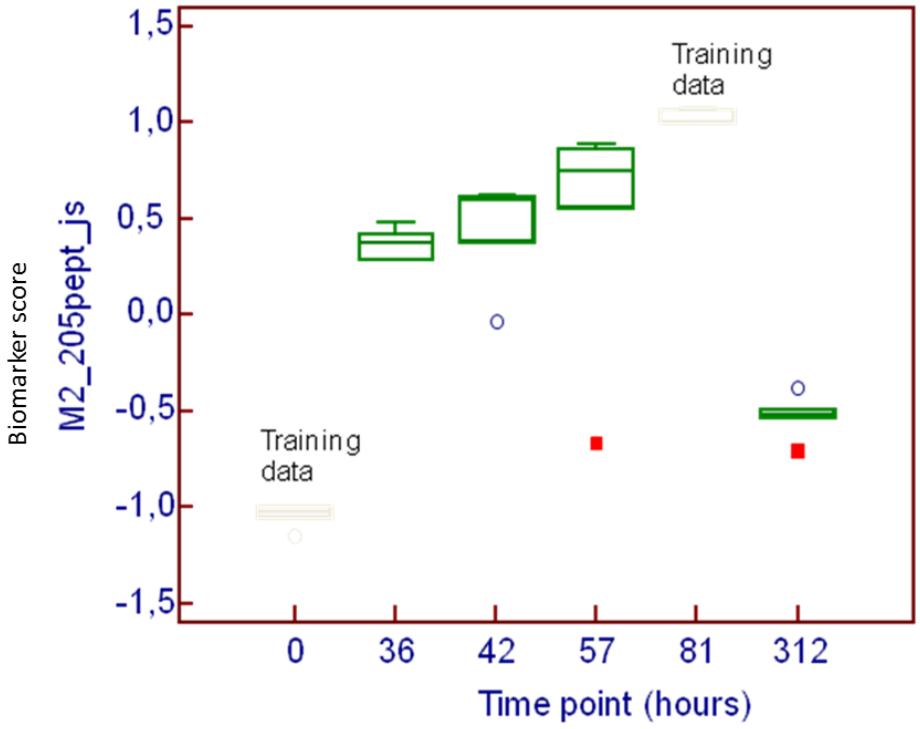

Figure 13-21: Box plot of training and validation set using CE-MS profile of control time and 81 h post-infection.
This was used to identify polypeptide biomarker panel (205 polypeptides); Biomarker scores; Control= -1.0, Diseased= 1.0

The panel of 205 polypeptides obtained were further matched from multi-consensus file of 3 reports of CE-MS carried out on milk samples including results obtained from Mansor *et al*. (2013). Out of the total number, seventy seven peptides were obtained (criteria used was number of basic amino acids + less than 100 ppm allowed). A model was then created using the training set described above and the 77 polypeptides. Thus a box plot (Figure 5-22) was plotted to show the different scores in progression of infection based on this model (training set plus 77 matched polypeptides). Time point 312 h presents scores closer to the beginning of the infection.

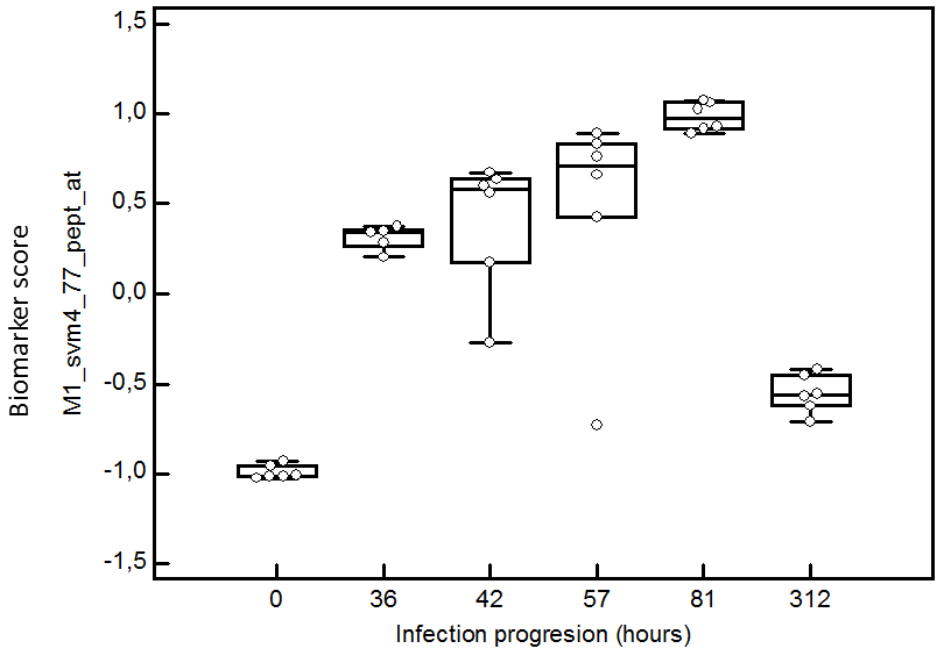

Figure 13-22: Box plot of training and validation set using CE-MS profile of control time and 81 h post-infection
Used to identify polypeptide biomarker panel (77 polypeptides); Biomarker scores; Control= -1.0, Diseased= 1.0

The peptide maps (CE/LS/MS peptide peaks) following the peptidomic analysis of 6 time points during the course of the mastitis challenge are shown in Figures 5-23, 5-24 and 5-25. It can be seen from the peptide maps, that as infection progressed there was a slight shift in peptides from higher to lower molecular weight and from earlier to later migration time upon capillary electrophoresis.

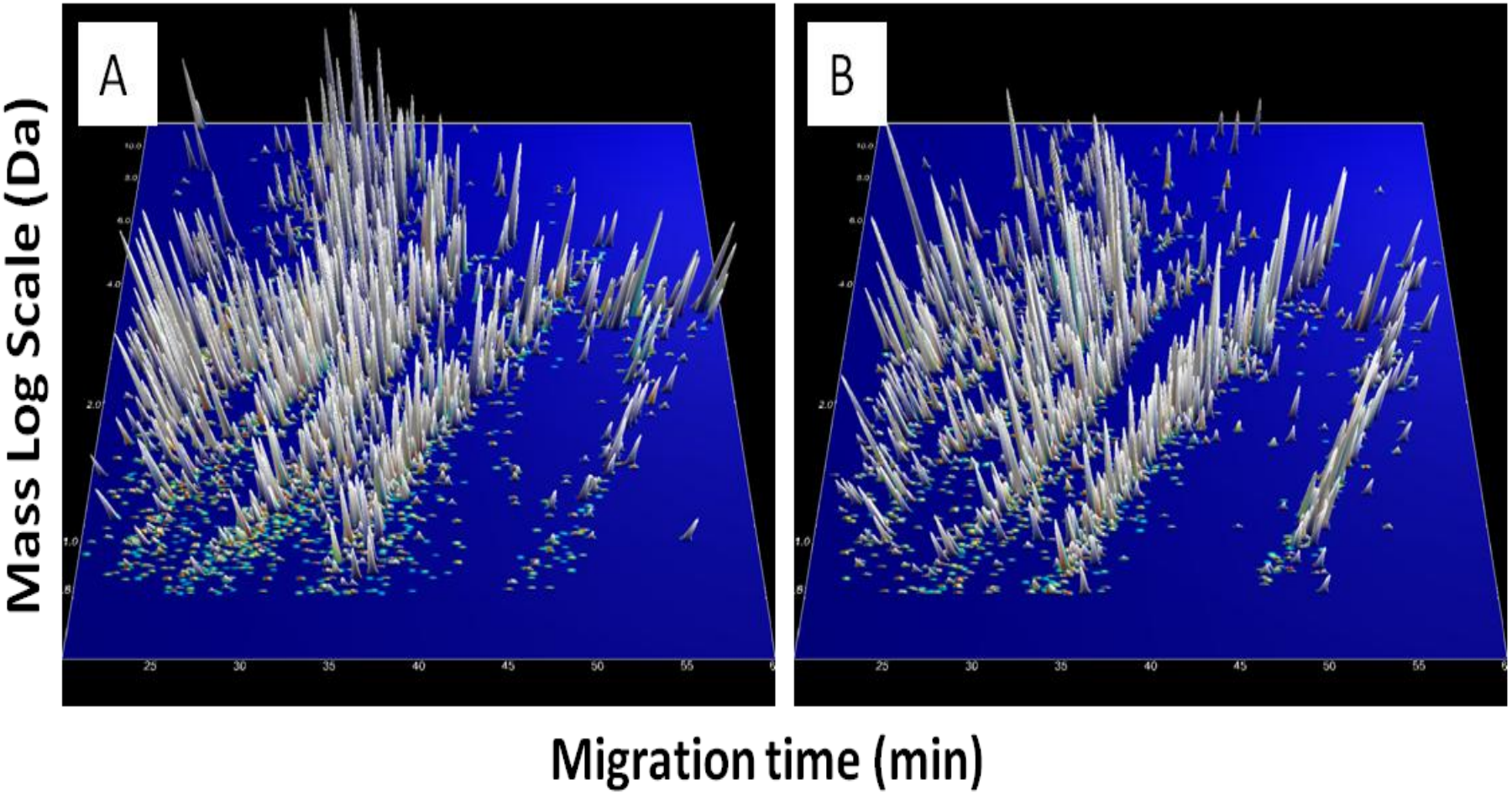

Figure 13-23: Composite peptide maps of CE/LC/MS peaks of milk at time points 0 h (A) and 36 h (B) h post infection respectively.
Z –axis represents the mean signal intensity.

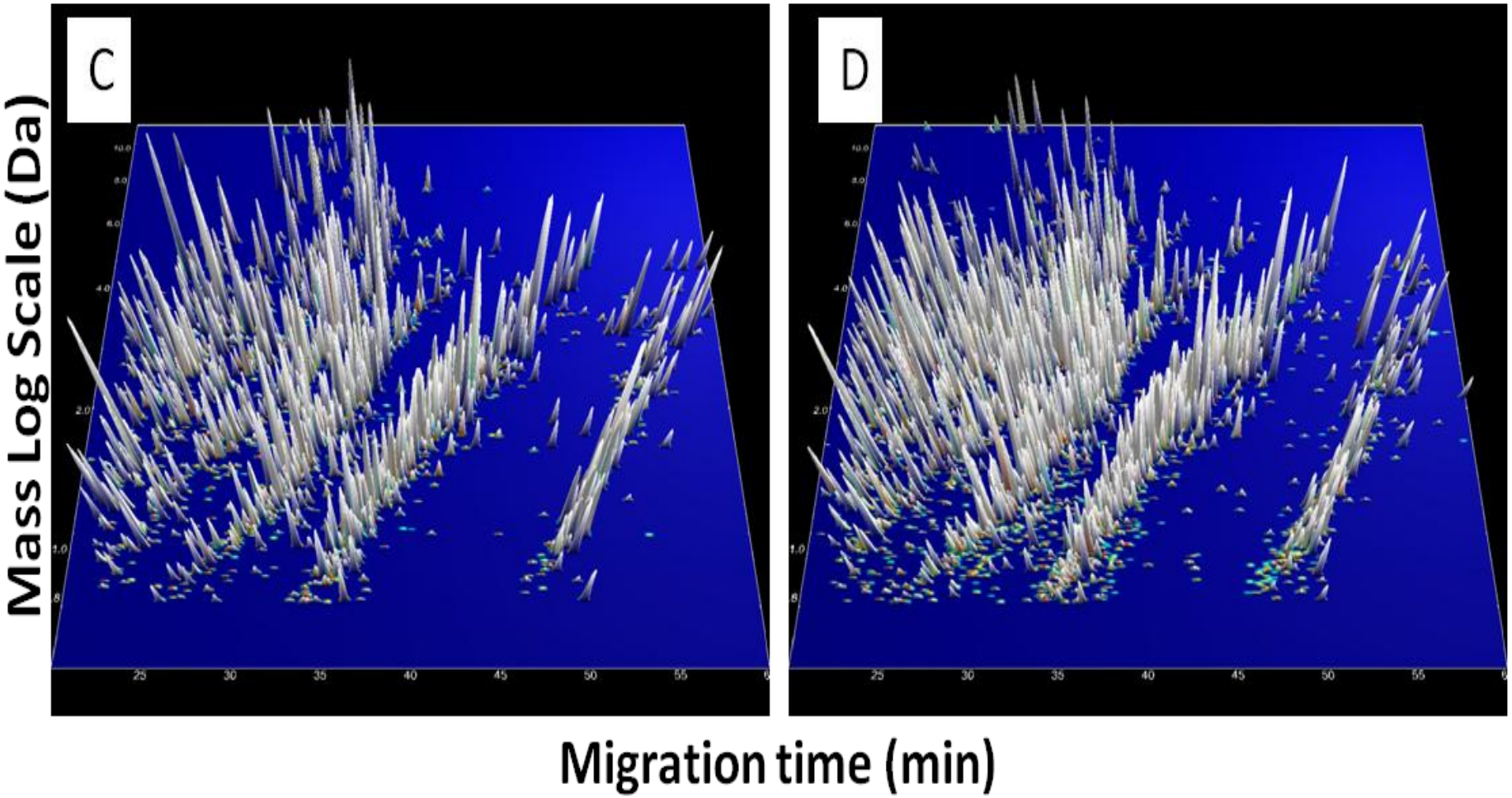

Figure 13-24: Composite peptide maps of CE/LC/MS peaks of milk at time points 42 h (C) and 57 h (D) post infection respectively.
Z –axis represents the mean signal intensity.

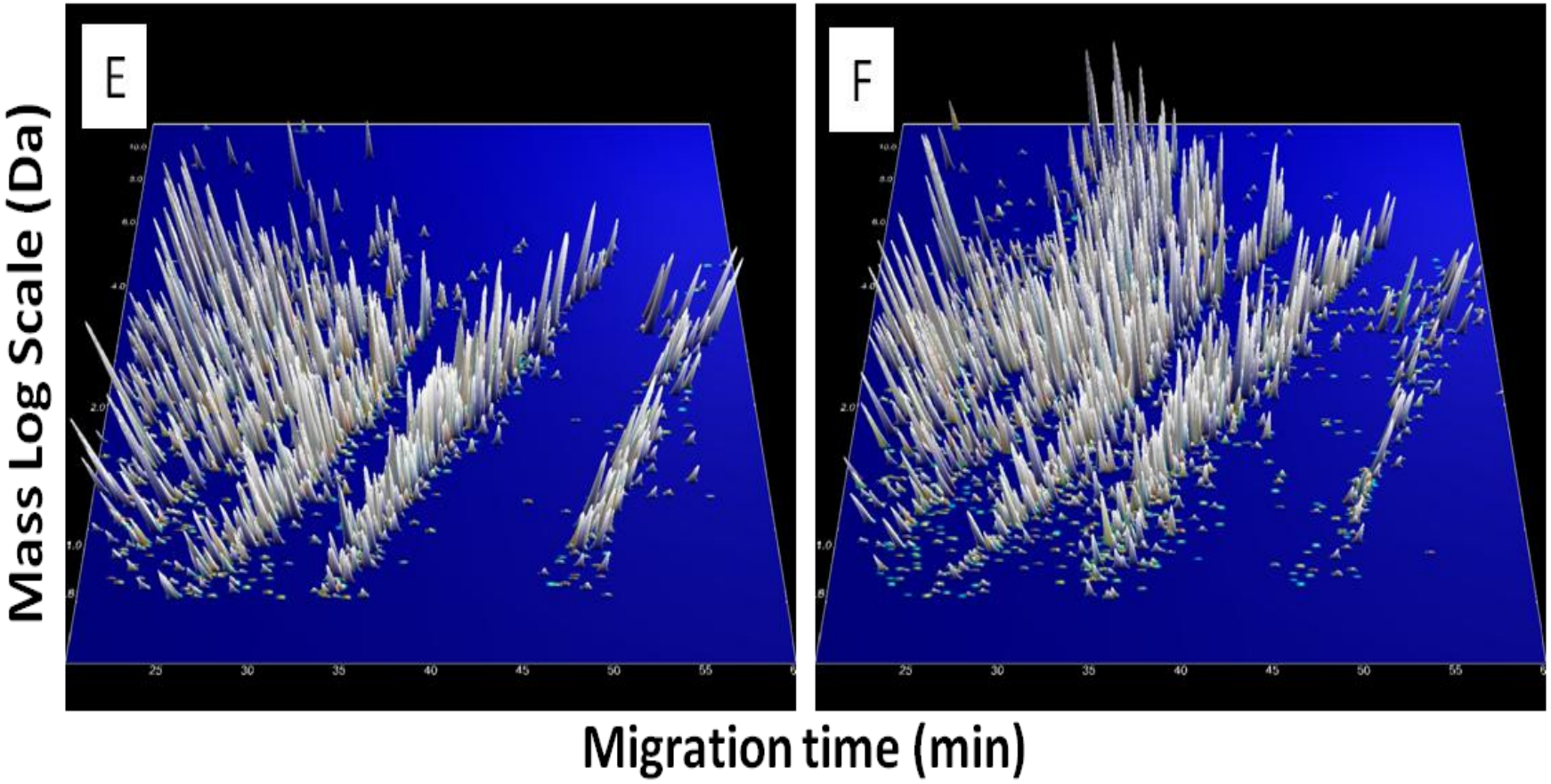

Figure 13-25: Composite peptide maps of CE/LC/MS peaks of milk at time points 81 (E) and 312 (F) h post infection respectively.
Z –axis represents the mean signal intensity.

Peptide maps (CE/LC/MS peaks) of potential biomarkers of *S. uberis* mastitis which were up-regulated or down regulated during infection time (36, 42, 57 and 81 h) relative to 0 h (pre-infection) are shown in Figure 5-26.

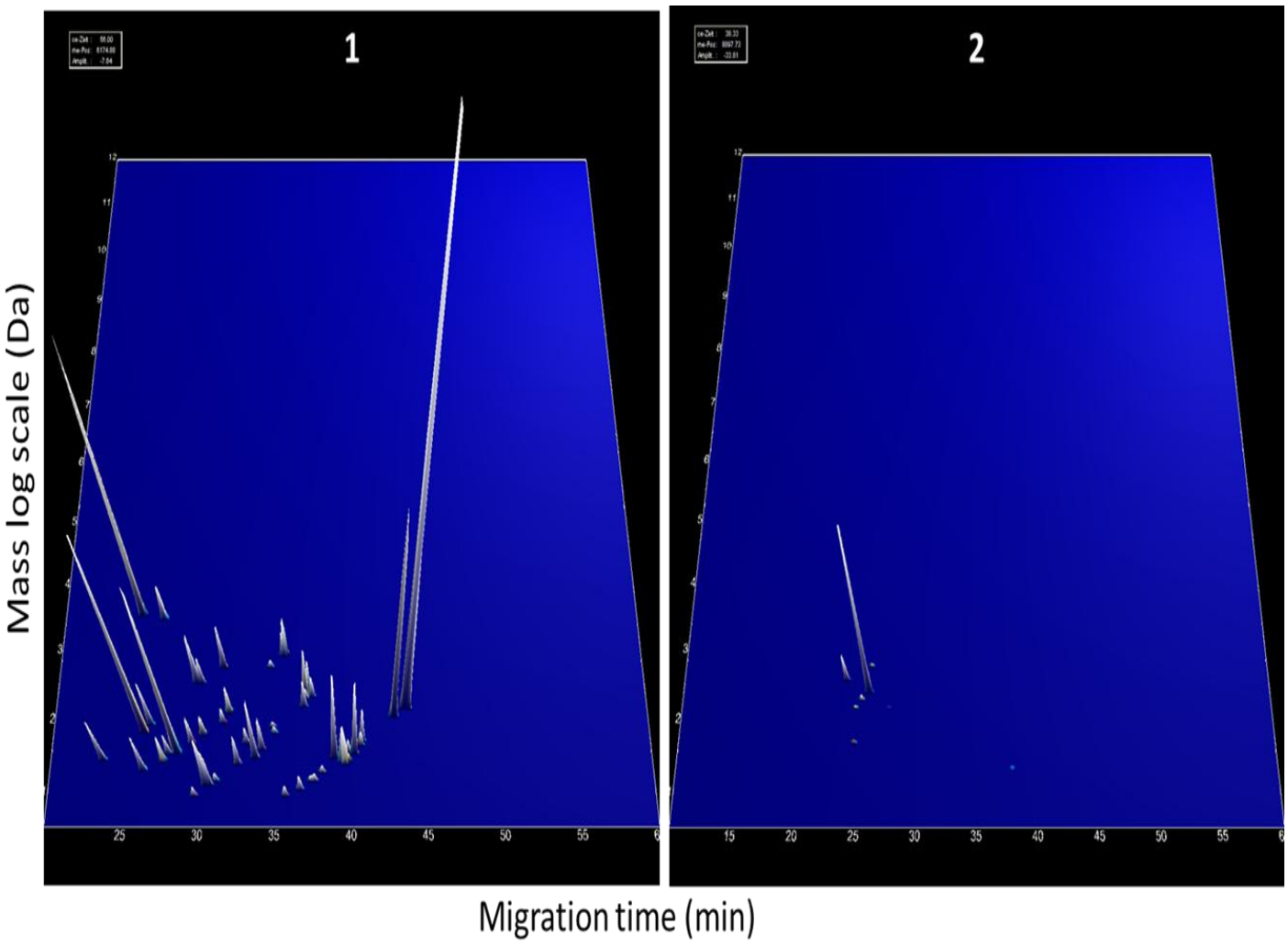

Figure 13-26: Differential peptide maps (CE/LC/MS peaks)
Showing peptides which were up-regulated (1) and down-regulated (2) during infection time (36 h, 42 h, 57 h and 81 h) relative to pre-infection time (0 h). Z –axis represents the mean signal intensity.

13.4.3.2 LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry sequences of the first 40 polypeptides in serial order out of 77 which were matched with 3 multi-consensus reports and also from report of Mansor *et al*. (2013), along with some of their characteristics, are listed in Table 5-4. Mass to charge ratio (m/z) range of the sequenced peptides was from 498.93 to 1008.88 Da and mass range from 1016.5 to 3610.74. Most of the sequenced peptides arose from cleavages of alpha-S1-casein and other caseins. A few were from SAA and glycosylation-dependent cell adhesion molecule (GDCAM) proteins. Some of the peptides derived from SAA protein were up regulated to several thousand folds during peak of infection, for example; GADKYFHARGNYDAA, GADKYFHARGNYDAAQRGPGGAWAA and SGKDPNHFRPAGLPDKY.

The greatest fold change occurred with the polypeptide GWRLPEYTVTQESGPAHRKEFTMTCRVERF which had sequences matching into the RISC-loading complex subunit protein. This peptide was the most up regulated peptide identified followed by SGKDPNHFRPAGLPDKY derived from SAA protein. There were 22 peptides which were down regulated among the total 77 sequenced and these were derived mainly from alpha-caseins and GDCAM proteins.

Table 13-3: Amino acid sequences of the first 50 polypeptides showing biomarker value and their protein characteristics
Peptide rows highlighted in green were identified in the present study, in 3 multi-consensus reports and also in report by Mansor *et al*. (2013) while the purple highlighted row indicates peptide identified only in present study and in Mansor *et al*. (2013), but not in the multi-consensus reports.

Number model	Mass	CE-migration time	Freq 0h	Freq 81h	Direction (peak infection)	Sequence	Charge	Protein Source
11	1177.6	30.25	1	0	down-regulated	NELSKDIGSES	2	Alpha-S1-casein
17	1250.74	31.01	1	0	down-regulated	TKVIPYVRYL	3	Alpha-S2-casein
29	1493.79	33.04	1	0	down-regulated	FVAPFPEVFGKEK	3	Alpha-S1-casein
68	1768.84	40.46	1	0	down-regulated	LYQGPIVLNPWDQVK	2	Alpha-S2-casein
102	2149.18	31.66	1	0	down-regulated	SSRQPQSQNPKLPLSILKE	4	Glycosylation-dependent cell adhesion molecule
104	2153.17	24.53	1	0	down-regulated	RGSKASADESLALGKPGKEPR	6	Fibroblast growth factor -binding protein 1
122	2277.28	27.65	1	0	down-regulated	SSRQPQSQNPKLPLSILKEK	5	Glycosylation-dependent cell adhesion molecule 1
123	2298.2	37.31	1	0	down-regulated	KNTMEHVSSSEESIISQETY	3	Alpha-S2-casein
135	2429.24	32.95	1	0	down-regulated	HIQKEDVPSERYLGYLEQLL	4	Alpha-S1-casein
140	2453.18	38.12	1	0	down-regulated	ILNKPEDETHLEAQPTDASAQF	3	Glycosylation-dependent cell adhesion molecule 1
146	2527.44	25.13	1	0	down-regulated	SSRQPQSQNPKLPLSILKEKHL	6	Glycosylation-dependent cell adhesion molecule 1
162	2708.46	26.6	1	0	down-regulated	LKKYKVPQLEIVPNSAEERLHSM	6	Alpha-S1-casein
163	2765.34	29.82	1	0	down-regulated	RTPEVDDEALEKFDKALKALPMHI	6	Beta-lactoglobulin
165	2788.43	30.64	1	0	down-regulated	EERLHSMKEGIHAQQKEPMIGVNQ	6	Alpha-S1-casein
118	2234.22	27.51	1	0.17	down-regulated	HPIKHQGLPQEVLNENLLR	5	Alpha-S1-casein
168	2826.54	25.95	1	0.17	down-regulated	HIQKEDVPSERYLGYLEQLLRLK	6	Alpha-S1-casein
22	1384.71	37.93	1	0.33	down-regulated	IPNPIGSENSEKT	2	Alpha-S1-casein
116	2233.23	24.72	1	0.33	down-regulated	RPKHPIKHQGLPQEVLNEN	6	Alpha-S1-casein
62	1744.99	24.67	1	0.67	down-regulated	HKEMPFPKYPVEPF	4	Beta-casein
126	2346.3	25.22	1	0.67	down-regulated	RPKHPIKHQGLPQEVLNENL	6	Alpha-S1-casein
158	2615.5	23.84	1	0.83	down-regulated	RPKHPIKHQGLPQEVLNENLLR	7	Alpha-S1-casein
141	2459.39	25.81	1	0.83	down-regulated	RPKHPIKHQGLPQEVLNENLL	6	Alpha-S1-casein
112	2215.06	43.52	1	1	up-regulated	SDIPNPIGSENSEKTTMPLW	2	Alpha-S1-casein
41	1560.77	39.33	0.17	1	up-regulated	GNYDAAQRGPGGAWAA	2	Serum amyloid A protein
33	1499.76	27.43	0.17	1	up-regulated	HIQKEDVPSERY	4	Alpha-S1-casein
21	1381.74	26.16	0.33	1	up-regulated	HPIKHQGLPQEV	4	Alpha-S1-casein

25	1456.75	39.53	0.17	1	up-regulated	YKVPQLEIVPNSA	2	Alpha-S1-casein
23	1445.8	32.42	0.17	1	up-regulated	VAPFPEVFGKEKV	3	Alpha-S1-casein
44	1595.83	27.76	0.5	1	up-regulated	SEETKENERFTVK	4	Complement C3
174	2989.64	35.25	0.17	1	up-regulated	LSLSQSKVLPVPQKAVPYPQRDMPIQA	4	Beta-casein
67	1767.84	40.66	0.17	1	up-regulated	FPKYPVEPFTESQSL	2	Beta-casein
85	1961.95	34.68	0	1	up-regulated	SRYPSYGLNYYQQKPV	3	Kappa-casein
13	1217.62	37.27	0.17	1	up-regulated	YPQRDMPIQA	2	Beta-casein
99	2125.03	42.81	0.33	1	up-regulated	GIHAQQKEPMIGVNQELAY	3	Alpha-S1-casein
3	1016.54	29.63	0.33	1	up-regulated	SHAFEVVKT	3	Glycosylation-dependent cell adhesion molecule 1
167	2823.44	34.41	0	1	up-regulated	SQSKVLPVPQKAVPYPQRDMPIQAF	4	Beta-casein
77	1887.97	34.79	0	1	up-regulated	QKAVPYPQRDMPIQAF	3	Beta-casein
32	1499.73	39.75	0	1	up-regulated	DIPNPIGSENSEKT	2	Alpha-S1-casein
20	1334.65	38.03	0	1	up-regulated	LSSSEESITRIN	2	Beta-casein

13.5 Discussion

13.5.1 Acute phase proteins during a *Streptococcus uberis* challenge

13.5.1.1 Haptoglobin

In this study milk Hp was first noticed to increase slightly from basal values at 30 h post challenge in 4 cows and at 36 h in two cows. The peak of the mean Hp in 6 cows was at time 57 h. The time of increase Hp coincided with the times of increasing SCC, peak bacteria CFU, and increases in most cytokines as previously described (Tassi *et al*., 2013), thus Hp levels can be said to reflect the stage of inflammation.

In comparison with other experimental mastitis challenge studies, for example that of Hiss *et al*. (2004) who induced an intramammary endotoxin challenge using LPS in dairy cows, Hp levels increased as early as 3 h in milk suggesting that the virulence or pathogenic factor of invading pathogens can play a crucial role on the onset and possible magnitude of Hp secretion into milk. In that study, the amount of pathogenic virulence factors injected may also have played a role in the quicker onset of rise in milk Hp. Following the experimental challenge of mammary glands with LPS, with an increase in Hp after 3 h in milk, and a rise at 9 h in serum, it was apparent that Hp in milk was not from seepage from serum. However the source(s) of Hp in mastitic and healthy milk samples has been subject of subsequent investigations mainly by RT-PCR (Lai *et al*., 2009). In the study of Pedersen *et al*. (2003), the same organism, *S. uberis,* was used for a mastitis challenge and earlier increases in milk Hp (10 h PC) were also observed compared to that in this study. Since that study only measured APP in milk following a short duration after experimental infection 0-12 h PC, the maximum increase from pre-challenge levels (tenfold) was observed at the 12th hour PC. The discrepancy observed in the onset of milk Hp increase in the present study and that reported by Pedersen *et al*. (2003) may be due to differences in strains of *S. uberis* used i.e. U103 used in the Pedersen *et al*. (2003) study whereas FSL Z1-048 was used in this study. The U1O3 was isolated from a case of clinical mastitis, while the FSL Z1-048 is a host adapted strain known to evade the host immune response and survive in the host causing chronic subclinical infections (Tassi *et al*., 2013). However following the

experimental challenge, clinical mastitis resulted. Therefore it can be concluded that, as suggested in the study of Kim *et al*. (2011), strain differences can influence the innate immune response and specifically APR and APP secretion patterns.

Up to a thousand fold increase in milk Hp was observed at the peak infection time in this study, highlighting the sensitivity of milk Hp to severity of infection.

13.5.1.2 M-SAA3

The response of M-SAA3 to the mammary challenge was more pronounced (in terms of total level of increase by μg/ml than Hp (~8000 μg/ml for M-SAA3 and ~500 μg/ml for Hp), although these changes occurred slightly later for M-SAA3 than Hp. There have been reports that SAA is a more sensitive APP to inflammation than Hp (Alsemgeest *et al*., 1994; Humblet *et al*., 2006), this has not been confirmed in the present study. M-SAA3 values were seen to increase to several thousand folds above basal levels at the period of peak infection (96 h). The profile of M-SAA3 in the present *S. uberis* mastitis challenge study can be said to reflect the severity and stage of the infection. Similar to the findings for Hp, M-SAA3 in two cows remained high at the last sampled time point (312 h), which indicated the presence of on-going infection, as confirmed by the positive detection of bacterial CFU in milk, from those quarters.

Unlike earlier observations, in this model of mastitis challenge, the APP response was slightly delayed, with the earliest noticeable increase in M-SAA3 occurring at 36 h as against previous reports of 6 h PC (Pedersen *et al*., 2003). In the study of Pedersen *et al*. (2003), SAA increased earlier than Hp whereas in the present study, Hp tended to raise earlier (average 36 h) than M-SAA3 (average 48 h) and also peaked earlier. The study of Pedersen *et al*. (2003) also reported that SAA increased in milk before SCC and also preceded onset of clinical signs. The variance in APP response under the same pathogen mastitis may be explained by strain differences. In a similar study, this time in challenged heifers, SAA increased slightly in milk at 24 h post challenge, but more appreciably at 42 h PC (de Greeff *et al*., 2013).

During an *E. coli* experimental infection study, various isoforms of SAA were detected in milk and appeared as early as 12 h post infection (Jacobsen *et al*.,

2005). In that study *S. uberis* mastitis milk expressed about 4 different isoforms of SAA ranging from 5.8-7.4 in pI.

Following a *Staphylococcus chromogenes* experimental infection, a CNS organism which is known to cause milk mastitis infections, MAA increased to peak levels at 54 h post challenge (Simojoki *et al*., 2009), but the milking interval was reported to affect M-SAA3 levels.

Pedersen *et al*. (2003) and Jacobsen *et al*. (2005) demonstrated an earlier rise in M-SAA3 than Hp suggesting that M-SAA3 may be more useful in early mastitis diagnosis. However other studies have not found M-SAA3 to be useful in relation to mastitis; Petersen *et al*. (2005) observed no significant correlation. In a recent study, Kalmus *et al*. (2013) did not find milk amyloid A (M-SAA3) as useful in indicating inflammation as Hp in mastitis caused by *A. pyogenes*. Although, Petersen *et al*. (2005), found that M-SAA3 was not correlated to SCC in mastitis milk, it can be inferred from the observations in the present study, that stage of infection when milk samples are collected for assay can significantly influence findings on both APP response and that of other innate immunity indices.

13.5.1.3 CRP

In this study, the first reports of milk CRP dynamics during the course of an experimental mastitis challenge have been presented. There was an initial noticeable increase in milk CRP at 30 h post infection in 4 out of 6 quarter-milk samples examined. These first increases were in the order of a 100 fold although rising from the undetectable (<1.8 ng/ml; LOD) concentration to an average of about 500 ng/ml in 3 samples, but was as high as a 4000 fold in one sample being undetectable at 24 h, but 4530 ng/ml by 30 h. However the maximum CRP at this level was less than that of Hp at ~500 µg/ml or M-SAA3 at ~8000 µg/ml.

The CRP concentration first peaked at 48 h post infection; however a biphasic pattern was seen, resulting in a second (slightly higher) peak of CRP concentration at 120 h post infection. The cause or significance of this biphasic pattern is not clear, but it may indicate changing phases in the bacterial growth and/or stimulation of cytokine response. The values of CRP, as was characteristic of the other two major APP in bovine milk, fell with the time

course of infection but remained significantly high at 312 h in all but one quarter sample which became undetectable.

Concentration increases as high as above 20,000 times the LOD were achieved at the peak of CRP levels. This level of increase in CRP in milk following a mastitis challenge clearly corresponds to the dynamics expected of a major acute phase protein and confirms the manufacturer's data (Life Diagnostics *Inc.*), of high CRP levels from mastitic milk samples. However, due to the presence of relatively small quantity of CRP in milk compared to the other APP (Hp and M-SAA3), which were measured in μg/ml, Hp and M-SAA3 have had more importance as indicators of inflammation in bovine milk. Nonetheless, using a proportionately sensitive assay with a sufficiently lower LOD may help to uncover potential of CRP in detecting mastitis.

From this study, CRP was found to rise earlier, than the other two APP and this could portend a potential advantage of CRP over Hp and M-SAA3 in the diagnosis of bovine mastitis. Other factors, such as the mastitis causing bacterial species, and the experimental model of the disease may however influence the rate of APP secretions, hence the behaviour of CRP in this study would require further validation using various disease models (natural and experimental) and mastitis causing pathogens.

Combinations of APP results into a mastitis diagnosis algorithm may add value to the use of these biomarkers, and would be suitable for future investigation.

13.5.2 Proteomics of milk during an *S. uberis* mastitis challenge

13.5.2.1 1DE SDS-PAGE

One dimensional gel electrophoresis is a robust and reproducible technique for protein separation of especially samples of simple mixture. However, it has low resolution. From the 1DE gel image, it was possible to identify visual differences in milk samples across the time points during the challenge period. Bands representing the major milk proteins (caseins) were observed to be absent at 36 h and 48 h, and only slightly visible at 42 h and 57 h, and then gradually increase in intensity towards resolution. In addition there was the gradual increase in intensity of bands identified as bovine lactoferrin with infection from 42 h up

until 312 h. There were also changes in serum albumin and immunoglobulin bands. Compared to the 1DE gel image of samples from control quarters, no obvious changes in protein bands could be observed across the time points spanning the entire challenge period. These changes in the high abundance proteins of milk have been documented previously (Hogarth *et al.*, 2004; Smolenski *et al.*, 2007), but here there is a clear demonstration of the transition of milk protein production from health to mastitis and resolution. It can therefore be inferred that 1DE is a good pre-fractionation method that can be employed for identifying broad changes in milk proteins during mastitis.

13.5.2.2 2DE: IEF and SDS-PAGE

Samples from 6 time points (0, 36, 57, 81, 168 and 312 h) during the course of the *S. uberis* mastitis challenge were further investigated to resolve proteins by both the isoelectric points and molecular mass. Spots corresponding to milk proteins as well as serum proteins were observed upon staining of 2DE gels. Although no mass spectrometry of gels spots were carried out in this study, inferences on the constituents of each spot was made by comparing gels spots analyses of similar whey samples carried out by Henderson (2013) and Mansor (2012) (Appendix Chapter 5).

The 2DE electrophoretogram of the various time points clearly delineated the stages of the infection through to abundance levels of milk proteins and Igs spots as well as presence and abundance of bovine serum albumin spots. Pre-infection samples (0 h), had abundant milk proteins spots and sparse Igs and BSA spots. During peak of infection (81 h), milk protein spots (caseins and whey proteins) were depleted, and BSA and Igs spots became abundant as samples came closer to the composition of blood (Hogarth *et al.*, 2004). Expectedly, as resolution of infection commenced (from 168 h), milk proteins spots began to increase while Igs and BSA spots diminished in size.

The 2DE methodology can be used for quantification of protein (spots) changes between different samples by the application of 2D gel analysis software such as SameSpots® (Totallab Ltd) and PDQuest analysis software package (Bio-Rad laboratories, Hemel Hempstead, UK), but would require duplicate or even replicate gels to allow statistical analysis. However, a more robust method for determining quantitative changes in proteins between gels is the DiGE method

which employs the comparison of spots of different samples run in the same gel, hence it eliminates technical errors (between gel variation) that may arise from running separate gels (Minden, 2007).

13.5.2.3 DiGE

In this study, 3 time points representing pre-infection, peak infection and resolution phase of the experimental mastitis model were analysed using the DiGE technique. The DiGE procedure provides a platform for comparison of similar samples and in this study it was possible to identify small changes in protein spots from different samples run on a single gel.

Using the Decyder™ 2-D image analysis software (DIA), it was possible to match and recognize differing spots across the three time points considered within the same gel using the internal pooled standard. In particular, quantitative changes in protein across the pre-infection, peak infection and resolution of infection stages were recognizable. The BVA module of the Decyder software also made it possible to reproducibly compare the spots and their variation across the 3 different gels, thus improving the robustness of the DiGE proteomic methodology.

From this study however, there were a number of spots observed in the DiGE gels which were not detected in the preparative gel possibly due to the relative lower protein concentration of each time point sample in the prep gel. This may pose a problem for identification of key proteins which are relevant to the diagnosis of IMI, as they cannot be picked for further MS analysis.

The technique enabled the detection of up to 728 protein spots which changed across the pre-infection, peak infection and resolution phase of the challenge course. Two hundred and thirty eight protein spots were up regulated during the course of infection while 283 protein spots were down regulated as the infection progressed. Among the 29 spots selected for LC-MS/MS identification 19 spots were of proteins up regulated during infection and were identified as serum related proteins such as albumin and serotransferrin which is known to increase in milk during mastitis. One other up regulated protein, benzodiazepine receptor (peripheral) associated protein 1, represents a rare finding in mastitic milk. It has been reported to have roles in steriodogenesis, apoptosis and oxidative

processes and being localized in the mitochondria (Casellas *et al.*, 2002), and this suggests the reason for its detection and up regulation in mastitic milk as probably being because of the part it plays in the phagocytic process and also as a consequence of seepage from serum into milk. Factor XIIa inhibitor precursor, a serine–type endopeptidase inhibitor with molecular roles in regulating important physiological pathways including complement activation, blood coagulation, fibrinolysis and the generation of kinins (www.uniprot.org/uniprot/P50448), was also identified as up regulated, likely as a results of seepage of from serum into milk. Acyl-protein thioesterase 1 isoform X1, leucine-rich repeat flightless-interacting protein 2, RAC-beta serine/threonine-protein kinase, RAC-beta serine/threonine-protein kinase and glial fibrillary acidic protein were all up regulated, are also likely related to the compromise of the blood milk barrier (Hogarth *et al.*, 2004).

Some of the down regulated proteins identified such as nuclear receptor ROR-alpha, thyroid receptor-interacting protein 6 and zinc finger protein have no clear significance in milk during mastitis.

Generally, DiGE is a proven useful and robust quantitative method for analysing protein differences between samples; however, this method is limited by the number of samples that can be compared per experiment, due to the number of dyes available for labelling samples. Newer approaches for quantitative proteomics studies are being developed to overcome these limitations, for example, label-free quantification proteomics, which does not require labelling of proteins prior to MS analysis but is rather based on precursor signal intensity or on spectral counting. This label-free method has been applied to study the proteomic changes in milk samples following this experimental *S. uberis* mastitis challenge (Dr Manikhandan Mudaliar, pers. comm.).

13.5.3 Peptidomics

The CE-MS analysis of bovine milk during the course of experimental mastitis revealed diverse changes in the milk peptidome across the time points spanning the mastitis challenge. This method of peptide analysis has been described as a powerful hyphenated technique for the study of peptidomic profiles (Herrero *et al.*, 2008), and has been exploited for the generation of biomarker panel of peptides for conditions such as renal (Good *et al.*, 2010) and cardiovascular

(Delles *et al.*, 2010) disorders. As observed in the study of Mansor *et al.* (2013), the technique was sensitive enough to detect peptide differences between milk samples from control and infected udders (31 polypeptides) and between milk from mastitic udders caused by two separate pathogens (14 polypeptides).

In order to identify potential biomarkers, a model that utilized training set from the pre-infection samples (0 h, with SCC levels <200,000 cells/ml reported in Tassi *et al.*, 2013) and from the time of peak of infection and greatest biochemical changes in milk (81 h post infection) was created. Statistical parameters such as significant BH p-value (0.01) and AUC=1 and correction for multiple testing were employed to screen polypeptides in order to determine those with biomarker potentials. The training set generated was then validated using the test set.

Overall, 205 peptides with significantly changing profile were detected, 77 peptides were able to be sequenced using an LC-MS/MS platform, using the peptide mass and number of basic amino acids link the peptides from the two separate systems (CE-MS and LC-MS/MS).

A majority of the successfully sequenced changing peptides arose from cleavages of alpha-S1-casein (n=31) and beta-casein (22 milk proteins confirming the reports of Dallas *et al.* (2014), Mansor *et al.* (2013) and Larsen *et al.* (2010b). This further explains the general decrease in milk caseins associated with clinical mastitis (Hogarth *et al.*, 2004).

A few of the peptides were from glycosylation-dependent cell adhesion molecule (GlyCAM-1) protein, (mainly down regulated), and serum amyloid A (SAA) protein (up regulated) cleavages. These two proteins have been identified as immune related proteins (Rocanda *et al.*, 2012; Boehmer *et al.*, 2010: Danielsen *et al.*, 2010). Proteases play a central role in the type and amounts of peptides detected in milk during mastitis and endogenous peptides such as plasmin, cathepsins, elastase, and amino- and carboxypeptidases have been suggested as the as crucial during the IMI as they are increased in milk due to release from the influx of neutrophils (PMNs) and other phagocytic cells (SCC) that occurs during mastitis (Larsen *et al.*, 2010b; Moussaoui *et al.*, 2003). These proteases were also reported to have specificities towards alpha-S1 and beta caseins.

Pathogen related proteases have also been suggested to contribute to the proteolysis observed in milk during mastitis (Larsen *et al.*, 2010b).

As reported in the study of Wedholm *et al.* (2008), peptides from alpha-S1, alpha-S2 and beta-caseins were identified in this study, additionally in the present study; two kappa-caseins fragments (sequenced) were seen during infection (absent at pre-infection samples). This followed the trend seen in the study of Moussaoui *et al.* (2003) who utilized LPS infusion to generate an experimental mastitis and studied the proteolytic changes of milk over time.

Out of the 77 peptides sequenced, 55 were increased in abundance (since they are mostly casein breakdown products) at peak infection time pointing to the increased proteolysis that occurs in milk during mastitis (Larsen *et al.*, 2010b). Fifty polypeptides showed qualitative differences between the 0 and 81 h post infection (being totally absent at one time as against the other), 27 peptides displayed quantitative changes with the course of infection.

Three polypeptides sequenced in this study, were similarly identified in both the multi-consensus and Mansor *et al.* (2013) reports. Two of these peptides were fragments from GlyCAM-1 protein and one was from cleavage of fibroblast growth factor-binding protein (FGFBP). All of these three polypeptides were found in pre-infection samples and absent during infection, and in the study of Mansor *et al.* (2013), these polypeptides only differentiated between healthy and mastitic samples and not between the two different mastitis pathogen species studied (i.e. *E. coli* and *S. aureus*). The matching of these peptides from the present study, the study of Mansor *et al.* (2013) and with reports from previous CE-MS milk analysis substantiates their probability as peptide markers of mastitis irrespective of the causal agent of mastitis.

As a time-point-based peptidomic study of mastitis progression, this study offers additional advantage over other previous peptidomic analysis of milk samples in relation to bovine mastitis, of detecting and identifying peptide(s), showing significant difference from pre-infection controls, as early as 36 h post infection. The probability exists that the peptidomic profile at an earlier time (before 36 h) may significantly differentiate pre-infection samples from commencement of infection, however, 36 h was the earliest post infection time assayed in this study. As an objective for future studies, it would be useful to determine the

earliest time point during which peptide changes are able to significantly differentiate healthy form infected samples.

In respect of peptide panel that differentiate mastitis caused by *S. uberis* from other pathogens, 72 of the polypeptides which were sequenced in this study, did not match any of the polypeptides detected in Mansor *et al*'s study or any of the multi-consensus reports. Therefore, these 72 peptides could represent a panel of peptides specific to *S. uberis* mastitis. Validation of this claim would be required using other *S. uberis* mastitis models such as natural infection and infections by different strains of *S. uberis*.

13.6 Conclusion

In this chapter, the APP profile of milk during an experimental mastitis challenge was studied and results clearly indicate the value of the 3 APP, Hp, M-SAA3 and CRP in diagnosing and monitoring the stage of mastitis infections.

Gel based proteomic studies, further carried out on the samples, gave clearer understanding on protein pattern variation with course of infection and resolution.

Analysis of the peptide profile in milk across selected time points of the experimental challenge, showed a panel of peptides, cross matched with reports from previous studies, which as early as 36 h post infection could significantly differentiate infected from non-infected milk, thus suggesting potential as biomarkers of bovine mastitis and specifically that of *S. uberis* origin.

14 Metabolomic investigation of milk following an experimental *Streptococcus uberis* mastitis challenge

14.1 Overview

In this chapter, a metabolomics approach to biomarker identification for bovine mastitis is described. The *S. uberis* experimental mastitis challenge discussed in the proceeding chapter is used as the time point based model of mastitis.

14.2 Introduction

14.2.1 Bovine milk metabolomics

Following the advances in instrumentation for metabolomic analyses, metabolomics of milk samples have been applied in a growing number of studies for increased understanding of the physiology of the mammary gland and lactation. This has included studies on the associations of milk metabolites with technological properties of different milk (traits), relationship with disease conditions (somatic cell counts), nutritional quality and composition, as well as authentication and screening for adulteration or detection of the origin of milk samples (Lamanna *et al.*, 2011; Lachenmeier *et al.*, 2009) and even for recognizing the association of blood with milk metabolites (Maher *et al.*, 2013).

In bovine milk, metabolomics experiments by Belloque and Ramos, (2002) used an NMR based approach for a targeted analysis of caseins in commercial milk samples while Hu *et al.* (2007) successfully used NMR (^{1}H 1D and ^{1}H-13C HSQC 2D) to quantify organic compounds in milk including the low quantity milk N-acetyl carbohydrate compounds which had rarely been quantified in commercial milk, before that study. Milk volatile constituents were identified and differentiated based on ration composition using GC-MS and discriminant analysis by Toso *et al.* (2002).

Likewise, Hettinga *et al.*, 2008 employed a solid phase micro extraction (SPME) GC-MS approach for the elucidation of volatile metabolites in milk in relation to

five common causative organisms of bovine mastitis. In order to confirm the origin of the volatile metabolites in mastitis milk, a further study was carried out by inoculating milk from healthy quarters with selected bacteria. Results from these studies not only demonstrated the specificity of the milk metabolome for intramammary infections, but also pathogen specific mastitis.

Furthermore, studies by Boudonck *et al*. (2009) showed the capability of a metabolomics approach using GC-MS and LC-MS in differentiating milk samples based on ten different characteristics that were sources of variability. Consequently, it was possible to differentiate milk samples coming from different farming systems. This study also highlighted metabolite differences that are present in various processed milks.

Another metabolomics experiment on milk by Klein *et al*. (2010) identified Acetone and ß-hydroxybutyric acid (BHBA) as indicators of metabolic status for cows, and also classified stage of lactation based on the milk metabolome. In that study NMR and GC-MS data were combined for further statistical analysis after confirmation of agreement of the analysis by the two methods, demonstrating the versatility that could be achieved with a combined approach to the study of the metabolome.

Metabolomic experiments in bovine milk have encompassed the identification of a diverse range of metabolites and compounds from different pathways. For example, phosphorylated saccharides accounted for the most significant changes in metabolites of milk with days of lactation in studies by Ilves *et al*. (2012), who utilized a global untargeted metabolomics approach in order to study the variation in milk and blood metabolome in different lactation stages of dairy cows. Changes in citrate and lactose were also observed to be significant with days in milk (DIM) and the duration of the current lactation. Harzia *et al*. (2013) were able to identify several marker compounds that indicated different coagulation abilities in milk through a metabolic profiling using LC-MS.

The ratio of glycerophosphocholine to phosphocholine was recognized as a prognostic marker for determining the risk of development of ketosis in cows using milk metabolomics (Klein *et al*., 2012), thus highlighting another area of use of milk metabolomics as not just in diagnosis, but also having prognostic

value. Similarly BHBA was recognized as one of the metabolites that had a significant increase in milk with high SCC (Sundekilde *et al.*, 2013).

An untargeted metabolomics (and proteomics) technique was used to analyse the profile of milk samples from dairy cows with different energy balances and at different stages of lactation by Lu *et al.* (2013). They found a correlation between acute phase response proteins, unsaturated fatty acids, and galactose-1-phosphate and negative energy balance. Since negative energy balance is a stress inducing condition especially in lactating dairy cows, the positive association with acute phase proteins (APP) is expected as the latter are normally induced in response to inflammation, infection or stress. Stomatin, a hormone that influences body growth, was also found to be strongly associated with a positive energy balance status of the cows as were cholesterol and other proteins.

Compounds shown to have antimicrobial properties, including N-alky diethanolamines, N-2-alkoxyethyl diethanolamines and N-alkyl ethanolamine were also elucidated in milk in up to 2.3-30 ppm using an electrospray ionisation MS procedure directly following protein precipitation (Vadla *et al.*, 2013). Melzer *et al.* (2013) have also shown a relationship between metabolite profile and specific milk traits, for instance lactic acid, uracil and nine other major metabolites were demonstrated to be correlated to high SCC, this agreed with findings of Sundekilde *et al.* (2013) who also uncovered the significant association of lactate to high SCC. Melzer *et al.,* 2013 have also used the tool of metabolomics to relate genetics to milk traits. Metabolite profiles of three specific milk traits were first determined as his was used to identify single nucleotide polymorphism (SNP) that could be used to predict these metabolic traits..

The use of metabolomics in order to detect viable biomarkers for mastitis has been explored by a number of researchers. Mansor *et al.* (2013) studied the metabolite profiles of milk samples from *E. coli* and *S. aureus* infected udder quarters in natural cases of clinical mastitis and compared these profiles to those from healthy milk samples. Fifteen metabolites, mostly tripeptides and dipeptides were found to be differentially expressed in infected samples compared to healthy samples. In particular, 3- nitrotyrosine, deoxyguanosine

and 3-methoxy-4-hydroxylphenyl were significantly increased in *E. coli* infections, while Leu-Arg, Ser-His and Lys-Ala-Gly were significantly increased in *S aureus* infections as against healthy. Pathway analysis showed that the lipids, protein and carbohydrate metabolism pathways were altered through the arachidonic acid, arginine and galactose metabolites, respectively. These metabolites were significantly increased in mastitis samples.

Sundekilde *et al*. (2013) using NMR spectroscopy of milk elucidated differences in metabolite profile between samples with high and low SCC. Multivariate data analysis techniques revealed distinct differences in the metabolite profiles of milk with low or high SCC (with lactate, butyrate, isoleucine, acetate and BHBA being increased in high and decreased in low SCC groups, while hippurate and fumarate decreased in high and increased in low respectively). In an earlier study by Hettinga *et al* (2009), some metabolites such as acetic acid (acetate), butyric acid (butyrate), were detected in milk samples from udders with mastitis caused by different pathogens. These studies have so far demonstrated that evaluating the metabolome of milk, can, if further explored and properly validated, be used to identify markers of metabolic-disease risks, mammary inflammation and outcomes of mastitis.

More work, therefore needs to be carried out in order to comprehensively elucidate the metabolome of milk during an IMI. Especially important is the relation to a time course of such an intramammary disease, which would enable the capturing and recognition of metabolite profile in the critical stages during a typical mastitis episode, such as the early, peak and the resolution phases of the infection.

14.3 Objectives of study

The aim of this study was to assess the variation in the metabolome in bovine milk samples following progression of experimental intramammary challenge with a host-adapted strain of *Streptococcus uberis* (FSL Z1–048).

Streptococcus uberis represents an important cause of mastitis in dairies in the UK and it has been shown to cause severe disease which is often difficult to control (Zadoks, 2007).

The ultimate goal of this experiment is to identify metabolites or compounds, having the potential for use as biomarkers of general mammary inflammation or infection specific to the *S. uberis* pathogen and strain, using a time course from infection free to resolution of infection. This will help to gain a better understanding of metabolic pathways altered with this infection and provide valuable information on the nature of the bacterial infection and host response.

14.4 Materials and methods

14.4.1 Samples

Milk samples used for this study were obtained following an experimental challenge of six udder quarters in six different cows with a host adapted strain of *Streptococcus uberis* (strain FSL Z1-048) as described in section 2.3.4.5. Samples collected at time 0, 36, 42, 57, 81 and 312 h after intramammary challenge from each infected quarter (n=36) were used for metabolomic studies.

APP profiles of the milk samples were determined as described in sections 2.3.4.5 and 3.3.6.4 and results reported in sections 2.4.5 and 3.4.4 respectively.

14.4.2 Sample extraction

The skimmed milk samples were extracted using chloroform: methanol (1:3 v/v) mixture (Canelas *et al.*, 2009; Beltran *et al.*, 2012) by adding 400 µl of this mixture to 100 µl of skimmed milk sample and mixing vigorously for 2 h at 4ºC (on a vortex). After this, the samples were centrifuged at 13 000 g, 4ºC for 5 min, then the supernatant was separated and stored at -80ºC until analysed.

14.4.3 Separation and detection

Liquid chromatography mass spectrometry (LC-MS) was carried out using a Dionex UltiMate 3000 RSLCnano in-line HPLC coupled to a Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. The separation of metabolites was performed using a 4.6 x 150 mm SeQuant ZIC-pHILIC (Merck KGaA, 6427 Darmstadt, Germany) column at 300 µl/min. The mobile phase consisted of (A) 20 mM Ammonium Acetate, pH 9.9 and (B) 80 % acetonitrile (ACN). Gradient B: 80 % ACN to 5 % ACN in 15 min then held at 5 % for 3 min, returned to 80 % in 1 min, equilibrated for 6 min. The total run time was 25 min.

Samples were prepared in H_2O: ACN (1:3) and 10 μl was injected in every run. The MS acquisition was performed in both negative and positive ionisation modes with full scan. The MS was set at 50,000 resolutions with the scan range from 70-1400 amu. The LC-MS analysis was performed with the assistance of Dr Manikhandan Mudaliar under the supervision of Dr. Karl Burgess at Glasgow Polyomics, College of Medical, Veterinary and Life Sciences, University of Glasgow, UK.

14.4.4 Data processing and statistical analysis

The data generated after the LC-MS analysis was processed using Ideom software version 18 (http://mzmatch.sourceforge.net/ideom.php). Briefly, the raw data was manually sorted into folders according to study groups and using the automated peak picking, grouping and filtering option in Ideom, the raw data was converted from 'RAW' file format to mzXML file format, peaks were identified and converted to peakML files using XCMS and peaks from all samples were combined and annotated into one peakML file using mzMatch imported into Ideom for metabolite identification, data from positive and negative modes were combined and comparison between the groups was performed using unpaired t-test (Darren *et al.*, 2012). Settings and thresholds used in the analysis is given in the Table 6-1. Table 6-2 shows the identification confidence for the LC-MS data used.

Table 14-1: Settings and threshold values used for running the LC-MS metabolomics experiment on milk

IDEOM SETTINGS		
Polarity:	Combined	
XCMS (Centwave)		
Method (file type):	mzXML	
Parts per million (ppm):	2	
Peak width (min):	5	seconds
Peak width (max):	100	seconds
S/N threshold:	3	
Prefilter (# points):	3	
Prefilter (intensity):	1000	
Mzdiff:	0.001	
mzMatch		
Mzmatch grouping RT window:	0.5	min
Mzmatch grouping m/z ppm:	5	ppm
Relative Standard Deviation (RSD) filter:	**0.80**	GENEROUS
Noise filter (coded):	0.80	
Intensity filter (LOQ):	**1000**	
Minimum detections #	**3**	
Retention time (RT) window for related	0.10	min
IDEOM		
RT for id of authentic standards:	**5.0**	**%**
RT for id for calculated RT:	**50.0**	**%**
PPM for mass identification:	**3.0**	**ppm**
Ignore related peaks before RT:	0.0	min
RT window for complex adducts:	0.50	min
RT window for Duplicate peaks:	1.00	min
RT window for Shoulder peaks:	2.0	min
Intensity ratio for Shoulder peaks:	5	to 1
Intensity limit duplicate peaks:	1	%
r^2 limit for duplicate peaks	0.99	

Table 14-2: Identification confidence for LC-MS data

IDENTIFICATION CONFIDENCE:	
Confidence levels	**Arbitrary**
Standard RT within 5%	9
Calculated RT within 50%	7
ID-dependent rejection	
Xenobiotics	4.5
RT outside window	3
Peak-dependent rejection	
Below intensity filter	0.5
RSD filter	0.4
Shoulder/duplicate peak	0.2
common adducts/fragments/isotopes	0.1
not more than blank control	0
Confidence modifiers	**add**
Preferred DB	1
Related peak (mzMatch)	-2
Filtering threshold: (adjust in macro)	5

14.4.5 Metabolite analysis

A spread sheet of metabolite mass, retention time (RT), formula, isomers, putative metabolite, pathway, map, max intensity and confidence obtained for each time point was obtained and is provided as supplementary information data. The mass spectra data were normalized and descriptive statistics including mean, standard deviation of metabolites in each time point for the 6 infected quarters as well as relative standard deviation, Fisher test and P-values of t-test of means at point 0, compared with other time points were also displayed on the excel spread sheet (supplementary information).

Structure, chemical and physical properties of all identified metabolites were assessed by linking to the Chemspider website that displays these details on the webpage (http://www.chemspider.com/Chemical-Structure). To further differentiate and quantify the changes to metabolites at different times and based on pathways, false discovery rate (FDR)-adjusted $P \leq 0.05$ $\log_2$ fold (at specific time points) was computed.

14.4.6 Pathway assignment

Pathway assignment of metabolites was carried out by following the link of the putative metabolite to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) map available on http://www.genome.jp/kegg-bin/show_pathway identifying pathways in which each metabolite is involved.

14.4.7 Advanced data analysis

The large data generated is to be also analysed by advanced stratified and bioinformatics methodologies but is beyond the scope of this thesis and will be reported elsewhere (Thomas and Mudaliar, in preparation).

14.5 Results

14.5.1 General

A total of 9681 different metabolites were detected over all 36 samples analysed, 5545 metabolites were in the positive ionisation mode while 4136 were in the negative ionisation mode. Of all the compounds detected, 3164 were provisionally identified (shown in supplementary data) and 1109 of these showed variation with time during the course of experimental challenge and were thus used for the comparison between time points. Six hundred and forty (640) of these varying metabolites were identified with a confidence score of ≥ 7. The confidence score (CS) of metabolites identification is based on the minimum reporting standards for metabolomics (Sumner *et al.*, 2007). Metabolites identified by mass and retention time (RT) with authentic standards are highlighted yellow in the following Tables and given a CS of 10 (n=57, supplementary data), all others are putative identifications. Putative identification is by an untargeted method whereby high-resolution LC-MS signals are matched to a database of theoretical masses and predicted retention times. It is recognized that a CS of 7 upwards is acceptable for biochemical interpretation (Sumner *et al.*, 2007), therefore all results presented and discussed, were based on metabolites having CS of ≥ 7 that changed over time.

A total of 57 metabolites were identified with a CS of 10, 3 with CS of 9, 152 with 8 and 428 with 7 making the total 640 metabolites. A summary of the number of metabolites detected and identified is presented in table 6-3. Figure

6-1 shows the proportion (percentage) of metabolites identified with CS of 7-10 from each of the different metabolic pathways, while Figure 6-2 shows the proportion of the metabolites which were identified using authentic standards with a CS of 10 (n=57) from the different pathways. In tables some metabolites are highlighted in yellow. The metabolites highlighted in yellow signify those that were identified using the authentic standards and given confidence score of 10.

Table 14-3: Summary of the number of metabolites in different categories
(CS-Confidence score; IM-ionisation mode)

Characteristic	Positive IM	Negative IM	Total
Peaks Detected	5545	4136	9681
Peaks Annotated	1849	1315	3164
Peaks non-annotated	3696	2821	6517
Metabolites Identified	628	481	1109
All metabolites compared across time points	628	481	1109
Metabolites compared across time points with CS of ≥ 7	373	267	640
Metabolites compared across time points with CS of 10	31	26	57

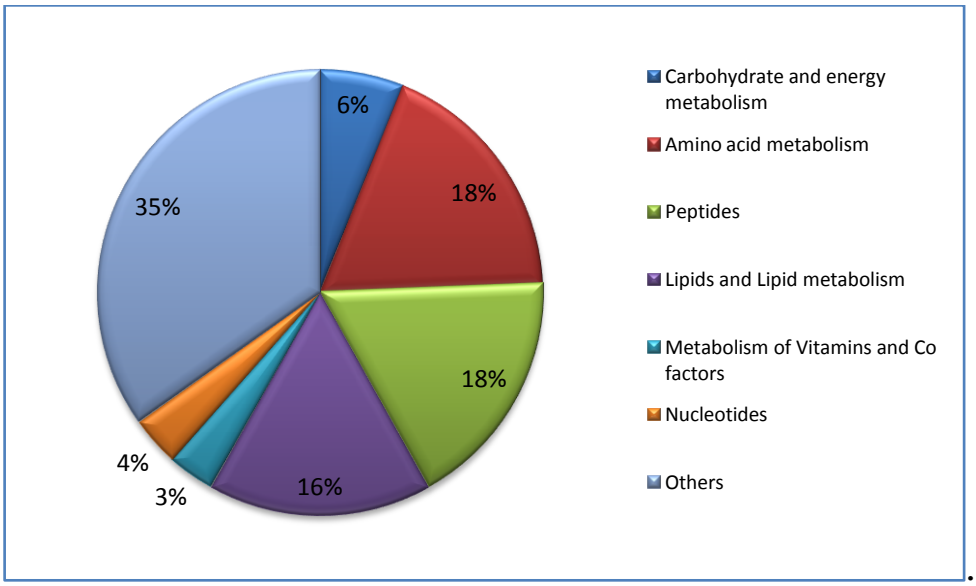

Figure 14-1: Percentage of metabolites in specific pathways identified with confidence score of 7 and above (n=640).

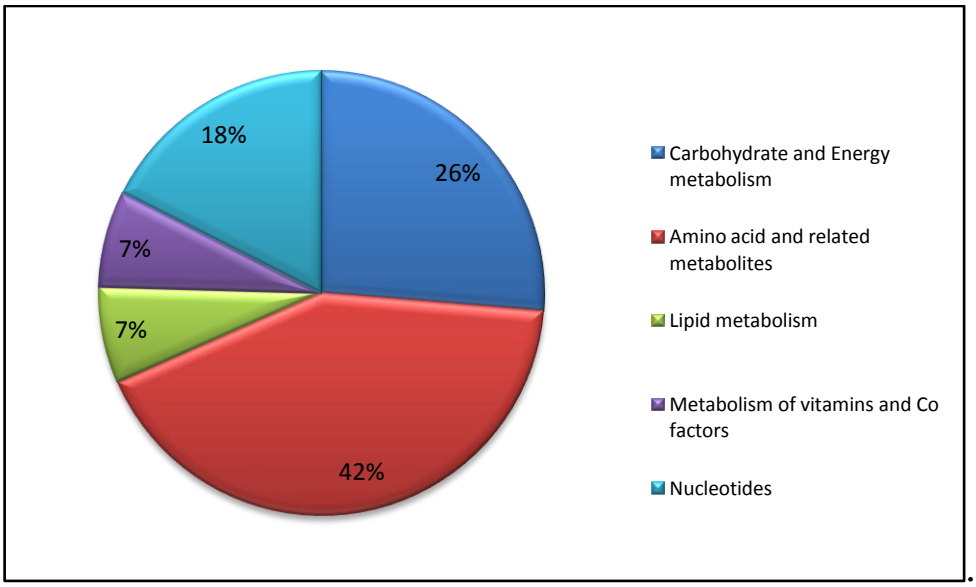

Figure 14-2: Percentage of metabolites in specific pathways identified with confidence score of 10 (n=57).

Altogether, the mass of metabolites identified that showed variation across time, ranged from 75 to 886 Da, whilst a retention time range of 0.29 to 21.6 s was seen. Metabolites were further examined based on their m/z intensity levels and level of change (increase or decrease) based on $\log_2$ intensity fold change (P-value of <0.05). Trends of specific metabolites with time were also investigated. Full lists of the change in the metabolome during experimental

infection are given in the supplementary data. The focus of this chapter is on the metabolites which showed the greatest change following infection. Table 6-4 shows the first 20 metabolites from the highest m/z intensity that changed in intensity with time.

Table 14-4: First 20 metabolites with highest m/z intensity (20/640)
Showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; p-positive, n-negative. Compounds highlighted in yellow are those with a confidence score of 10.

Metabolite	Formula	Pathway	m/z intensity	IM/peak
Creatine	C4H9N3O2	AAs	1.92E+09	p7
Betaine	C5H11NO2	AAs	1.61E+09	p24
L-Carnitine	C7H15NO3	AAs	1.36E+09	p78
sn-glycero-3-Phosphocholine	C8H20NO6P	LLs	1.33E+09	p91
Choline	C5H13NO	AAs	1.08E+09	p112
L-Proline	C5H9NO2	AAs	6.05E+08	p68
Citrate	C6H8O7	C/E	5.99E+08	n6
Creatinine	C4H7N3O	AAs	5.08E+08	p125
Hexadecanoic acid	C16H32O2	LL	5.03E+08	n85
2-Dehydro-3-deoxy-L-rhamnonate	C6H10O5	C/E	4.04E+08	n162
Orotate	C5H4N2O4	NT	3.53E+08	n205
Choline phosphate	C5H14NO4P	LL	3.24E+08	p270
Hippurate	C9H9NO3	AAs	2.77E+08	n332
(S)-Malate	C4H6O5	C/E	2.64E+08	n139
L-Leucine	C6H13NO2	AAs	2.4E+08	p153
8-keto-7-aminoperlagonate	C9H15NO3	0	1.84E+08	n345
4-Trimethylammoniobutanoate	C7H15NO2	AA	1.84E+08	p1
Methyloxaloacetate	C5H6O5	CE	1.69E+08	n368
(R)-3-Hydroxybutanoate	C4H8O3	LL	1.62E+08	n393
Asp-Cys-Ser-Tyr	C19H26N4O9S	PP	1.59E+08	p372

There were four distinct trends observed among metabolites as the infection progressed;

Trend A; decline in intensity from 0 h with the lowest levels at one of the points between 0 and 312 h (coinciding with peak clinical disease) and a restoration back to normal levels (as at 0 h; control/healthy samples), at the resolution time

312 h. Examples of metabolites showing this trend are creatine, betaine and sn-glycero-3-Phosphocholine which were lowest at 81 h, while others such as L-carnitine, pyridoxal and L-glutamine were lowest at 57 h.

Trend B; an increase in m/z intensity of compound(s) present at 0 h, from initial levels (0 h) rising to a maximum during the peak periods of clinical mastitis and then dropping back at 312 h. Examples are hexadecanoic acid (peaked at 57 h and then drops), (R)-3-hydroxybutanoate (peaked at 57 h) and decanoic acid (peak at 57 h). N-acetyl-L-aspartate (peaked at 42 h) and then drops almost to levels recorded at 0 h by 312 h. This occurred with a total of 73 metabolites.

Trend C; Metabolites that were initially absent in healthy samples (0 h), appearing after 0 h and peaking at some point between 42-81 h, while disappearing (or almost) again at 312 h. Examples are thiomorpholine 3-carboxylate, Leu-Ala-Gln and L-ala-L-glu.

Trend D; Metabolites in which the levels fluctuated irregularly and in no specific pattern in relation to progression of disease. This occurred with urate, N-ethylmaleimide and D-ornithine.

The specific metabolites with the most fold increase or decrease at different time points after 0 h are listed in Table 6-5.

Table 14-5: List of metabolites that showed the greatest changes at different time points. The changes were based on Log_2 (fold) and were of two types; increasing (up regulation) or decreasing (down regulation).

Time point	Highest fold decrease	Highest fold increase
36 h	Deoxycytidine (-5.43)	7-methyladenine (10.27)
42 h	Deoxycytidine (-5.33)	Trp-Ala (8.22)
57 h	Choline phosphate (-9.54)	Leu-Ala-Gln (12.24)
81 h	Choline phosphate (-10.83)	Puromycin (10.92)
312 h	5-Methyl-2'-deoxycytidine(-4.12)	Thiomorpholine3-carboxylate (10.87)

14.5.2 Time points analysis of metabolites

The metabolites were assessed by comparing mean m/z intensities at the five different time points to their mean intensities at 0 hour (0 h) which was used as

the control time. Student's T-test was carried out to compare means of the different time points with mean at time 0 h. Frequency distributions of metabolites at specific time points relative to control time (0 h) are shown in Figures below 6-3 to 6-8.

At each time point, the tables show metabolites with significant change from 0 h (first twenty), trend of metabolites and metabolites with the highest m/z intensity (first twenty) are listed with their major characteristics (Table 6-6 to 6-26). In these tables metabolites are listed in descending order of m/z intensity and metabolites with a yellow highlight are those that were identified using authentic standards and given confidence score of 10. Figures 6-1 to 6-12 also show the distribution of metabolites present and absent in samples at various time points relative to the 0 h time point sample.

14.5.2.1 Thirty six hours post infection

At this time point some substantial changes had occurred in the milk metabolome. Details of the distribution of changes relative to time 0 h and lists of first 20 metabolites with most significant changes in m/z intensity are shown in Figure 6-3 and tables 6-6 to 6-9.

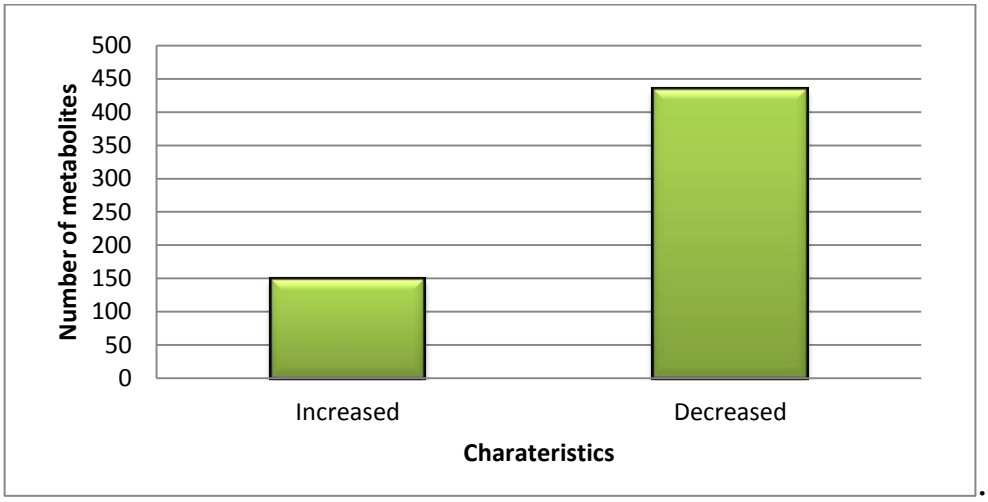

Figure 14-3: Proportion of metabolites present at 0 h, which have either increased or decreased in m/z intensity by 36 h.
Of 640 metabolites identified, 581 were present at 0 h, histogram shows how many increased (149) and decreased (422) with course of infection.

Table 14-6: Two compounds that were present at time 0 h but absent at 36 h
PP-small peptides; 0-unknown pathway

Metabolite	Mass	Formula	Pathway	IM/peak ID
Glu-Leu-Lys-Lys	258.16	C23H44N6O7	PP	p2033
Alpha-N-Acetylneuraminyl -2,6-beta-D-galactosyl-1,4-N -acetyl-beta-D- glucosamine	674.24	C25H42N2O19	0	n3554

Table 14-7: The 20 Metabolites with highest m/z intensities of n=433, present at 0 h, and reduced at 36 h.
In descending m/z intensity, (Trend A) showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; p-positive, n-negative (ion detection).

Metabolite	Mass	Formula	15 Path	m/z Intensity	16 IM/p
Creatine	131.07	C4H9N3O2	AAs	1919896320	p7
Betaine	117.08	C5H11NO2	AAs	1610266496	p24
L-Carnitine	161.11	7H15NO3	AAs	1356191488	p78
sn-glycero-3-Phosphocholine	257.1	C8H20NO6P	LL	1331854592	p91
Choline	103.1	C5H13NO	AAs	1080505600	p112
Citrate	192.03	C6H8O7	C/E	598937344	n6
Creatinine	113.06	C4H7N3O	AAs	507809600	p125
2-Dehydro-3-deoxy-L-	162.05	C6H10O5	C/E	403872448	n162
Orotate	156.02	C5H4N2O4	NT	352815456	n205
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
Hippurate	179.06	C9H9NO3	AAs	276560320	n332
(S)-Malate	134.02	C4H6O5	C/E	263881568	n139
8-keto-7-aminoperlagonate	185.11	C9H15NO3	0	184082784	n345
4Trimethylammoniobutanoate	145.11	C7H15NO2	AAs	184079424	p1
Methyloxaloacetate	146.02	C5H6O5	C/E	168854336	n368
Asp-Cys-Ser-Tyr	243.07	C19H26N4O9S	PP	158657744	p372
Lactose	342.12	C12H22O11	C/E	152418496	n396
N-(octanoyl)-L-homoserine	245.16	C12H23NO4	0	147646064	p410
cis-Aconitate	174.02	C6H6O6	C/E	145240640	n339
N-Acetyl-D-glucosamine	221.09	C8H15NO6	AAs	143361424	p373

Table 14-8: The 20 Metabolites with highest m/z intensities out of 148, present at 0 h, increased by 36 h

In descending m/z intensity, (Trend B) showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	17 m/z	18 IM/
L-Proline	115.06	C5H9NO2	AAs	605189696	p68
Hexadecanoic acid	256.24	C16H32O2	LL	502962240	n85
L-Leucine	131.09	C6H13NO2	AAs	239876032	p153
(R)-3-Hydroxybutanoate	104.05	C4H8O3	LL	162329008	n393
Decanoic acid	172.15	C10H20O2	LL	150188064	n484
L-Phenylalanine	165.08	C9H11NO2	AAs	134699168	p418
N-Acetyl-L-aspartate	175.05	C6H9NO5	AAs	134420832	n518
L-1-Pyrroline-3-hydroxy-5-carboxylate	129.04	C5H7NO3	AAs	108133304	n224
Leu-Leu-Val	343.25	C17H33N3O4	PP	106793240	n543
5-Hydroxypentanoate	118.06	C5H10O3	LL	95705432	n556
L-Tyrosine	181.07	C9H11NO3	AAs	73216184	p541
L-Methionine	149.05	C5H11NO2S	AAs	64114592	p412
2-C-Methyl-D-erythritol 4-phosphate	216.04	C5H13O7P	LL	59665204	n572
D-Lysine	146.11	C6H14N2O2	AAs	50868420	p594
5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-	193.07	C10H11NO3	0	50290708	n671
Leu-Val	230.16	C11H22N2O3	PP	46736928	p623
(9Z)-Tetradecenoic acid	226.19	C14H26O2	LL	44001420	n776
Ala-Val-Val-Pro	384.24	C18H32N4O5	PP	42998740	p651
Ethyl 3-oxobutanoate	130.06	C6H10O3	0	42456888	n784
[ST] (5Z,7E)-9,10-seco-5,7,10(19)-	368.34	C27H44	LL	39139004	p720

Table 14-9: The 20 Metabolites with highest m/z intensities out of 44, absent at 0 h but present at 36 h.
In descending m/z intensity, (Trend A) showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites: SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak ID
Thiomorpholine3 -carboxylate	147.04	C5H9NO2S	0	23337922	p880
Leu-Val-Gly	287.18	C13H25N3O4	PP	12892019	n1341
Leu-Ala-Gln	330.19	C14H26N4O5	PP	11301985	p1536
L-Ala-L-Glu	218.09	C8H14N2O5	PP	6445378	p2019
gamma-L-Glutamyl putrescine	217.14	C9H19N3O3	AAs	4641422	p2259
7-Methyladenine	149.07	C6H7N5	NT	4261534	p1948
Puromycin	471.22	C22H29N7O5	SM	3969427	p2546
Ile-Met-Met-Val	492.24	C21H40N4O5S2	PP	3730136	p2617
Leu-Leu-Ser	331.21	C15H29N3O5	PP	2811824	n2295
ZAPA	146.01	C4H6N2O2S	0	2713729	p2843
Ala-His	226.11	C9H14N4O3	PP	2673113	p2858
Ala-Leu-Ser-Ser	376.2	C15H28N4O7	PP	2541258	n2336
Glu-Lys-Lys-Gln	265.65	C22H41N7O8	PP	2335129	p3005
Ser-Arg	261.14	C9H19N5O4	PP	2331511	p3010
N2-(D-1-Carboxyethyl) -L-arginine	246.13	C9H18N4O4	AAs	2257569	p3042
Ala-Leu-Asn-Ser	403.21	C16H29N5O7	PP	2019028	p3162
Taurodeoxycholate	499.3	C26H45NO6S	LL	1970903	n2494
Lys-Val-Ser	332.21	C14H28N4O5	PP	1530063	p3506
Convolvine	291.15	C16H21NO4	0	1434941	p3577
Asp-Leu-Gln-Gln	502.24	C20H34N6O9	PP	1340881	p3739

18.1.1.1 Forty two hours post-infection

One compound was found present at 0 h but absent at 42 h; N-ethylethanolamine phosphate (a lipid metabolism intermediate detected in negative ionisation mode). Pattern of changes in metabolites relative to 0 h and a list of the first 20 metabolites with most changing m/z intensity at this point are shown in Figure 6-4 and Tables 6-10 to 6-13 respectively.

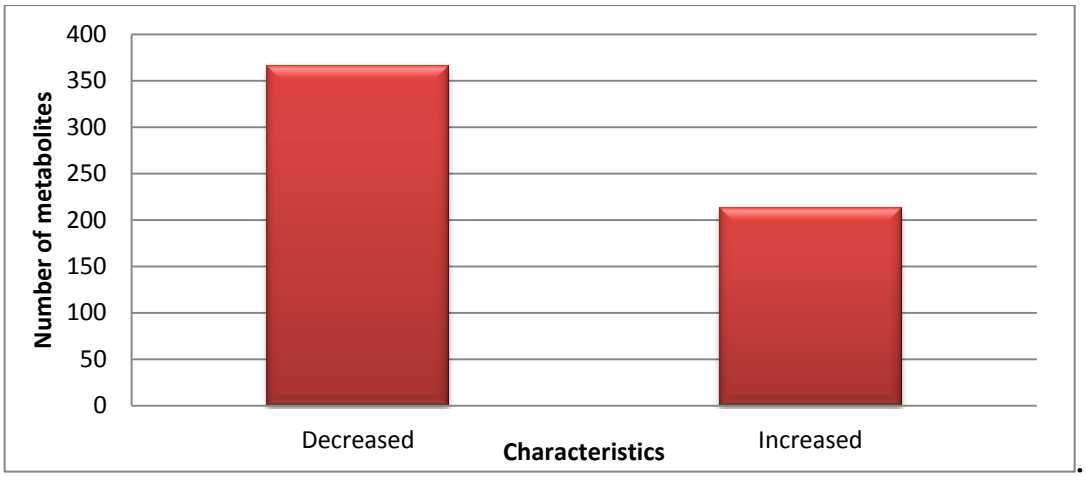

Figure 14-4: Proportion of metabolites present at 0h, increasing or decreasing at 42 h

Table 14-10: The 20 Metabolites with highest m/z intensities out of 366 present at 0 h having decreased m/z intensity at 42 h (Trend A)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak ID
Creatine	131.07	C4H9N3O2	AAs	1919896320	p7
Betaine	117.08	C5H11NO2	AAs	1610266496	p24
L-Carnitine	161.11	C7H15NO3	AAs	1356191488	p78
sn-glycero-3-Phosphocholine	257.1	C8H20NO6P	LL	1331854592	p91
Choline	103.1	C5H13NO	AAs	1080505600	p112
Citrate	192.03	C6H8O7	C/E	598937344	n6
Creatinine	113.06	C4H7N3O	AAs	507809600	p125
2-Dehydro-3-deoxy-L-rhamnonate	162.05	C6H10O5	C/E	403872448	n162
Orotate	156.02	C5H4N2O4	NT	352815456	n205
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
Hippurate	179.06	C9H9NO3	AAs	276560320	n332
(S)-Malate	134.02	C4H6O5	C/E	263881568	n139
8-keto-7-aminoperlagonate	185.11	C9H15NO3	0	184082784	n345
4-Trimethylammoniobutanoate	145.11	C7H15NO2	AAs	184079424	p1
Methyloxaloacetate	146.02	C5H6O5	C/E	168854336	n368
Asp-Cys-Ser-Tyr	243.07	C19H26N4O9S	PP	158657744	p372
Lactose	342.12	C12H22O11	C/E	152418496	n396
N-(octanoyl)-L-homoserine	245.16	C12H23NO4	0	147646064	p410
cis-Aconitate	174.02	C6H6O6	C/E	145240640	n339
N-Acetyl-D-glucosamine	221.09	C8H15NO6	AAs	143361424	p373

Table 14-11: The 20 Metabolites with highest m/z intensities out of 215 present at time 0 h having increased m/z intensity by time 42 h (Trend B)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z	IM/peak ID
L-Proline	115.06	C5H9NO2	AAs	605189696	p68
Hexadecanoic acid	256.24	C16H32O2	LL	502962240	n85
L-Leucine	131.09	C6H13NO2	AAs	239876032	p153
(R)-3-Hydroxybutanoate	104.05	C4H8O3	LL	162329008	n393
Decanoic acid	172.15	C10H20O2	LL	150188064	n484
L-Phenylalanine	165.08	C9H11NO2	AAs	134699168	p418
N-Acetyl-L-aspartate	175.05	C6H9NO5	AAs	134420832	n518
L-1-Pyrroline-3-hydroxy-5-	129.04	C5H7NO3	AAs	108133304	n224
Leu-Leu-Val	343.25	C17H33N3O4	PP	106793240	n543
5-Hydroxypentanoate	118.06	C5H10O3	LL	95705432	n556
L-Histidine	155.07	C6H9N3O2	AAs	79849448	p512
Hexanoic acid	116.08	C6H12O2	LL	77407704	n604
L-Tyrosine	181.07	C9H11NO3	AAs	73216184	p541
L-Arginine	174.11	C6H14N4O2	AAs	68342504	p529
L-Methionine	149.05	C5H11NO2S	AAs	64114592	p412
2-C-Methyl-D-erythritol4-	216.04	C5H13O7P	LL	59665204	n572
D-Lysine	146.11	C6H14N2O2	AAs	50868420	p594
5,6-Dihydroxy-3-methyl-2-oxo-	193.07	C10H11NO3	0	50290708	n671
Leu-Val	230.16	C11H22N2O3	PP	46736928	p623
(9Z)-Tetradecenoic acid	226.19	C14H26O2	LL	44001420	n776

Table 14-12: The 20 Metabolites with highest m/z intensities out of 54, that were absent at 0 h but present at 42 h (Trend C)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative.

Metabolite	Mass	Formula	Pathway	m/z	IM/peak ID
Thiomorpholine 3-carboxylate	147.04	C5H9NO2S	0	23337922	p880
Leu-Val-Gly	287.18	C13H25N3O4	PP	12892019	n1341
Leu-Ala-Gln	330.19	C14H26N4O5	PP	11301985	p1536
L-Ala-L-Glu	218.09	C8H14N2O5	PP	6445378	p2019
Leu-Val-Val	329.23	C16H31N3O4	PP	6164059	n1852
Gamma-L-Glutamylputrescine	217.14	C9H19N3O3	AAs	4641422	p2259
7-Methyladenine	149.07	C6H7N5	NT	4261534	p1948
Puromycin	471.22	C22H29N7O5	SM	3969427	p2546
Ile-Met-Met-Val	492.24	C21H40N4O5S2	PP	3730136	p2617
beta-Alanyl-L-arginine	245.15	C9H19N5O3	AAs	2845797	p2788
Leu-Leu-Ser	331.21	C15H29N3O5	PP	2811824	n2295
ZAPA	146.01	C4H6N2O2S	0	2713729	p2843
Ala-His	226.11	C9H14N4O3	PP	2673113	p2858
Ala-Leu-Ser-Ser	376.2	C15H28N4O7	PP	2541258	n2336
Glu-Lys-Lys-Gln	265.65	C22H41N7O8	PP	2335129	p3005
Ser-Arg	261.14	C9H19N5O4	PP	2331511	p3010
N2-(D-1-Carboxyethyl)-L-arginine	246.13	C9H18N4O4	AAs	2257569	p3042
Lys-Tyr	309.17	C15H23N3O4	PP	2213790	p3059
Glu-Arg	303.15	C11H21N5O5	PP	2065376	p3157
Ala-Leu-Asn-Ser	403.21	C16H29N5O7	PP	2019028	p3162

Table 14-13: Ten metabolites absent at 0 h and 36 h, but present at 42 h (Trend C-2)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z intensity	IM/peak ID
Leu-Val-Val	329.23	C16H31N3O4	PP	6164059	n1852
beta-Alanyl-L-arginine	245.15	C21H35N5O6	AAs	2845797	n2899
Lys-Tyr	309.17	C7H10N2O4	PP	2213790	n2470
Glu-Arg	303.15	C22H28N4O10	PP	2065376	n4029
Lys-Lys-Tyr	218.63	C15H23N3O4	PP	1229814	p3059
Gln-Leu-Pro-Pro	453.26	C21H35N5O5	PP	999610	p3812
Leu-Thr	232.14	C9H19N5O3	PP	972397	p2788
Ala-Asp-Pro	301.13	C10H20N2O4	PP	336479	p2124
(S)-AMPA	186.06	C11H21N5O5	0	320437	p3157
Asp-Asp-Pro-Tyr	508.18	C12H19N3O6	PP	88687	p4747

18.1.1.2 Fifty seven hours post infection

Choline phosphate was the metabolite with the highest fold (log_2 fold change) decrease at this time point (-9.54) while Leu-Ala-Gln had the highest fold increase (12.24). Other significantly decreasing compounds at this time include (S)-dihydroorotate propanoyl phosphate and pyridoxal. Furcatin was another metabolite which at 57 h decreased very significantly (3.43 fold decrease with p-value of 3.38×10^{-8}) from 0 h levels, but was being restored by 312 h, trend A.

Leu-Ala-Gln, Puromycin, beta-Alanyl-L-arginine, Ala-His and Ala-Leu-Ser-Ser has the highest fold increase (in decreasing order) among all changing metabolites at 57 h. Thr-Tyr (L-Threonyl-L-tyrosine) was also significantly increased (6.43 fold) at 57 h following a trend C. A hydrophobic dipeptide, peaked at 57 h, and was absent again at 312 h, just as it was at 0 h. Details of distribution of metabolites and listings of metabolites with significant changes at this time are shown in Figures 6-5 and Tables 6-14 to 6-17.

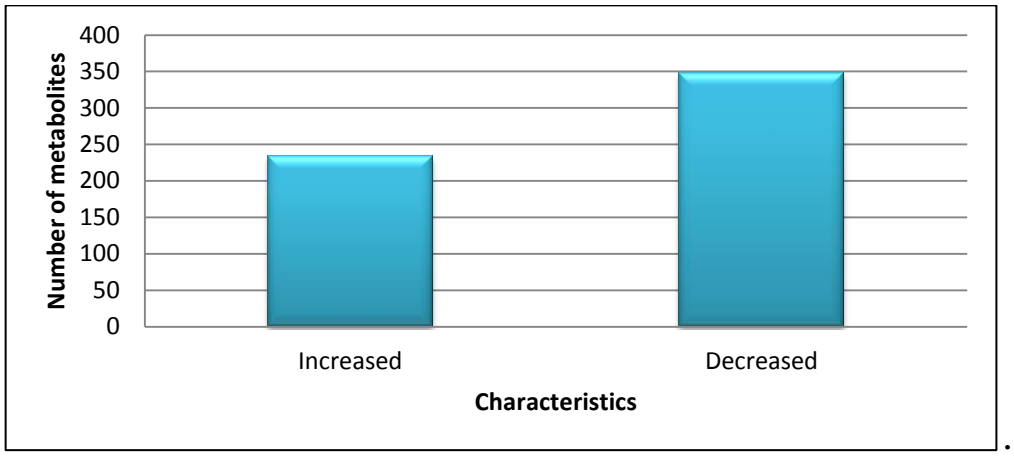

Figure 14-5: Proportion of metabolites present at 0h, decreasing or increasing at 57 h

Table 14-14: The 20 Metabolites with highest m/z intensities out of 348 which were present at 0 h and decreased in m/z intensity by 57 h (Trend A)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	19 m/z	IM/peak ID
Creatine	131.07	C4H9N3O2	AAs	1919896320	p7
Betaine	117.08	C5H11NO2	AAs	1610266496	p24
L-Carnitine	161.11	C7H15NO3	AAs	1356191488	p78
sn-glycero-3-Phosphocholine	257.1	C8H20NO6P	LL	1331854592	p91
Choline	103.1	C5H13NO	AAs	1080505600	p112
Citrate	192.03	C6H8O7	C/E	598937344	n6
Creatinine	113.06	C4H7N3O	AAs	507809600	p125
2-Dehydro-3-deoxy-L-rhamnonate	162.05	C6H10O5	C/E	403872448	n162
Orotate	156.02	C5H4N2O4	NT	352815456	n205
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
Hippurate	179.06	C9H9NO3	AAs	276560320	n332
(S)-Malate	134.02	C4H6O5	C/E	263881568	n139
8-keto-7-aminoperlagonate	185.11	C9H15NO3	0	184082784	n345
4-Trimethylammoniobutanoate	145.11	C7H15NO2	AAs	184079424	p1
Methyloxaloacetate	146.02	C5H6O5	C/E	168854336	n368
Asp-Cys-Ser-Tyr	243.07	C19H26N4O9S	PP	158657744	p372
Lactose	342.12	C12H22O11	C/E	152418496	n396
N-(octanoyl)-L-homoserine	245.16	C12H23NO4	0	147646064	p410
cis-Aconitate	174.02	C6H6O6	C/E	145240640	n339
N-Acetyl-D-glucosamine	221.09	C8H15NO6	AAs	143361424	p373

Table 14-15: The 20 Metabolites with highest m/z intensities out of 233 present at 0 h and increasing in m/z intensity at 57 h (Trend B)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	20 m/z	IM/peak
L-Proline	115.06	C5H9NO2	AAs	605189696	p68
Hexadecanoic acid	256.24	C16H32O2	LL	502962240	n85
L-Leucine	131.09	C6H13NO2	AAs	239876032	p153
(R)-3-Hydroxybutanoate	104.05	C4H8O3	LL	162329008	n393
Decanoic acid	172.15	C10H20O2	LL	150188064	n484
L-Phenylalanine	165.08	C9H11NO2	AAs	134699168	p418
N-Acetyl-L-aspartate	175.05	C6H9NO5	AAs	134420832	n518
L-1-Pyrroline-3-hydroxy-5-carboxylate	129.04	C5H7NO3	AAs	108133304	n224
Leu-Leu-Val	343.25	C17H33N3O4	PP	106793240	n543
5-Hydroxypentanoate	118.06	C5H10O3	LL	95705432	n556
L-Histidine	155.07	C6H9N3O2	AAs	79849448	p512
Hexanoic acid	116.08	C6H12O2	LL	77407704	n604
L-Tyrosine	181.07	C9H11NO3	AAs	73216184	p541
L-Arginine	174.11	C6H14N4O2	AAs	68342504	p529
3-Methyleneoxindole	145.05	C9H7NO	0	66962672	n611
L-Methionine	149.05	C5H11NO2S	AAs	64114592	p412
2-C-Methyl-D-erythritol 4-phosphate	216.04	C5H13O7P	LL	59665204	n572
D-Lysine	146.11	C6H14N2O2	AAs	50868420	p594
5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-	193.07	C10H11NO3	0	50290708	n671
Orthophosphate	97.977	H3O4P	C/E	48464996	n685

Table 14-16: The 20 Metabolites with highest m/z intensities out of 59, absent at 0 h but present at 57 h (Trend C).
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak ID
Thiomorpholine 3-carboxylate	147.04	C5H9NO2S	0	23337922	p880
Leu-Val-Gly	287.18	C13H25N3O4	PP	12892019	n1341
Leu-Ala-Gln	330.19	C14H26N4O5	PP	11301985	p1536
L-Ala-L-Glu	218.09	C8H14N2O5	PP	6445378	p2019
Leu-Val-Val	329.23	C16H31N3O4	PP	6164059	n1852
gamma-L-Glutamylputrescine	217.14	C9H19N3O3	AAs	4641422	p2259
7-Methyladenine	149.07	C6H7N5	NT	4261534	p1948
Puromycin	471.22	C22H29N7O5	SM	3969427	p2546
Ile-Met-Met-Val	492.24	C21H40N4O5S2	PP	3730136	p2617
beta-Alanyl-L-arginine	245.15	C9H19N5O3	AAs	2845797	p2788
Leu-Leu-Ser	331.21	C15H29N3O5	PP	2811824	n2295
ZAPA	146.01	C4H6N2O2S	0	2713729	p2843
Ala-His	226.11	C9H14N4O3	PP	2673113	p2858
Ala-Leu-Ser-Ser	376.2	C15H28N4O7	PP	2541258	n2336
Glu-Lys-Lys-Gln	265.65	C22H41N7O8	PP	2335129	p3005
Ser-Arg	261.14	C9H19N5O4	PP	2331511	p3010
N2-(D-1-Carboxyethyl)-L-arginine	246.13	C9H18N4O4	AAs	2257569	p3042
Lys-Tyr	309.17	C15H23N3O4	PP	2213790	p3059
Glu-Arg	303.15	C11H21N5O5	PP	2065376	p3157
Ala-Leu-Asn-Ser	403.21	C16H29N5O7	PP	2019028	p3162

Table 14-17: The 20 Metabolites with highest m/z intensities out of 20 present at 0 h but absent at 57 h listed by m/z intensity.
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; LG-Lipids Glycerophospholipids; LP-Lipids: Polyketides; p-postive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak ID
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
N-Acetyl-D-glucosamine 6-phosphate	301.06	C8H16NO9P	AAs	61189868	n622
D-Glucose 6-phosphate	260.03	C6H13O9P	C/E	17423838	n1193
(S)-Dihydroorotate	158.03	C5H6N2O4	NT	8641255	n1688
Ethanolamine phosphate	141.02	C2H8NO4P	AAs	4438335	n1194
CMP	323.05	C9H14N3O8P	NT	828210	p3851
[PC ethyl,acety] 1-ethyl-2-acetyl-sn-glycero-3-phosphocholine	327.14	C12H26NO7P	LG	702942	p4232
Oxaloacetate	132.01	C4H4O5	C/E	475359	n3324
[Fv] Kurzichalcolactone	263.1	C32H30O7	LP	453875	p4544
U 50488	368.14	C19H26N2OCl2	0	429468	n3395
3',5'-Cyclic AMP	329.05	C10H12N5O6P	NT	313534	p617
Glu-Asp-Asp	377.11	C13H19N3O10	PP	308783	n3573
4-Nitrophenylsulfate	218.98	C6H5NO6S	0	213780	n3747
4-Hydroxyaminoquinoline N-oxide	176.06	C9H8N2O2	0	154301	p5256
Malvin	327.09	C29H34O17	0	150250	n3882
Fexaramine	248.14	C32H36N2O3	0	145077	p4542
3-Methoxy-4-hydroxyphenylethyleneglycolsulfate	264.03	C9H12O7S	0	122686	n3946
N-Methylethanolamine phosphate	155.03	C3H10NO4P	LL	104954	n3997
5-Carboxy-2-oxohept-3-enedioate	216.03	C8H8O7	AAs	77691	n4057
ubiquinone-1	250.12	C14H18O4	0	48992	p5099

20.1.1.1 Eighty one hours post infection

Lactose a disaccharide, which is involved in the galactose metabolism pathway and the major milk sugar, was one of the major milk metabolites most significantly reduced at 81 h. It had a 4 fold decrease while other metabolites such as choline phosphate, alpha-D-Galactosyl-1, 3-beta-D-galactosyl-1, 4-N-acetyl-D-glucosamine, 3-Dehydrocarnitine, and sn-Glycerol 3-phosphate had the highest fold decreases (10, 7, 6 and 5 folds) respectively. D-glucose also decreased by about 5 fold at this time. Overall, more metabolites were significantly decreased at this time point (having up to 5 fold change) than at other time points showing trend A, (reducing gradually up to 81 h) and by 312 h, it was almost back to levels recorded at 0 h. Puromycin, beta-Alanyl-L-arginine, Ala-His, [ST hydrox] N-(3alpha, 7alpha-dihydroxy-5beta-cholan-24-oyl)-taurine, Arg-Lys-Asp-Gln, Ala-Trp-Gln-Gln and Leu-Thr increased from point 0 h. N-Acetyl-beta-alanine was one other metabolite that increased in several folds above its concentration recorded at 0 h but showed irregular fluctuations not consistent with infection course. N-Acetyl-beta-alanine is involved in amino acid metabolism as a precursor of alanine. Distributions of metabolites at this time point relative to 0 h are shown in Figures 6-6 while Tables 6-18 to 6-21 shows lists of metabolites with significant changes at this time.

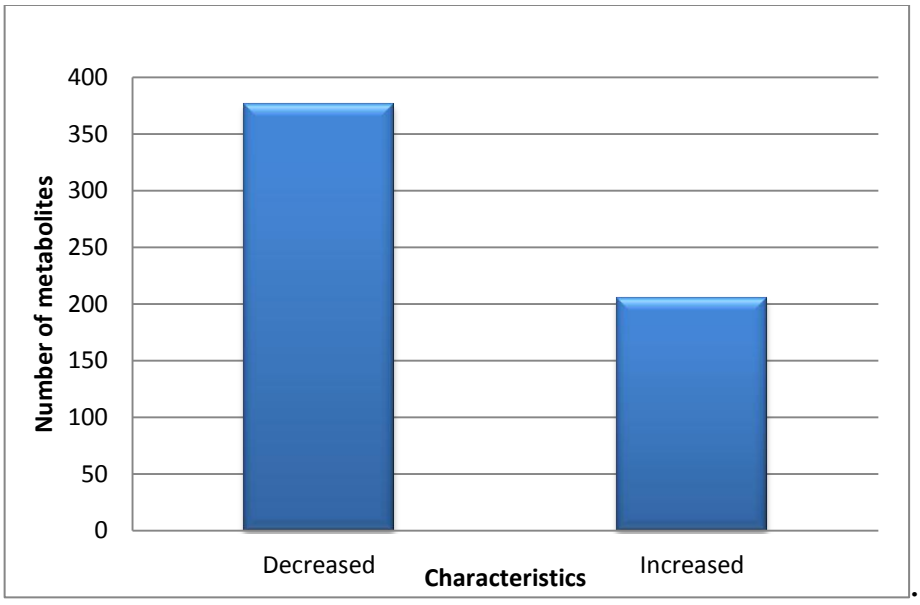

Figure 14-6: Proportion of metabolites present at 0 h which decreased or increased at 81 h.

Table 14-18: The 20 Metabolites with highest m/z intensities out of 376 present at 0 h with decreased m/z intensity at 81 h (Trend A).
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative.

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak
Creatine	131.07	C4H9N3O2	AAs	1919896320	p7
Betaine	117.08	C5H11NO2	AAs	1610266496	p24
L-Carnitine	161.11	C7H15NO3	AAs	1356191488	p78
sn-glycero-3-Phosphocholine	257.1	C8H20NO6P	LL	1331854592	p91
Choline	103.1	C5H13NO	AAs	1080505600	p112
Citrate	192.03	C6H8O7	C/E	598937344	n6
Creatinine	113.06	C4H7N3O	AAs	507809600	p125
2-Dehydro-3-deoxy-L-rhamnonate	162.05	C6H10O5	C/E	403872448	n162
Orotate	156.02	C5H4N2O4	NT	352815456	n205
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
Hippurate	179.06	C9H9NO3	AAs	276560320	n332
(S)-Malate	134.02	C4H6O5	C/E	263881568	n139
8-keto-7-aminoperlagonate	185.11	C9H15NO3	0	184082784	n345
4-Trimethylammoniobutanoate	145.11	C7H15NO2	AAs	184079424	p1
Methyloxaloacetate	146.02	C5H6O5	C/E	168854336	n368
Asp-Cys-Ser-Tyr	243.07	C19H26N4O9S	PP	158657744	p372
Lactose	342.12	C12H22O11	C/E	152418496	n396
N-(octanoyl)-L-homoserine	245.16	C12H23NO4	0	147646064	p410
cis-Aconitate	174.02	C6H6O6	C/E	145240640	n339
N-Acetyl-D-glucosamine	221.09	C8H15NO6	AAs	143361424	p373

Table 14-19: The 20 Metabolites with highest m/z intensities out of 205, present at 0 h with increased m/z intensity at 81 h (Trend B)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak
L-Proline	115.06	C5H9NO2	AAs	605189696	p68
Hexadecanoic acid	256.24	C16H32O2	LL	502962240	n85
L-Leucine	131.09	C6H13NO2	AAs	239876032	p153
(R)-3-Hydroxybutanoate	104.05	C4H8O3	LL	162329008	n393
Decanoic acid	172.15	C10H20O2	LL	150188064	n484
L-Phenylalanine	165.08	C9H11NO2	AAs	134699168	p418
N-Acetyl-L-aspartate	175.05	C6H9NO5	AAs	134420832	n518
L-1-Pyrroline-3-hydroxy-5-carboxylate	129.04	C5H7NO3	AAs	108133304	n224
Leu-Leu-Val	343.25	C17H33N3O4	PP	106793240	n543
5-Hydroxypentanoate	118.06	C5H10O3	LL	95705432	n556
L-Histidine	155.07	C6H9N3O2	AAs	79849448	p512
Hexanoic acid	116.08	C6H12O2	LL	77407704	n604
L-Tyrosine	181.07	C9H11NO3	AAs	73216184	p541
L-Arginine	174.11	C6H14N4O2	AAs	68342504	p529
L-Methionine	149.05	C5H11NO2S	AAs	64114592	p412
D-Lysine	146.11	C6H14N2O2	AAs	50868420	p594
5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-	193.07	C10H11NO3	0	50290708	n671
Leu-Val	230.16	C11H22N2O3	PP	46736928	p623
(9Z)-Tetradecenoic acid	226.19	C14H26O2	LL	44001420	n776
Ala-Val-Val-Pro	384.24	C18H32N4O5	PP	42998740	p651

Table 14-20: The 20 Metabolites with highest m/z intensities out of 58, absent at 0 h present at 81 h (Trend C)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative.

Metabolite	Mass	Formula	Pathway	M/z Intensity	IM/peak ID
Thiomorpholine 3-	147.04	C5H9NO2S	0	23337922	p880
Leu-Val-Gly	287.18	C13H25N3O4	PP	12892019	n1341
Leu-Ala-Gln	330.19	C14H26N4O5	PP	11301985	p1536
L-Ala-L-Glu	218.09	C8H14N2O5	PP	6445378	p2019
Leu-Val-Val	329.23	C16H31N3O4	PP	6164059	n1852
gamma-L-	217.14	C9H19N3O3	AAs	4641422	p2259
7-Methyladenine	149.07	C6H7N5	NT	4261534	p1948
Puromycin	471.22	C22H29N7O5	SM	3969427	p2546
Ile-Met-Met-Val	492.24	C21H40N4O5S2	PP	3730136	p2617
beta-Alanyl-L-arginine	245.15	C9H19N5O3	AAs	2845797	p2788
Leu-Leu-Ser	331.21	C15H29N3O5	PP	2811824	n2295
ZAPA	146.01	C4H6N2O2S	0	2713729	p2843
Ala-His	226.11	C9H14N4O3	PP	2673113	p2858
Ala-Leu-Ser-Ser	376.2	C15H28N4O7	PP	2541258	n2336
Glu-Lys-Lys-Gln	265.65	C22H41N7O8	PP	2335129	p3005
Ser-Arg	261.14	C9H19N5O4	PP	2331511	p3010
N2-(D-1-Carboxyethyl)-L-	246.13	C9H18N4O4	AAs	2257569	p3042
Lys-Tyr	309.17	C15H23N3O4	PP	2213790	p3059
Glu-Arg	303.15	C11H21N5O5	PP	2065376	p3157
Ala-Leu-Asn-Ser	403.21	C16H29N5O7	PP	2019028	p3162

Table 14-21: The 20 Metabolites with highest m/z intensities out of 29, present at 0 h and absent at 81 h (Trend A-2)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	M/z	IM/peak
Choline phosphate	183.07	C5H14NO4P	LL	324032768	p270
N-Acetyl-D-glucosamine 6-phosphate	301.06	C8H16NO9P	AAs	61189868	n622
D-Glucose 6-phosphate	260.03	C6H13O9P	C/E	17423838	n1193
(S)-Dihydroorotate	158.03	C5H6N2O4	NT	8641255	n1688
Propanoyl phosphate	154	C3H7O5P	C/E	5321211	n1914
Ethanolamine phosphate	141.02	C2H8NO4P	AAs	4438335	n1194
CMP	323.05	C9H14N3O8P	NT	828210	p3851
D-Ribose 5-phosphate	230.02	C5H11O8P	C/E	726186	n3070
[PC ethyl,acety] 1-ethyl-2-acetyl-sn-glycero-3-	327.14	C12H26NO7P	LG	702942	p4232
Oxaloacetate	132.01	C4H4O5	C/E	475359	n3324
[Fv] Kurzichalcolactone	263.1	C32H30O7	LP	453875	p4544
U 50488	368.14	C19H26N2OCl2	0	429468	n3395
[PC acetyl(4:2)] 1-butyryl-2-acetyl-sn-glycero-3osphocholine	355.18	C14H30NO7P	LG	375129	p4664
P-DPD	212.01	C5H9O7P	0	369553	n3480
2-Deoxy-D-ribose 5-phosphate	214.02	C5H11O7P	C/E	323946	n2843
3',5'-Cyclic AMP	329.05	C10H12N5O6P	NT	313534	p617
Glu-Asp-Asp	377.11	C13H19N3O10	PP	308783	n3573
4-Nitrophenylsulfate	218.98	C6H5NO6S	0	213780	n3747
urate-3-ribonucleoside	300.07	C10H12N4O7	NT	213725	n3749
4-Hydroxy-3-nitrosobenzamide	166.04	C7H6N2O3	0	196354	n3219

20.1.1.2 Three hundred and twelve hours post infection

5-Methyl-2'-deoxycytidine has the greatest fold decrease at 312 h. 3, 5/4-Trihydroxycyclohexa-1, 2-dione, an intermediary of inositol phosphate metabolism (carbohydrate metabolism), was also found to have significant fold reduction. Thiomorpholine 3-carboxylate, Puromycin, [FA] O-Palmitoyl-R-carnitine and tetradecanoylcarnitine were most significantly increased metabolites. Ala-Gly-Tyr, a hydrophobic tripeptide was one metabolite which also increased from 0 h levels in a significant order of folds. Distribution of metabolites present and absent at this time point relative to 0 h are shown in Figures 6-7 while Tables 6-22 to 6-26 gives a list of the metabolites in with m/z intensity changes at this time.

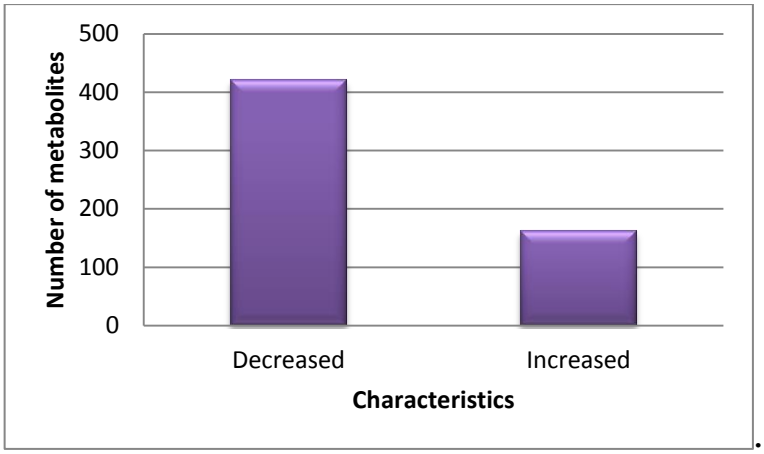

Figure 14-7: Proportion of metabolites present at 0 h, decreasing or increasing at 312 h

Table 14-22: The 20 Metabolites with highest m/z intensities of 27 present at 0 h, reduced during infection going back to normal levels at 312 h (trend A)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative.

Metabolites	Mass	Formula	Pathway	m/z intensity	IM/peak ID
Creatine	131.0694	C4H9N3O2	AAs	1919896320	p7
Choline	103.0996	C5H13NO	AAs	1080505600	p112
Hippurate	179.0583	C9H9NO3	AAs	276560320	n332
Asp-Cys-Ser-Tyr	243.0716	C19H26N4O9S	PP	158657744	p372
D-Glycerate	106.0266	C3H6O4	C/E	32857964	n895
Ecgonine	185.1052	C9H15NO3	SM	10426968	p1634
2-Ethylhexyl phthalate	278.1515	C16H22O4	0	2596841	p1707
L-Formylkynurenine	236.0794	C11H12N2O4	AAs	1750220	p3371
Ala-Met-Ala-Pro	388.1771	C16H28N4O5S	PP	1317536	p3751
Imidazol-5-yl-pyruvate	77.01912	C6H6N2O3	AAs	1295559	n2720
Asp-Phe-Cys-Pro	240.0846	C21H28N4O7S	PP	1180533	n2784
Lactosamine	341.1323	C12H23NO10	C/E	1028320	n2882
Formylpyruvate	116.0109	C4H4O4	0	727474	n3059
Leu-Pro-Tyr	391.2102	C20H29N3O5	PP	641105	p4296
Nalpha-Dimethyl-L-histidine	183.1008	C8H13N3O2	0	524394	p4447
Ginsenoside Rh2	311.2222	C36H62O8	0	289180	p4878
3-Hydroxy-2-methylpyridine-4,5-dicarboxylate	197.0325	C8H7NO5	VC	285486	n2192
Phenethylamineglucuronide	297.1213	C14H19NO6	0	271261	n3614
Trimethylaminoacetone	115.0997	C6H13NO	0	221264	p4867
21 4,5-dihydro-5,5-dimethyl-4-(3-	184.1101	C10H16O3	0	219496	p4500

Table 14-23: All (19) metabolites present at 0 h, increased during infection and falling back to 0 h levels at 312 h (trend B)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Formula	Pathway	22 m/z	23 IM/peak
(L-Seryl)adenylate	C13H21N6O9P	0	19214912	n1082
[FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid	C4H8O5	C/E	18440210	n1047
Nonanoic acid	C9H18O2	LL	11126324	n1442
Ile-Tyr	C15H22N2O4	PP	8091055	p1795
Ala-Pro	C8H14N2O3	PP	5502632	p2145
[FA hydroxy(18:2)] 9S-hydroxy-10E,12Z-octadecadienoic acid	C18H32O3	LL	5268704	n1920
5,6-Dihydrothymine	C5H8N2O2	NT	5194296	p528
N,N-Dimethylglycine	C4H9NO2	AAs	3219889	n2211
Glycine	C2H5NO2	AAs	1850985	p2260
Ethyl (R)-3-hydroxyhexanoate	C8H16O3	0	1087381	n2841
Pro-Pro	C10H16N2O3	PP	1042433	p3381
Maleamate	C4H5NO3	VC	722413	n2887
Phenylacetic acid	C8H8O2	AAs	648813	n774
2-Aminophenol	C6H7NO	AAs	633281	p2047
Caffeic aldehyde	C9H8O3	SM	441880	n2946
2-Hydroxyethanesulfonate	C2H6O4S	AAs	344144	n3529
di-n-Undecylamine	C22H47N	0	197438	p5140
[FA (9:2)] 2,6-nonadienoic acid	C9H14O2	LL	191842	n3792
Suberic acid	C8H14O4	LL	179402	n3824
3-Dehydroteasterone	C28H46O4	SM	125943	p4479

Table 14-24: All (18) metabolites which were absent at 0 h, present during infection (36-81 h) and then absent again at 312 h (Trend C)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	24 IM/pea
Leu-Val-Gly	287.184	C13H25N3O4	PP	12892019	n1341
Leu-Ala-Gln	330.19	C14H26N4O5	PP	11301985	p1536
gamma-L-Glutamylputrescine	217.142	C9H19N3O3	AAs	4641422	p2259
7-Methyladenine	149.070	C6H7N5	NT	4261534	p1948
Glu-Lys-Lys-Gln	265.650	C22H41N7O8	PP	2335129	p3005
Lys-Val-Ser	332.205	C14H28N4O5	PP	1530063	p3506
Convolvine	291.146	C16H21NO4	0	1434941	p3577
Asp-Leu-Gln-Gln	502.238	C20H34N6O9	PP	1340881	p3739
Ala-Thr-Thr-Tyr	454.206	C20H30N4O8	PP	1180278	n2786
N-Formimino-L-glutamate	174.064	C6H10N2O4	AAs	1094068	p3902
Lys-Phe-Cys	396.182	C18H28N4O4	PP	1059106	n2859
Arg-Leu-Lys-Asn	264.666	C22H43N9O6	PP	1038579	p3933
Met-Val	248.119	C10H20N2O3	PP	994687	n2901
Guanosine	283.091	C10H13N5O5	NT	677377	p1097
Ala-Lys-Ala-Pro	385.231	C17H31N5O5	PP	540860	p4425
Ala-Asn-Gly	260.111	C9H16N4O5	PP	530324	n3256
Asp-Leu-Leu-Gln	487.264	C21H37N5O8	PP	283327	n3604
Thr-Tyr	282.121	C13H18N2O5	PP	161941	n3855

Table 14-25: Metabolites present at 0 h but absent at 312 h
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): 0-unknown pathway; NT-nucleotides and related metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z Intensity	IM/peak ID
CMP	323.05	C9H14N3O8P	NT	828210	p3851
25 N1,N8-	229.18	C11H23N3O2	0	356028	p4706
3',5'-Cyclic AMP	329.05	C10H12N5O6P	NT	313534	p617

Table 14-26: The 20 Metabolites with highest m/z intensities out of 31 absent at 0 h but present at 312 h (Trend C)
In descending m/z intensity, showing their associated pathways and ionisation mode (IM): AAs-amino acids and related metabolites; PP-small peptides; C/E-carbohydrate and energy metabolites; LL-lipids and lipid related metabolites; 0-unknown pathway; VC-vitamins, co-factors and related metabolites; NT-nucleotides and related metabolites; SM-biosynthesis of secondary metabolites; p-positive; n-negative

Metabolite	Mass	Formula	Pathway	m/z	IM/peak
Thiomorpholine 3-carboxylate	147.04	C5H9NO2S	0	23337922	p880
L-Ala-L-Glu	218.09	C8H14N2O5	PP	6445378	p2019
Puromycin	471.22	C22H29N7O5	SM	3969427	p2546
Ile-Met-Met-Val	492.24	C21H40N4O5S2	PP	3730136	p2617
beta-Alanyl-L-arginine	245.15	C9H19N5O3	AAs	2845797	p2788
Leu-Leu-Ser	331.21	C15H29N3O5	PP	2811824	n2295
ZAPA	146.01	C4H6N2O2S	0	2713729	p2843
Ala-His	226.11	C9H14N4O3	PP	2673113	p2858
Ala-Leu-Ser-Ser	376.2	C15H28N4O7	PP	2541258	n2336
Ser-Arg	261.14	C9H19N5O4	PP	2331511	p3010
N2-(D-1-Carboxyethyl)-L-arginine	246.13	C9H18N4O4	AAs	2257569	p3042
Lys-Tyr	309.17	C15H23N3O4	PP	2213790	p3059
Glu-Arg	303.15	C11H21N5O5	PP	2065376	p3157
Ala-Leu-Asn-Ser	403.21	C16H29N5O7	PP	2019028	p3162
Taurodeoxycholate	499.3	C26H45NO6S	LL	1970903	n2494
Lys-Lys-Tyr	218.63	C21H35N5O5	PP	1229814	p3812
Glu-Lys-Asn-Arg	272.65	C21H39N9O8	PP	1087936	p3903
Ovothiol A-cysteine disulfide	160.03	C10H16N4O4S2	0	776263	p4157
Ala-Gly-Ser	233.1	C8H15N3O5	PP	756449	p4166
Ala-Gly-Arg	302.17	C11H22N6O4	PP	718070	p4219

25.1.1 The changes in metabolites in relation to pathways of metabolism

Overall, there were more compounds which significantly reduced with time from the control time (0 h) than compounds which increased or newly emerge, at all-time points considered. Specifically, most carbohydrate metabolism compounds showed a trend of reducing with time as infection progressed (trend A), while most peptide molecules increased with time (trend C).

25.1.1.1 Carbohydrate and energy metabolism

A total of thirty nine (39) carbohydrate and energy metabolites changed with time and all were present at 0 h. Having the peak intensity among these was citrate. Figure 6-8 shows the proportion of metabolites and their changes with time. Fold change in carbohydrate and energy metabolites during the course of infection were computed and Table 6-27 shows a list of first 10 metabolites, having greatest fold change, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$). Examples of carbohydrate and energy metabolism metabolites displaying the various trends are listed in table 6-28.

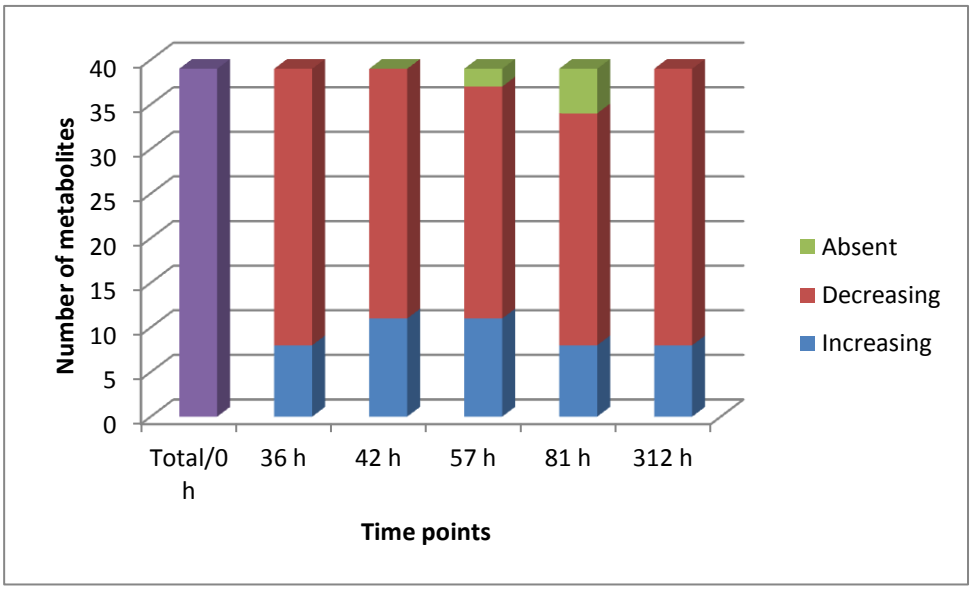

Figure 14-8: Carbohydrates and energy metabolism metabolites and their changes at different time points
Showing total metabolites present at 0 h, and proportion of the metabolites absent (green); decreasing (red) or increasing (blue) at the various time points.

Table 14-27: First 10 carbohydrate and energy metabolites having significant fold change in time (P<0.05) in order of decreasing fold change.
Time of the highest fold change (THFC), the value of fold change (FC) log_2 and type of change that occurred are also depicted in the table, P-value (at that time point)

Metabolites	Mass	Formula	Specific Pathway	m/z intensity	P-value	THFC	Log_2 FC(Fold change)	Type of change
2-Dehydro-3-deoxy-L-rhamnonate	162.05283	C6H10O5	Fructose & mannose	403872448	7.805E-07	81	-5.01	Down regulation
Methyloxaloacetate	134.02151	C5H6O5	C5-Branched dibasic acid	168854336	0.0001349	81	-5.53	Down regulation
cis-Aconitate	146.0215	C6H6O6	Citrate cycle (TCA cycle)	145240640	9.434E-05	81	-5.84	Down regulation
D-Glucose	342.11621	C6H12O6	Glycolysis / Gluconeogenesis	112635952	8.567E-08	81	4.93	Down regulation
D-Glucuronolactone	176.03209	C6H8O6	Ascorbate & aldarate metabolism	10795787	0.0226727	36	4.85	Up regulation
D-Glucuronolactone	176.03209	C6H8O6	Ascorbate and aldarate metabolism	10795787	0.0226727	42	4.81	Up regulation
N-Acetylneuraminate	309.10615	C11H19NO9	Amino sugars metabolism	13092637	0.0007315	81	-4.65	Down regulation
2-Deoxy-D-ribose 5-phosphate	214.02436	C5H11O7P	Pentose phosphate pathway	323946	0.00	57	-4.13	Down regulation
Lactose	342.11621	C12H22O11	Galactose metabolism	152418496	6.107E-09	81	-4.18	Down regulation
2-Dehydro-3-deoxy-D-gluconate	174.01641	C6H10O6	Pentose phosphate pathway	1487273	4.064E-07	81	-4.09	Down regulation

Table 14-28: Examples of Carbohydrate and Energy metabolites displaying different trends during the course of infection

Trend A	Trend B	Trend C	Trend D
Lactose	**Pyruvate**	N/A	**Orthophosphate**
2-Dehydro-3-deoxy-L-rhamnonate	**[FA trihydroxy(4:0)] 2,3,4-trihydroxy-**	N/A	**D-Gluconic acid**
(S)-Malate	**D-Glucuronolactone**	N/A	**Succinate**
Methyloxaloacetate	**3-Ethylmalate**	N/A	**D-Sorbitol**
Lactose	**L-Arabinose**	N/A	**Deoxyribose**

25.1.1.2 Protein metabolism

Amino acids and Related Metabolites

A total of 117 compounds differed in intensity with time course in this study. The compound with the highest intensity of this pathway was creatine, however the metabolite with the greatest fold change was N-Acetyl-D-glucosamine 6-phosphate which had an approximately 13 fold decrease at 57 h from its concentration at 0 h. Beta-Alanyl-L-arginine on the other hand was up regulated by the most folds (10.5) at 81 h post infection.

Fold changes in the first ten highest altered amino acids and related metabolites are presented and Table 6-29 while Table 6-30 displays examples of metabolites with different trends in this metabolic pathway. Figure 6-9 shows proportion of changes (increase or decrease) in metabolites of this pathway at different time points.

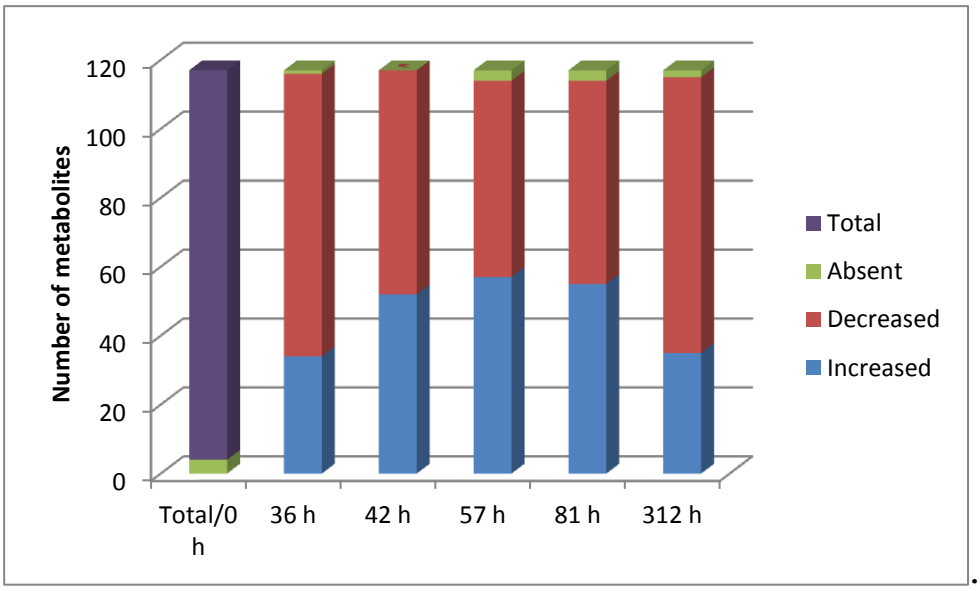

Figure 14-9: Amino acids and related metabolites and their changes at different time points
Showing total metabolites present at 0 h (purple), and proportion of the metabolites absent (green); decreasing (red) or increasing (blue) at the various time points.

Table 14-29: First 10 amino acids and related metabolites with significant change in time (P<0.05) in order of decreasing fold change.

Metabolites	Mass	Formula	Pathway	m/z intensity	P-value	THFC	Log_2 Fold	Type of change
N-Acetyl-D-glucosamine 6-phosphate	301.0563	C8H16NO9P	Glutamate	61189868	0.00	57	-13.08	Down regulation
Beta-Alanyl-L-arginine	245.14854	C9H19N5O3	beta-Alanine	2845797	0.0236861	81	10.55	Up regulation
Beta-Alanyl-L-arginine	245.14854	C9H19N5O3	beta-Alanine	2845797	0.03	57	10.03	Up regulation
N2-(D-1-Carboxyethyl)-L-arginine	246.13269	C9H18N4O4	Arginine and proline	2257569	0.010944	57	7.97	Up regulation
N-Acetyl-L-aspartate	175.04804	C6H9NO5	Alanine and aspartate	134420832	0.0436121	57	7.28	Up regulation
N-Formimino-L-glutamate	174.06406	C6H10N2O4	Histidine	1094068	0.0451602	57	6.63	Up regulation
N6-Acetyl-L-lysine	188.11598	C8H16N2O3	Lysine degradation	28109198	0.0293803	57	5.82	Up regulation
L-Tyrosine	181.07387	C9H11NO3	Tyrosine	73216184	0.0113784	57	5.76	Up regulation
L-Methionine	149.05104	C5H11NO2S	Methionine	64114592	0.0206183	57	5.58	Up regulation
N-Acetyl-L-phenylalanine	207.08948	C11H13NO3	Phenylalanine	386711	0.0085574	81	-5.57	Down regulation

Table 14-30: Examples of amino acid related metabolites displaying the various trends with course of infection.
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	Trend B	Trend C	Trend D
Creatine	**L-Proline**	**gamma-L-Glutamylputrescine**	**N-Acetyl-beta-alanine**
Betaine	**L-Leucine**	**beta-Alanyl-L-arginine**	**2-Isopropylmaleate**
L-Carnitine	**L-Phenylalanine**	**N2-(D-1-Carboxyethyl)-L-**	**Urocanate**
Choline	**N-Acetyl-L-aspartate**	**N-Formimino-L-glutamate**	**S-Methyl-L-methionine**
L-Threonine	**L-1-Pyrroline-3-hydroxy-5-carboxylate**	-	**Gentisate aldehyde**

Peptides

One hundred and twelve (112) peptides having 2 to 4 amino acid residues were identified, varying in concentration with time. The highest intensity metabolite in this group is the tetra-peptide Asp-Cys-Ser-Tyr, followed by Leu-Leu-Val and Leu-Val. The greatest fold change occurred with tri-peptide Leu-Ala-Gln (~12 fold) at the 57 h point.

Fold change in small peptides during the course of infection were computed and Table 6-31 shows a list of first 10 peptides, having greatest fold change, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$), Table 6-32 displays examples of different trending peptides and Figure 6-10 presents the proportion of peptides changes at different time points.

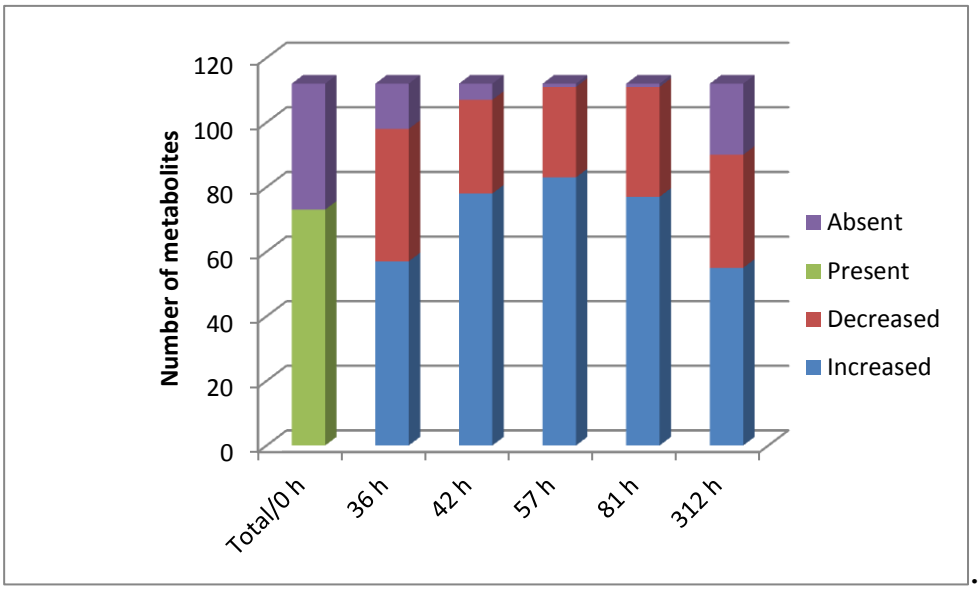

Figure 14-10: Peptides and related metabolites and their changes at different time points
Showing total metabolites present at 0 h (green), and proportion of the metabolites absent (purple); decreasing (red) or increasing (blue) at the various time points.

14-31: First 10 Peptides and related metabolites with significant change in time (P<0.05) in order of decreasing fold change.

Metabolites	Mass	Formula	Pathway	m/z intensity	P-value	THFC	Fold change	Type of change
Leu-Ala-Gln	330.19	C14H26N4O5	Hydrophobic peptide	11301985	0.03354	57	12.25	Up regulation
Ala-His	226.1064	C9H14N4O3	Basic peptide	2673113	0.015318	57	10.45	Up regulation
Ala-Leu-Ser-Ser	376.1959	C15H28N4O7	Hydrophobic peptide	2541258	0.023883	57	10.12	Up regulation
Val-Gly-Pro	271.1529	C12H21N3O4	Hydrophobic peptide	8562357	0.047323	57	9.64	Up regulation
Ile-Met-Met-Val	492.2426	C21H40N4O5S2	Hydrophobic peptide	3730136	0.045738	57	9.58	Up regulation
Leu-Phe	278.163	C15H22N2O3	Hydrophobic peptide	12801995	0.011838	57	9.35	Up regulation
Lys-Val-Ser	332.2058	C14H28N4O5	Basic peptide	1530063	0.027428	57	9.30	Up regulation
Arg-Lys-Asp-Gln	272.6457	C21H39N9O8	Basic peptide	1375922	0.04312	57	9.16	Up regulation
Ala-His	226.1064	C9H14N4O3	Basic peptide	2673113	0.024157	81	9.16	Up regulation
Ala-Trp-Gln-Gln	531.244	C24H33N7O7	Hydrophobic peptide	1096478	0.049699	81	8.95	Up regulation

Table 14-32: Examples of peptides displaying the various trends during the course of infection
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	Trend B	Trend C	Trend D
Asp-Cys-Ser-Tyr	**Leu-Leu-Val**	**Leu-Val-Gly**	**Ala-Val-Val-Pro**
Lys-Val	**Leu-Val**	**Leu-Ala-Gln**	**Leu-Pro**
Ile-Phe-Thr-Pro	**Ile-Val**	**L-Ala-L-Glu**	**Lys-His**
Asp-Phe-Cys-Pro	**Leucyl-leucine**	**Leu-Val-Val**	**Asp-Asp-Pro-Ser**
Leu-Lys-Asp	**Leu-Phe**	**Ile-Met-Met-Val**	**Ala-Tyr**

25.1.1.3 Lipids and lipid metabolism Metabolites

A total of one hundred and four (104) compounds were identified in these pathways. Three of these were absent at 0 h, while all others were present. The metabolite with highest intensity in this group was sn-glycero-3-Phosphocholine.

Figure 6-11 shows the frequency distribution of metabolites in this pathway with time points. Fold change in lipids during the course of infection were computed and Table 6-33 shows a list of first 10 metabolites, having greatest fold change, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$) and Table 6-34 gives examples of metabolites in this pathway following different trends.

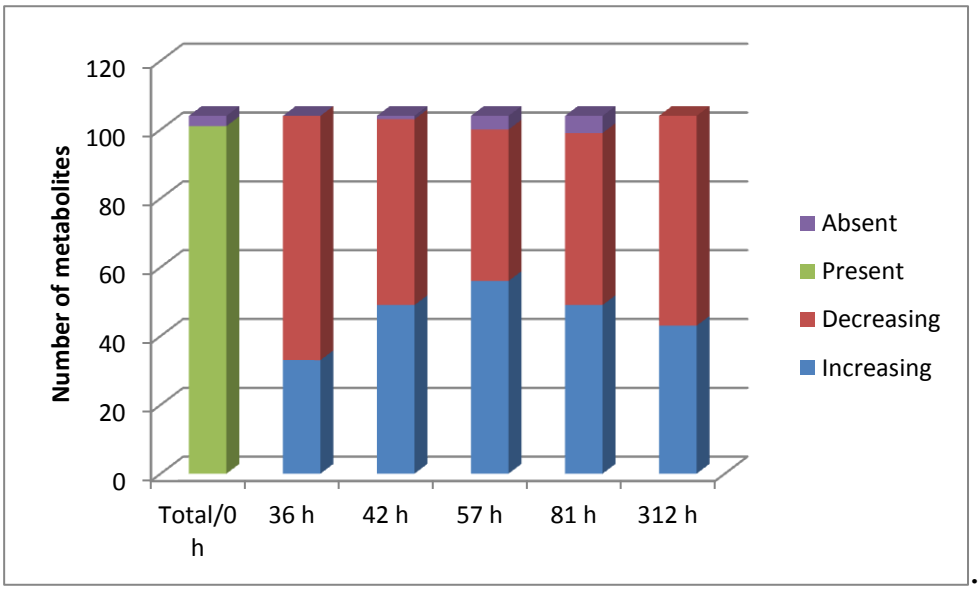

Figure 14-11: Lipids and lipid metabolites and their changes at different time points
Showing total metabolites present at 0 h (green), and proportion of the metabolites absent (purple); decreasing (red) or increasing (blue) at the various time points.

Table 14-33: First 10 Lipids, Lipid metabolism and related metabolites having significant fold change in time (P<0.05) in order of decreasing fold change.

Metabolites	Mass	Formula	Pathway	m/z	P-value	THFC	Fold	Type of change
Choline phosphate	183.065955	C5H14NO4P	Glycerophospholipid metabolism	324032768	0.0015	81	10.83	Down regulation
[ST hydrox] N-(3alpha,7alpha-dihydroxy-5beta-cholan-24-oyl)-taurine	499.296836	C26H45NO6S	Bile acid biosynthesis	1970903	0.035	57	9.54	Up regulation
[FA] O-Palmitoyl-R-carnitine	399.334472	C23H45NO4	Fatty acyl carnitine	490806	0.0189	81	7.60	Up regulation
[PE (18:1/18:1)] 1-(1Z-octadecenyl)-2-(9Z- otadecenoyl)-sn-glycero-3-osphoethanolamine	729.567453	C41H80NO7P	Glycerophospho-ethanolamines	265814	0.0116	81	6.98	Up regulation
[FA (22:0)] 13Z-docosenoic acid	338.318825	C22H42O2	Biosynthesis of unsaturated FA	829085	0.0136	57	6.61	Up regulation
[FA] O-Palmitoyl-R-carnitine	399.334472	C23H45NO4	Fatty acyl carnitines	490806	0.0409	57	6.35	Up regulation
sn-Glycerol 3-phosphate	172.013729	C3H9O6P	Glycerolipid metabolism	18743178	0.0021	81	5.82	Down regulation
[FA (16:0/2:0)] Hexadecanedioic acid	143.107179	C16H30O4	Fatty Acids and Conjugates	569451	0.0163	57	5.58	Up regulation
L-Serine-phosphoethanolamine	228.050985	C5H13N2O6P	Glycerophospholipid metabolism	366023	0.0114	81	5.27	Down regulation
sn-glycero-3-Phosphoethanolamine	215.056024	C5H14NO6P	Glycerophospholipid metabolism	48044336	0.0028	81	5.26	Down regulation

Table 14-34: Examples of Lipid metabolites displaying the various trends in the course of infection.
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	Trend B	Trend C	Trend D
sn-glycero-3-Phosphocholine	**[FA (16:0/2:0)] Hexadecanedioic acid**	**[ST hydrox] N-3alpha,7alpha-**	**Hexanoic acid**
[FA hydroxy(9:0)] 2-hydroxy-nonanoic acid	**[FA methyl(6:1)] 2-methyl-2Z-hexenoic acid**	**[FA] O-Palmitoyl-R-carnitine**	**2-C-Methyl-D-erythritol 4-phosphate**
[Fv hydroxy,hydrox] (S)-2,3-Dihydro-7-	**LysoPE(0:0/20:3(11Z,14Z,17Z)**	**[PE (18:1/18:1)] 1-(1Z-ctadecenyl)-2-**	**[SP hydroxy,hydroxy,methyl(10:2/2:0)] 6R-(8-**
[SP (17:0)] heptadecasphinganine	**-**	**-**	**N-Butyryl-L-homoserine lactone**
[Fv] Kurzichalcolactone	**-**	**-**	**[FA oxo(16:0)] 3-oxo-hexadecanoic acid**

25.1.1.4 Nucleotide metabolism

There were a total of 23 compounds in this category. 20 were present at 0h while 3 were not. Orotate had the highest m/z intensity of all metabolites in this pathway. Figure 6-12 shows the frequency distribution of metabolites these pathways. Fold change in nucleotides and related metabolites during the course of infection were computed and Table 6-35 shows a list of first 10 metabolites, having greatest fold change, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$) and Table 6-36 shows examples of compounds displaying different trends with course of infection belonging to this group of metabolites.

Only two compounds were significantly increased (from 0 h) at times 57 h and 81 h respectively which were 5,6-dihydrothymine and trimetaphosphate.

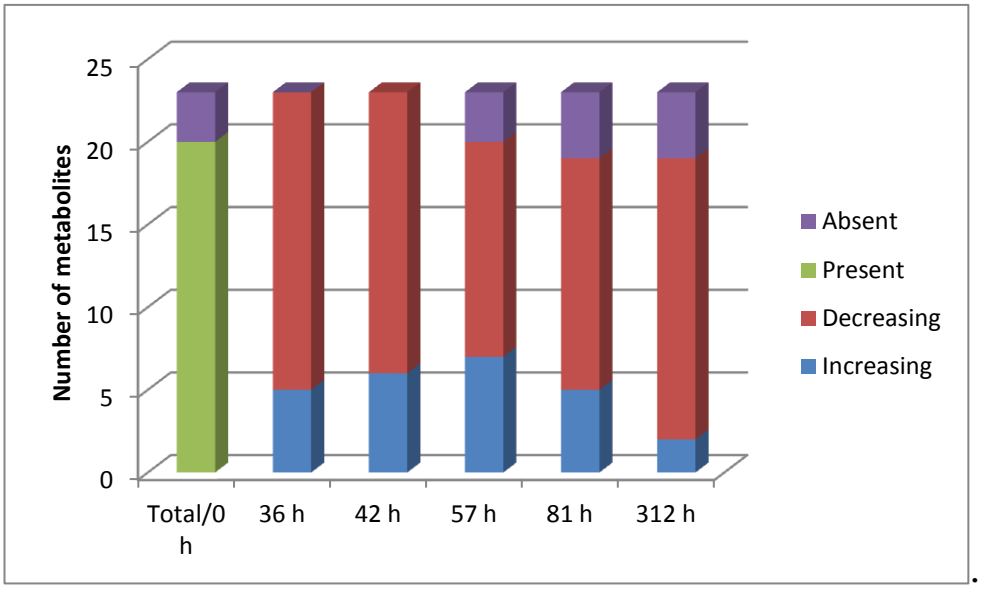

Figure 14-12: Nucleotides and related metabolites and their changes at different time points
Showing total metabolites present at 0 h (green), and proportion of the metabolites absent (purple); decreasing (red) or increasing (blue) at the various time points

Table 14-35: First 10 nucleotides and related metabolites having significant fold change in time (P<0.05) in order of decreasing fold change.
THFC-time of highest fold change

Metabolites	Mass	Formula	Pathway	m/z intensity	P-value	THFC	Fold change	Type of change
7-Methyladenine	149.0701	C6H7N5	Base excision repair	4261534	0.032	36	10.27	Up regulation
Inosine	268.0804	C10H12N4O5	Purine metabolism	598516	0.005	57	8.29	Up regulation
(S)-Dihydroorotate	158.0327	C5H6N2O4	Pyrimidine metabolism	8641255	0.002	57	-8.24	Down regulation
Guanosine	283.0913	C10H13N5O5	Purine metabolism	677377	0.003	57	8.19	Up regulation
7-Methyladenine	149.0701	C6H7N5	Base excision repair	4261534	0.023	57	7.73	Up regulation
Inosine	268.0804	C10H12N4O5	Purine metabolism	598516	0.038	81	7.26	Up regulation
Deoxycytidine	227.0903	C9H13N3O4	Pyrimidine metabolism	3535116	0.015	57	-5.79	Down regulation
Orotate	156.0171	C5H4N2O4	Pyrimidine metabolism	352815456	0.000	81	-5.57	Down regulation
Deoxycytidine	227.0903	C9H13N3O4	Pyrimidine metabolism	3535116	0.015	36	-5.43	Down regulation
(S)-Dihydroorotate	158.0327	C5H6N2O4	Pyrimidine metabolism	8641255	0.015	42	5-.33	Down regulation

Table 14-36: Examples of Nucleotides and related metabolites displaying the various trends with course of infection
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	Trend B	Trend C	Trend D
Cytidine	5,6-Dihydrothymine	Guanosine	Hypoxanthine
Cytosine	N/A	7-Methyladenine	Urate

N/A-not applicable

25.1.1.5 Vitamins and Co-factors metabolism

The total number of compounds that changed in the course of infection in this metabolic group was 22. All these were present at 0 h. Nicotinamide had the highest m/z intensity. Fold (level of) change in vitamins, co-factors and related metabolites during the course of infection were computed and Table 6-37 and shows a list of the six metabolites, with significant fold change with time, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$) while Table 6-38 is a list of metabolites showing different trends in this group.

Figure 6-13 shows the distribution trends of metabolites in this pathway with time.

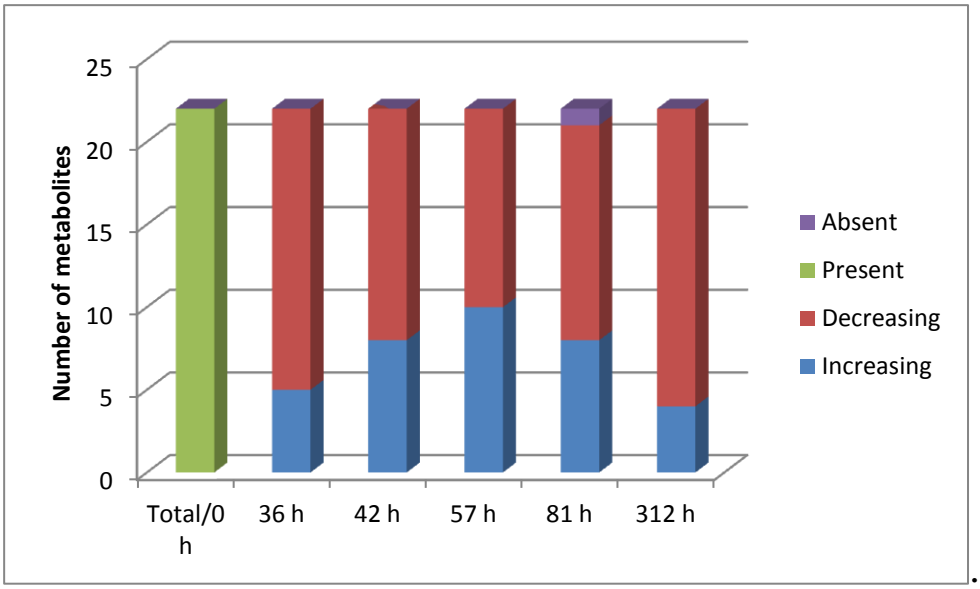

Figure 14-13: Vitamins, cofactors and related metabolites and their changes at different time points.
Showing total metabolites present at 0 h (green), and proportion of the metabolites absent (purple); decreasing (red) or increasing (blue) at the various time points

Table 14-37: First 7 Vitamins and Co-factors and related metabolites with significant change in time (P<0.05) in order of decreasing fold change.
Some metabolites displayed most significant fold change at different time points and are displayed at the different time points when the fold change occurred. THFC-time of highest fold change.

Metabolites	Mass	Formula	Pathway/Metabolism	m/z intensity	P-value	THFC	Fold change	Type of change
Pyridoxal	167.0583106	C8H9NO3	Vitamin B6	6655131	0.0005	57	-6.32	Down regulation
Pyridoxal	167.0583106	C8H9NO3	Vitamin B6	6655131	0.0005	81	-5.45	Down regulation
3-Hydroxy-2-ethylpyridine-4,5-dicarboxylate	197.032485	C8H7NO5	Vitamin B6	285486	0.0000	81	-5.14	Down regulation
Pyridoxal	167.0583106	C8H9NO3	Vitamin B6	6655131	0.0005	42	-4.59	Down regulation
Nicotinamide	122.047965	C6H6N2O	Nicotinate & nicotinamide	116364936	0.0013	42	-3.74	Down regulation
Pyridoxal	167.0583106	C8H9NO3	Vitamin B6	6655131	0.0005	312	-3.21	Down regulation
Nicotinamide	122.047965	C6H6N2O	Nicotinate & nicotinamide	116364936	0.0013	81	-3.09	Down regulation
Thiamin	264.10418	C12H16N4O	Thiamine	1124829	8.42E-06	81	-2.74	Down regulation
Pyridoxine	169.073852	C8H11NO3	Vitamin B6	658731	0.0041	57	-2.65	Down regulation
Pyridoxamine	168.089827	C8H12N2O2	Vitamin B6	1129846	0.0152	57	-2.56	Down regulation
1-Methylpyrrolinium	83.0734422	C5H9N	Nicotinate & nicotinamide	676540	0.0126	57	2.25	Up regulation
6-ydroxypseudooxynicotine	97.0527639	C10H14N2O	Nicotinate& nicotinamide	515716	0.0002	42	-2.07	Down regulation

Table 14-38: Examples of vitamins, co-factors and related metabolites displaying various trends during the course of infection
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	Trend B	Trend C	Trend D
3-Hydroxy-2-methylpyridine-4,5-dicarboxylate	1-Methylpyrrolinium	N/A	L-Urobilinogen
Riboflavin	Dethiobiotin	N/A	Maleamate

N/A-not applicable

25.1.1.6 All other minor pathways

Other pathways whose metabolites were represented in different time points of infection include metabolites of biosynthesis of polyketides and non-ribosomal peptides, biosynthesis of secondary metabolites, medium components, xenobiotic biodegradation and metabolism as well as unspecified pathways compounds. Compounds in all these categories totalled 223.

The compound with highest intensity of all other pathways metabolites is 8-keto-7-aminoperlagonate, belonging to the group of compounds with unknown metabolic map or pathway but it displayed a Trend A. Fold change in a combination of all other remaining groups of metabolite pathways during the course of infection were computed and Table 6-39 shows a list of first 10 metabolites, having greatest fold change, type of change (up or down regulation) and the corresponding time when it occurred ($P<0.05$) and Table 6-40 shows different trend metabolites. Proportional changes of metabolites with time are shown in Figure 6-14.

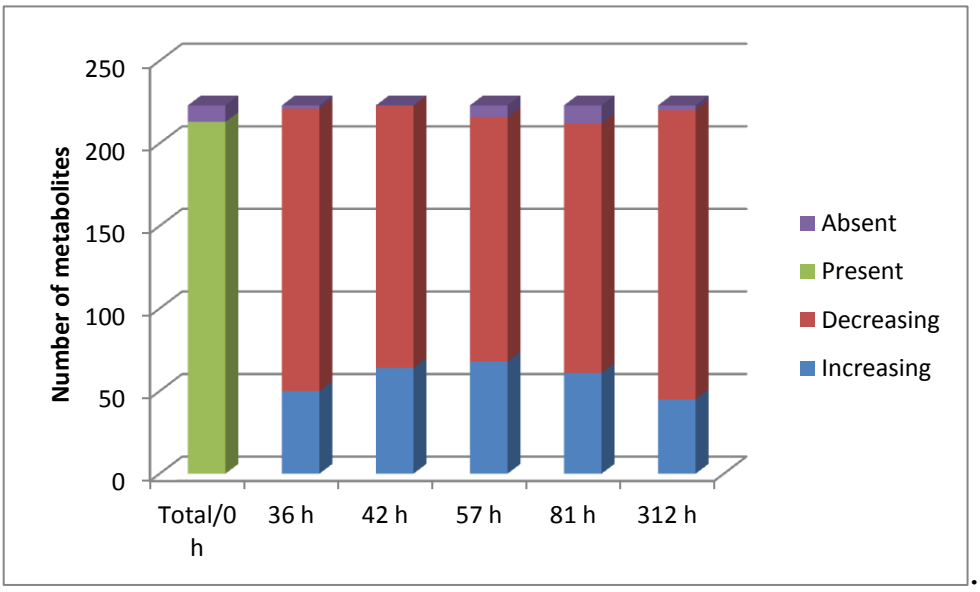

Figure 14-14: All other metabolites in different and unknown pathways and their changes at different time points
Showing total metabolites present at 0 h (green), and proportion of the metabolites absent (purple); decreasing (red) or increasing (blue) at the various time points

Table 14-39: First 10 metabolites from all other pathways having significant fold change in time (P<0.05) in order of decreasing fold change

Some metabolites displayed most significant fold change at different time points and are displayed at the different time points when the fold change occurred. * Other minor or unspecified pathways metabolites include metabolites involved in biosynthesis of secondary metabolites[b] and metabolites of unknown pathways[0].

Metabolites	Mass	Formula	Pathway	m/z intensity	P-value	THFC	Fold change	Type of change
Puromycin	471.22247	C22H29N7O5	Puromycin [b]	3969427	0.02479	81	10.92	Up regulation
Thiomorpholine 3-carboxylate	147.03534	C5H9NO2S	0^0	23337922	0.049398	312	10.87	Up regulation
Puromycin	471.22247	C22H29N7O6	Puromycin [b]	3969427	0.024614	57	10.47	Up regulation
Convolvine	291.14693	C16H21NO4	0^0	1434941	0.036735	57	9.35	Up regulation
ZAPA	146.01494	C4H6N2O2S	0^0	2713729	0.016248	57	9.01	Up regulation
Thiomorpholine 3-carboxylate	147.03534	C5H9NO2S	0^0	23337922	0.00363	57	8.93	Up regulation
Puromycin	471.22247	C22H29N7O5	Puromycin[b]	3969427	0.010308	312	8.20	Up regulation
Ovothiol A-cysteine disulfide	160.03059	C10H16N4O4S2	0^0	776263	0.022443	57	7.73	Up regulation
Convolvine	291.14693	C16H21NO4	0^0	1434941	0.030466	81	7.58	Up regulation
alpha-D-Galactosyl-1,3-beta-D-galactosyl-1,4-N-acetyl-D-glucosamine	545.19529	C20H35NO16	0^0	15667663	0.000164	81	-7.40	Down regulation

Table 14-40: Examples of all other metabolites pathways displaying the various trends with course of infection
(Trend A-decline in intensity with infection and rise at resolution; Trend B-increase in intensity with infection and drop at resolution; Trend C-absent from pre-infection, present at peak infection and absent at resolution; Trend D-irregular fluctuations from pre-infection to resolution).

Trend A	**Trend B**	**Trend C**	**Trend D**
Retronecine	5,6-Dihydroxy-3-methyl-2-oxo-1,2,5,6-tetrahydroquinoline	Convolvine	1,8-Diazacyclotetradecane-2,9-dione
Ecgonine	PIperidine	-	Capryloylglycine
2-Ethylhexyl phthalate	N5-Ethyl-L-glutamine	-	Valproylglycine
4-Nitroaniline	N-Acetylmuramate	-	Theophylline
Aspidinol	N-Acetyl-D-glucosamine 6-sulfate	-	N-Acetyl-L-histidine

25.2 Discussion

25.2.1 General

This study was an untargeted global metabolomics study of milk, carried out to investigate and describe the metabolite profile of milk and its changes with time during the course of an intramammary challenge with a host-adapted strain of *S. uberis*, an important environmental pathogen of mastitis.

A major goal was to identify potential biomarker candidates for mastitis in general and specifically for *S. uberis* mastitis. In addition, it was hoped that this study will provide information on metabolic pathways that change during the course of IMIs and possible measures that could be exploited for the enhancement of animal management, diagnosis, therapeutics and welfare of dairy cows.

Metabolomics produces 'big data' which can be analysed by sophisticated statistical and bioinformatics approaches. These are beyond the scope of this thesis but are to be addressed in the future. Here a molecular and biochemical approach was taken to identify the key metabolites and metabolic pathways altered during mastitis.

Clinical mastitis developed in all quarters challenged with this host adapted strain of *S. uberis* (Tassi *et al.*, 2013). This was unexpected as the host adapted strains were thought to be characterized by a more subclinical and persistent mammary infection (Zadoks, 2007). There was also a considerable cellular and cytokine response by the host to this challenge (Tassi *et al.*, 2013)

The time 0 (0 h) was the control time, which corresponded to absence of infection, as confirmed by SCC and bacteriology (Tassi *et al.*, 2013). Hence all data obtained from other time points were statistically compared with values of 0 h. Metabolites identified with a CS of 7 to 10 were selected to ensure that data were reliable. The method used for the analysis was found to be specific and the Ideom software was able to give provide a comparison of time points.

It is expected that metabolomics experiments yield a high number of metabolites (Sundekilde *et al.*, 2013; Leichtle *et al.*, 2013) so it was not surprising that in this analysis over 9000 metabolites were detected. Moreover

the methodology used, LC-MS, is known to be of higher sensitivity than other metabolomics techniques such as H-NMR spectroscopy, although having its own disadvantages (Boudonck *et al.,* 2009; Sundekilde *et al.,* 2011). Wang *et al.* (2012) constructed a metabolic network for the bovine mammary tissue using bovine genome information and uncovered up to 1,743 metabolites and 657 enzymes. They also identified 11 crucial enzymes whose expression changed in condition of mastitis by integrating the array from healthy and clinical mastitis.

About a third of the detected metabolites were recognised and identified using the KEGG maps associated with the programme, while in the comparison interface, a third of the identified metabolites showed variation with the course of infection. Previously, in a study by Mansor (University of Glasgow PhD thesis) over 15000 metabolites were detected and only 1356 of these were identified. The higher number of metabolites detected in Mansor's study may be by virtue of the fact that mastitis samples studied were from natural cases of mastitis, caused by more than one organism.

In studies of milk metabolomics using NMR spectroscopy, compounds found to be potential biomarkers for mastitis through correlation with elevated SCC such as lactose (Sundekilde *et al.*, 2013) were also found in this study.

Pathways represented by the metabolites detected and identified in this metabolomics experiment ranged from protein metabolism (amino acids, peptides), carbohydrate and energy metabolism, vitamins and cofactors metabolism, nucleotides, biosynthesis of secondary metabolites, as well as xenobiotic degradation intermediates. These findings were similar to those of Boudonck *et al.*, (2009).

Other metabolites found belonged to biochemical classes of medium components, polyketides and non-ribosomal peptides as well as compounds with unknown pathway groups.

Metabolites were classified in their super and sub pathways, retention times, intensity, ionisation modes, formula and number of isomers. In this study, creatine, betaine, L-carnitine and sn-glycero-3-phosphocholine were the metabolites with highest m/z intensity in that order.

Overall, in the comparisons of metabolites between time points, N-Acetyl-D-glucosamine 6-phosphate was the most significantly changed (fold log_2) compound, from the level it was at in time 0 h (trend A) and its most significant change (decrease) occurred at 57 h. Similarly, Leu-Ala-Gln was the most up regulated metabolite observed in the entire experiment.

Lactose (m/z intensity=152418496, negative ionisation mode) which was identified by the Ideom software with 42 different isomers including maltose and lactulose was also among the highly down regulated metabolites during the course of infection. In all the major metabolic pathways (carbohydrate, protein, lipid, nucleotide, vitamin and co-factor metabolism), metabolites showed greatest changes (fold levels) at times 57 h and 81 h. This suggests that peak inflammatory changes in this challenge study occurred at this period, hence the most profound metabolite changes milk are seen at this period. Whether this is true for natural infections as well and how this differs or reflects infections by other species or strains of *S. uberis* cannot be inferred from this study. The earliest (36 h) significant changes in metabolites were seen among the nucleotide group rather than in metabolites from any other metabolic pathway.

Compounds seen at 0 h are hypothetically metabolites which are normal constituents of healthy milk from normal mammary gland metabolism but become depleted, damaged or are no longer synthesized as a result of the presence and activities of pathogens and the body's reaction to get rid of the pathogens.

During mastitis (or other inflammatory conditions), physiological metabolic pathways are disrupted to accommodate the need to fight invading organisms. Pathways that enhance bacterial cell lysis such as production of free radicals are enhanced.

25.2.2 Time points comparisons

25.2.2.1 Thirty Six Hours (36 h) post infection

Thirty six h post infection (36 h) corresponds to the time of the maximum average bacteria colony forming units (CFU) isolated from milk samples in challenged quarters during the challenge study (Tassi *et al.*, 2013). Thus,

metabolites that appeared or increased at this time may relate to products or intermediates of peak bacterial activities and metabolism or the host's reaction to bacteria invasion. At 36 h, the earliest changes related to infection were noticed in clinical signs, APP and cytokine levels. Clinical signs of mastitis were first observed between 30-48 h PC, appearing first at 36 h for two of the six challenge cows (Tassi *et al.*, 2013). Other changes first seen at this time in some cows include increase in temperature for one cow, detection of high SCC in one cow and appearance of most cytokines in most cows. The APP Hp was also first increased from baseline values at 36 h in two cows.

Several metabolites were also found to have become significantly different at this time from their initial levels at 0 h. Some of these compounds which displayed fluctuation with the course of infection similar to bacterial count, SCC and clinical scores, suggests possible early markers of this *S. uberis* infection.

4-Hydroxy-3-nitrosobenzamide was one very significantly decreased metabolite from 0 h at this time (36 h), however, an irregular fluctuation in the level of this metabolite was observed between 42 to 312 h, and thus it may not be accurately predictive of the on-going IMI. Also, the pathway of this metabolite was unidentified by the KEGG software (denoted as 0), making it difficult to relate its biochemical significance to its presence and dynamics during the disease.

Deoxycytidine had the highest fold down regulation. Deoxycytidine is a nucleotide related compound and as was observed for most nucleotides, there was a general decrease in these compounds with course of infection. On the contrary and unlike other nucleotides and related compounds, the methylated nucleotide; 7-methyladenine was the most up-regulated metabolite of all identified compounds in this experiment, at 36 h; however it was a 'newly appearing' metabolite which was not present at 0 h but was detected first at 36 h. It is a compound involved in base excision DNA repair, indicating that while rapid decreases in nucleotides occurs at the early stages of mastitis in milk samples, a possible fate of the metabolites is the addition of substituent groups such as alkyl group. The presence of this compound in urine of rats has been shown to be indicative of exposure to methylating agents (Mandel *et al.*, 1994).

Ovothiol A-cysteine disulfide was another compound found with a high fold increase at 36 h compared to 0 h. It was also absent at 0 h and was not associated with any known pathway using the KEGG map. It peaked at 57 h but still remained significantly high at 312 h although the trend suggests levels were returning back to baseline as recorded for 0 h. The course of this compound with time post-infection, which has been designated trend C in this study, corresponds to the pattern of the IMI clinical signs and other indicators of inflammation such as APP (Hp which also peaked at 57 h), bacterial counts and to an extent SCC. Nothing is known of ovothiol A-cysteine disulfide's role in milk but it may be a by-product of bacterial or endogenous protease degradation of proteins or amino acids, or the use of sulphur from dithiotreol for the formation of cysteine (Ryan *et al.*, 2001).

Potential biomarker candidates, that appear early in the course of an infection can be early indicators of such mastitis inflammation, therefore a compound such as ovothiol A-cysteine can offer that advantage, if proven and validated.

Two compounds (Glu-Leu-Lys-Lys and alpha-N-Acetylneuraminyl-2, 6-beta-D-galactosyl-1, 4-N-acetyl-beta-D- glucosamine) were present at 0 h but absent at 36 h. However, these compounds reappeared again at 42 h and thereafter, suggesting a fluctuation that may not be related to infection course. Many other compounds showed significant variations in intensity between 0 h and 36 h, some being present at 0 h and absent at 36 h, or being absent at 36 h but present at 36 h, the basis of which mastitis biomarkers could be sourced.

25.2.2.2 Forty two hours post infection

The forty second hour post infection corresponded to the time of the highest mean SCC in challenged quarters as reported by Tassi *et al.* (2013). Somatic cells are increased in milk during mastitis as a result of the increased migration of leukocytes into the udder. SCC has thus been used as the 'gold standard' in many countries, for the recognition of subclinical mastitis in dairy farms.

Deoxycytidine was also the most down regulated metabolite at 42 h. It is a ribonucleoside involved in the metabolism of DNA. Although this metabolite followed trend A, it however still remained very significantly low at the

resolution time point thus may not be a sensitive gauge of reversal of inflammatory signs.

Another example of a down-regulated compound at this time is 4, 5-dihydro-5, and 5-dimethyl-4-(3-oxobutyl) furan-2(3H)-one, it displayed a distinct trend A and was almost back to normal (0 h levels) at 312 h. It was identified in the positive ionisation mode and is an intermediate in the terpenoid biosynthesis pathway with 9 isomers, many of which were non-esterified unsaturated fatty acids (C_{10}).

Terpenoids are precursors of sterols and steroids in animals, and these are useful mediators of inflammation, and it is likely that a reduction in this compound as infection progressed is related to its massive recruitment for the formation of terpenoids and consequently, steroids.

Out of all metabolites, the dipeptide Trp-Ala was the most up regulated at 42 h from 0 h. There were ten metabolites that appeared for the first time at 42 h (absent at 0 h and 36 h). These compounds were mainly di- and tripeptides (8 out of 10) and are a reflection of metabolites released into milk as a result of the peak activities and presence of somatic cells (mainly neutrophils), which are known to release proteolytic enzymes such as cathepsins and amino peptidases (Urech *et al.*, 1999). On the other hand, there was one compound present at time 0 and 36 h, but undetectable at time 42, N-Methyl ethanolamine phosphate. Many compounds were noticed to be present at 0 h and 36 h, but became either increasing (213) or decreasing (365); at 42 h. L-proline had highest intensity among such increasing metabolites with creatine, in the decreasing group.

25.2.2.3 Fifty seven hours post infection

This time point corresponds to time when peak average Hp levels in milk were recorded. Choline phosphate was the most down regulated compound and it is involved in the lipid metabolism (glycerophospholipid metabolism) and specifically in the formation of membrane lipid phosphatidylcholine, which is its inverse form and has been described as a universal biomembrane adhesive because of its peculiar properties (Yu *et al.*, 2012). The trend observed for this

metabolite may be due to it being used by pathogens in milk for formation of cell membranes as they multiply.

The compound Furcatin (m/z intensity = 479125), taken as one example of significantly down-regulated metabolites at 57 h, had an unknown pathway and displayed a trend A. It was detected in the negative ionisation mode and is a disaccharide which is a structural derivative of ß-D-apiofuranosyl-(1→6)-D-glucopyranose. Its role in milk is not known and the reasons for its decrease with time in course of infection may be related to the general decline in carbohydrate metabolites encountered during the course of the IMI, noticed in this study. A few other compounds (n=20) which were present in control samples became totally absent at 57 h, N-Acetyl-D-glucosamine 6-phosphate, D-Glucose 6-phosphate, (S)-Dihydroorotate Ethanolamine phosphate, CMP [PC ethyl, acetyl] 1-ethyl-2-acetyl-sn-glycero-3-phosphocholine and Oxaloacetate. Pyridoxal and Propanoyl phosphate also showed great reductions in intensity (but not complete absence). These metabolites constitute important co-factors that are essential for biochemical processes in the cell (for example transamination reactions), thus their depletion corresponds to their rapid conversion for use by invading bacteria.

The amino acid, glycine which was present at control time, was one of the most significantly increased compounds at 57 h (trend B). Among compounds not present at 0 h, the hydrophobic di-peptide, Thr-Tyr, greatly increased (trend C), first appearing at 36 h, peaking at 57 h and absent again at resolution time at 312 h, but the tri-peptide, Leu-Ala-Gln had the most fold increase among these newly emerging compounds. The appearance and rapid surge of these small peptides during the period of peak infective changes, is a pointer to enormous proteolytic changes going on in milk during an IMI. Proteolysis of milk proteins that occurs in mastitis, results in the rise in levels of small peptides in milk (Larsen *et al.*, 2010b).

25.2.2.4 Eighty one hours post infection

The cytokine, interleukin 17A (IL-17A) was first noticed by immunoassay to increase between 72 h and 144 h, with a peak of average at 81 h in 4/6 cows. At the same time, there was also a temporary increase in lymphocyte levels in

milk. IL-17A displayed a pattern unlike other interleukins such as IL-6, which showed earlier average peak and displayed a progression similar to APP (Tassi *et al.*, 2013). The detection of IL-17A was also associated with a decrease in bacteria CFU and spontaneous clearing of infections, which was observed in the cows with detectable IL-17A. Thus the metabolomic analysis carried out for this time point could determine if metabolites reflect a status of metabolism indicative of resolution.

Many of the metabolites identified with variation with time showed the highest levels of fold change (especially decrease) at this time point (in addition to 57 h). It can therefore be inferred that 81 h post infection, in this experimental mastitis model, matches the period of greatest alteration impacted by mastitis on milk metabolites and by extension the mammary gland.

Choline phosphate was the most 'fold reduced' metabolite at 81 h, lactose too was also very significantly reduced at this time compared to control time samples. It is the major product of galactose metabolism and the main sugar found in milk; a disaccharide of galactose and glucose.

Among metabolites present from the 0 h, Val-Gly-Pro had the greatest fold increase. Creatine had the highest intensity among initially present compounds. Betaine which is an oxidative product of choline involved in the glycine and serine metabolism has been shown to play a crucial role in the synthesis of milk fat, and in this experiment it had the highest m/z intensity (from pre-infection time) after creatine. Studies have shown its ability to increase milk yield when administered in feed to cows, without significantly altering milk composition (Peterson *et al.*, 2012). There is a possibility that betaine formed a component of the cows' feed, which was secreted into milk.

25.2.2.5 Three hundred and twelve hours post infection

This time corresponds to the stage of resolution of infection. Five out of six cows had cleared the infection at this time. This time point was generally characterized by restoration of levels of carbohydrate and energy metabolites to values as at 0 h and the disappearance of small peptides noticed in abundance during the peak periods of the infection (36 h to 81 h). Features consistent with

a molecule being a good biomarker include its ability to also correctly indicate the resolution stage of infection by reverting back to the level of characteristic at normal or healthy state. Many compounds were observed to show parallel at 312 h with 0 h after eitherincreasing, decreasing or disappearing at points between these two time points.

A few compounds, however still retained differences at this time from 0 h. It was observed that though some of these compounds differed significantly from 0 h at this point, their levels of fold change were not as high as seen during the peak infection periods (36-81 h).

5-Methyl-2'-deoxycytidine and thiomorpholine 3-carboxylate (newly emerging) had the highest fold decrease and increase respectively at this time point, while another hydrophobic dipeptide; Leu-Asn was the most (fold) increased amongst previously present compounds.

For instance, 3, 5/4-Trihydroxycyclohexa-1, 2-dione was one metabolite, a carbohydrate of the inositol phosphate pathway with 5 isomers, including one involved in the xenobiotic degradation pathway (3-oxadipate) and another in the metabolism of vitamins and co factors (2-formylglutarate), which had a high fold decrease. It was characterized by being reduced as infection progressed having lowest levels at 81 h, and then beginning to rise at 312 h (trend A).

25.2.3 Metabolic pathways

25.2.3.1 Carbohydrate metabolism

Milk is a secretion of the mammary gland whose components represents the end product of mammary gland metabolism, although milk itself does not normally contain metabolically active cells. Therefore, compositions of milk can reflect the metabolic/biochemical status of the gland and can thus be used to make inferences on the systems biology of the mammary gland cells in different physiological and pathological conditions.

In all, metabolites of carbohydrate and energy metabolism were most significantly different from 0 h levels (reduced) at time 81 h. Hence it may be deduced that the changes in these pathway metabolites, occur late in the course

of infection and as such may not offer good potentials for an early mastitis marker. The general trend with the carbohydrate and energy metabolome in milk observed during mastitis is a gradual decrease in concentration as infection progresses. This could be because they are used up by bacteria for energy during the course of infection, or their production may be inhibited as part of host response.

Lactose

Lactose is synthesized in the Golgi apparatus of the mammary epithelial secretory cells in a reaction catalysed by lactose synthetase complex, using glucose and galactose as substrates (DeWitt, 2010). Many bacteria utilize lactose as a source of their energy, especially the *Streptococcus* group, hence the trend observed for lactose (becoming depleted) with the peak of infection in this study. The lactose trend is discussed further under the carbohydrate metabolism section below, but lactose which is the main milk sugar and in addition plays a crucial role in the osmotic maintenance of milk volume, was found present at 0 h (healthy samples) but significantly reduced with time of infection but restored to normal levels at 312 h.

In a recent study of the milk metabolome during the first few months of lactation, it was discovered that lactose and citrate were the metabolites that contributed the most to changes observed in the milk metabolites between early lactation and other stages, decreasing with days in milk (Ilves *et al.,* 2012). Previous studies had highlighted the existence of lactose in milk within a narrow concentration range (Klein *et al.*, 2012), therefore, the marked decrease in lactose with time of infection is a significant but expected finding that is now well accepted (Bruckmaier *et al.*, 2004; Shuster *et al.*, 1991; Pyorälä, 2003).

Apart from the fact that many bacteria utilize lactose as source of energy (especially the *Streptococcus spp*), there could be pathogen induced damage to lactose synthesizing mammary gland cells, resulting in a lack of or diminished synthesis of lactose. Hence the progressive lactose depletion observed with time of infection and its restoration back to normal levels at time of low bacteria CFU. A number of studies have already described the correlation of lactose with mastitis or SCC levels in milk (Pyorälä, 2003; Berglund *et al.*, 2007; Malek dos

Reis *et al.*, 2013). Lactose level in milk is often measured as a quality trait of milk and an indicator of milk composition (Wittenburg *et al.*, 2013; Melzer *et al.*, 2013). In the study of Melzer *et al.* (2013), lactose (along with other milk traits) was found to be associated with specific metabolic profile of milk, and was thus identified as a characteristic that could be used to the represent the interrelations that occur between milk traits and the milk metabolome. Lactose was found to be correlated significantly to mostly carbohydrate metabolism compounds such as 1, 3-Dihydroxyacetone and Glucaric acid-1, 4-lactone. In that study, it was also observed that a negative correlation existed between somatic cell scores and lactose. This is congruent to findings in this study ofsignificant decreases in lactose levels at the time points with the peak SCC.

Lactose fluctuations in milk are so important that Bittante and Cecchinato (2013) demonstrated by the use of the milk lactose trait, the breeding values of cows.

Citrate

Citrate showed a very significant decrease from normal levels throughout the course of the IMC. By the time of remission of infection, levels of this compound were still very significantly lower than at 0 h. Citrate is an intermediate of the tricarboxylic acid cycle (TCA) formed from the condensation of oxaloacetate and acetyl Co A. Acetyl Co A is the product of the reaction catalysed by the enzyme complex pyruvate dehydrogenase on pyruvate. In the study by Klein *et al.* (2010), citrate did not show any significant variation throughout the lactation period studied, however previous studies have reported wide variation with lactation stage (Garnsworthy *et al.*, 2006) and based on other factors such as nutrition. Though variation was observed in citrate levels with disease in this study, a specific pattern that can be matched with the disease trend was not followed.

Pyruvate

In this study, pyruvate was found to be increased as the infection peaked, suggesting a correlation between pyruvate and SCC as reported by Chagunda *et al.* (2006a). These findings were in contrast to those of Klein *et al.* (2012) who found no association between pyruvate or lactate and SCC. Chagunda *et al.*

(2006b) had also reported an increase in activity of lactate dehydrogenase (LDH) with mastitis. The enzyme LDH, converts lactate to pyruvate in a reversible reaction. Pyruvate was the only intermediate of the glycolytic pathway which increased as infection peaked and this may be due to conversion of lactate produced by bacteria into pyruvate by LDH. This may explain the spiking of pyruvate levels, observed in their study and also in this investigation.

Fumarate

Another energy metabolite of the TCA cycle, fumarate showed a significant decrease from control levels as infection peaked, corroborating the findings of Sundekilde *et al*. (2013), who identified that fumarate (and hippurate) were significantly decreased in milk having high SCC. The lowest point for fumarate was at 81 h, and at 312 h, although beginning to increase was still highly significantly different from 0 h levels (P= 0.0006). Fumarate also takes part in the TCA and the significance of its decrease in milk during mammary infection is that energy synthesizing precursors are deficient.

Other energy metabolites

Other intermediates of the TCA such as oxaloacetate, cis–aconitate, succinate, S-malate and oxoglutarate were also observed to be significantly reduced as infection peaked, thus it can be concluded that the IMI generally leads to a down regulation of the TCA cycle. It was found that some intermediates of the pentose phosphate pathway, such as ribose, were up regulated during the peak of mastitis. As observed in the study of Mansor (2012), D-glucose levels also decreased at the peak of infection. It has been suggested that this occurs in order to maintain the osmotic balance in the mammary gland, following a loss of balance that occurs after leakage of ions such as Na^+ and Cl^- during inflammation-induced disruption of blood milk barrier (Marschke & Kitchen 1984). In addition, it was suggested that blood perfusion of the mammary gland is markedly reduced during mastitis; consequently uptake of glucose by the mammary gland is hampered (Mansor, 2012). The decrease in glucose concentration could also explain the drop in lactose during mastitis, given that glucose is a major substrate for the formation of lactose. Glucose-6-phospate was also found to be absent at peak infection time but high during pre-infection.

Lactate is one end product of energy metabolism in bacteria and it has been reported to correlate with mastitis. It also displayed a trend B, which is consistent with previous reports of lactate fluctuation with mastitis.

Hydroxybutyrate

Hydroxybutyrate (BHBA) was found to show an increasing trend with infection agreeing with many previous studies that have indicated that this metabolite is an indicator not only of mastitis or high SCC but also of metabolic status and ketosis in dairy cattle (Moyes *et al.*, 2009a; Klein *et al.*, 2012; Sundekilde *et al.* 2013). It was most significantly increased by 57 h, and then dropped slightly at 81 h. Levels at other times were not found to be significantly different from control time levels.

25.2.3.2 Proteins and peptides metabolism

It has been demonstrated that mastitis results in an increased concentration of soluble proteins of milk (whey) while there is reduction in the insoluble (caseins) fractions (Urech *et al.,* 1999). Many of the compounds that were absent at 0 h, present at 36 h, 42 h, 57 h and 81 h and absent again at 312 h were peptides and intermediates of protein metabolism. This observation is similar to findings of Mansor (2012) who noticed an increase of di- and tri-peptides in mastitic milk samples compared to healthy samples. In that study, it was also observed that certain di and tri-peptides were associated with one specific causative organism, more than another. *S. aureus* mastitis milk showed more small peptides than in milk from cows with *E. coli* mastitis.

It has also been proposed that the increase in small molecular weight peptides is due to the activities of plasma proteases such as plasmin, leukocyte associated proteases and cathepsins, as well as bacterial proteases (Haddadi *et al.*, 2005; Larsen *et al.,* 2010b). It has also been suggested that both endogenous proteases and bacterial proteases have different specificities in their cleavage of milk proteins (Napoli *et al.*, 2007; Haddadi *et al.*, 2005).

Amino acids

Amino acids (AAs) and related nitrogenous compounds in milk are obtained via direct absorption from the blood or protein degradation in the mammary gland

and are important for the synthesis of milk proteins in the gland. Methionine and lysine have been described as limiting AAs for milk protein synthesis. Glutamine (and glutamate) has also been hypothesized to be limiting AAs for milk production in dairy cows (Meijer *et al.*, 1993). Significant changes occurred in intensities of AAs and their related metabolites during the course of the IMI. These findings are in agreement with observations of Wang *et al.* (2012), who used a systems biology approach to study the bovine mammary tissue metabolism, and ascertained that enzymes related to amino acid metabolism were very significantly altered during mastitis. This leads to a compromise in the synthesis of milk proteins which makes up the major nutrient composition of milk, hence the reduced milk yield commonly associated with mastitis.

Creatine had the highest intensity out of all 117 AAs and related nitrogen containing compounds identified and among all 640 metabolites with a 7-10 CS. It followed a trend A; being most abundant at control time but decreased post challenge, to the lowest levels at 57 h. It was detected in the positive ionisation mode and was related to the glycine, serine and threonine metabolism pathway on the KEGG map. Even at 36 h, creatine had showed a highly significant reduction from 0 h levels and continued to reduce to lowest level at 81 h, but by the resolution time 312 h, it had almost returned back to same as at 0 h. The P-value between the mean of Creatine concentration at 0 h and at 312 h was not significant at $P<0.1$. Creatine is an energy metabolite (in form of energy releasing creatine phosphate) found in muscle and a product of amino acid degradation.

Another AA related molecule which reduced significantly starting from 36 h, but regained intensity to become almost the same as at 0 h levels was hippurate (0 h vs. 312 h ; $P=0.9$). Its lowest point was at 42 h. Hippurate is formed from the conjugation of benzoic acid with glycine, to facilitate the excretion of the former from the body. Hippurate was found by Sundekilde *et al.* (2013) to be significantly reduced in association with high milk SCC levels. Its reduction with time even as glycine increased in milk, is a pointer to a diversion of the glycine conjugation pathway of benzoic acid, whose antibacterial properties, as suggested by Sundekilde *et al.* (2013), is exploited to combat invading bacteria in the mammary gland. The trend of creatine and hippurate is consistent with

molecules that can be used to identify new infection while also indicating resolution at the appropriate time that corresponds to observations of clinical scores and bacteriological examination. An added advantage of creatine is its very high intensity at 0 h, as such making decreases during infection time(s) easy to recognize.

Betaine, the amino acid related metabolite which was next in peak intensity, is an oxidative product of choline which can ultimately be used for the synthesis of fatty acids in milk (Peterson *et al.*, 2012). Showing a similar trend to creatine in milk during mastitis, its high presence in healthy milk samples may be due to the secretion into milk from dietary sources in the rumen. Although reasons for its progressive reduction during intramammary infection and restoration in resolution of infection is unknown, but it can be speculated that the pathogens responsible for mastitis and present in milk, may possess enzymatic systems similar to the rumen microbes that can convert betaine to more useful energy deriving intermediates such as acetate (Peterson *et al.*, 2012). Thus, as bacterial counts peak, betaine levels decline and then begin to increase as the infection is resolved. This corresponds to time of fall in bacterial counts or absence of bacteria (Tassi *et al.*, 2013). Milk betaine levels have been reported to correlate with milk N-acetyl carbohydrate levels (Klein *et al.*, 2012). Supplementation of betaine in diet has been observed to cause an increase in milk yield in dairy cows (Peterson *et al.*, 2012).

N-Acetyl-D-glucosamine which is a normal constituent of milk was found to decrease with infection and increase back at 312 h (trend A), it can be suggested that since it is a substrate for NAGase, which increases in milk during IMI and has been recognized as an enzyme indicator of mastitis (Chagunda *et al.*, 2006b), decreased levels may be due to increased activity of the NAGase on this metabolite. Among all the AAs metabolites that changed throughout all the examined time points, the most significantly altered (lowered from control levels) was p-Benzenediol at 57 h. It is involved in the tyrosine metabolism pathway and has 5 isomers. 3-Methoxy-4-hydroxyphenylglycolaldehyde and 2-Methylglutaric acid were the next most significantly changing compounds, the former being also involved in the tyrosine metabolism pathway having 13 isomers with 182.06 Da MW, and the latter with 16 isomers and Mw of 146.02 Da. All

three metabolites named above, had the greatest changes at time 57 h and were detected in the negative mode.

Amongst the 22 standard amino acids (that can be encoded for by the genetic codons), L-Proline had the highest intensity followed by L-Leucine, L-Phenylalanine, L-Histidine and L-Glutamate in that order. All these amino acids except L-glutamate had a trend of increasing post challenge, which is a different trend from that observed for most of the non-standard AAs (standard AAs are the 20 AAs encoded directly by the codons of the universal genetic code and used for synthesis of proteins). Other standard amino acids found to decrease with time of infection included L-threonine and L-aspartate.

In the study of Csapó *et al.* (1995), most significantly increased free amino acids milk during mastitis included Ile, Ala, Pro, Leu and Asp. However, Asp was observed to decrease with infection in the present study, although the trend was somewhat irregular. L-Methionine was observed to increase with mastitis in this study; this confirms the findings of Mansor (University of Glasgow PhD thesis, 2012) who observed increased methionine in mastitis milk from *E. coli* and *S. aureus* infected quarters compared to controls. This increase in free methionine may arise from breakdown of proteins to small peptides and even amino acids as it is an important limiting amino acid required for metabolic reactions in the body.

At 36 h PC, the most significantly changed amino acid related compound was N-gamma-acetyldiaminobutyrate (NADA), which decreased to the lowest point at that 57 h. This metabolite was the earliest most significantly changed metabolite associated with progress of infection. By 312 h the intensity had returned towards control values, although there was still a very significant difference compared to 0 h levels, (P=0.0001). Studies by Wang *et al.* (2012) on the systems biology of mammary tissue demonstrated that an enzyme, glycine-C-acetyltransferase (GCAT) involved in glycine, serine and threonine metabolism was significantly affected in mastitis. NADA has been reported to be involved in the osmotic adaptation of bacteria through the action of one of its derivatives, ectoine, which helps organisms survive extremes of osmotic stress. It was also found to stabilize rabbit muscle LDH against inactivation by heat (Cánovas *et al.*, 1999). Fluctuating levels of NADA observed in this study probably occurred due

to its conversion to ectoine or hydroxyectoine, by bacterial biosynthetic enzymes. Ectoine itself was identified in this experiment, and showed a similar trend to NADA.

Contrary to findings of Mansor's study (University of Glasgow PhD thesis, 2012), phosphocholine levels did not increase as infection peaked in this study, even while glycine levels were increasing. Isoleucine and tyrosine were also found to increase with infection as observed by Mansor. Tyrosine levels have been associated with marked casein proteolysis (Murphy *et al.,* 1989).

Peptides

The major proteins of milk (caseins) and to a lesser extent α-lactalbumin, ß-lactoglobulin**,** immunoglobulins, bovine serum albumin as well as the minor ones such as lactoperoxidase, lysozyme and lactoferrin are a rich source of peptides upon hydrolysis. Peptide hydrolysates of milk proteins have been studied and established to have various bioactive properties (Ammendolia *et al.*, 2012) (Arruda *et al.*, 2012; Artym and Zimecki, 2013; Barzyk *et al.*, 2009; Cadee *et al.*, 2007). Mastitis causes an increase in the presence of peptides in milk due to the release of proteases from the blood somatic cells and or bacteria (Larsen *et al.*, 2010b; Lindmark-Mansson *et al.*, 2005; Moussaoui *et al.*, 2002; Wedholm *et al.*, 2008).

Although only small peptides of less than or equal to four amino acid residues in length were targeted in this study, there were 112 peptides that showed changes across time points assessed in the course of IMC study. The peptide with greatest fold change was Leu-Ala-Gln followed by Ala-His and Ala-Leu-Ser-Ser, all of these showed an increase in intensity from 0 h.

Asp-Cys-Ser-Tyr was present in healthy samples (0 h) and showed a trend A (lowest point at 81 h and almost restored to control levels at 312 h). Other peptides that showed a similar pattern include Ile-Phe-Thr-Pro, Asp-Phe-Cys-Pro and Leu-Lys-Asp, Asp-Cys-Ser-Tyr and Leu-Leu-Val.

Irregular fluctuations between time points were observed for most of the peptides that were initially present at 0 h. Some however, for example, Ile-Val,

Val-Gly-Pro and Cys-Lys-Pro-Pro distinctly increased with progress of infection, and decreased again by the resolution time.

The peptide most significantly different from 0 h was Glu-Met-Phe-His (at 81 h), and this peptide still remained significantly low at 312 h. In Mansor (2012)'s study, S. *aureus* milk also had a high peptide profile; however, the most abundant peptides observed were Gln-Ser-Ser, Leu-Asn-Tyr and Leu-Thr. This was different from the observation in the present study. This difference in peptide profiles of mastitis milk may indicate that pathogens could be crucial in the proteolysis of milk proteins during mastitis. Different varieties of proteases existing in different organisms (pathogens) may explain the varying specificities of cleavage sites on the proteins, hence the detection of different high intensity (small) peptides in milk from mastitis by different pathogens. Although studies by Dufour *et al.*(2009) was not able to attribute mastitis related caseinolysis, to the proteases of a mammopathogenic *E. coli* P4 strain, several other studies have suggested the role of bacterial proteases (Andrew, 1983; Grieve and Kitchen, 1985; Haddadi *et al.*, 2005; Johansson *et al.*, 2013). Moreover, in the study of Weldholm *et al.* (2008), several peptides with unknown enzyme specific cleavage sites were identified, and could probably result from bacterial protease cleavage. On the other hand, peptide variation in mastitis milk may be due to stage of the infection or as a result of the variation in level of SCC in the course of an infection of the mammary gland, as somatic cells are a major source of proteases which act on milk proteins. Weldholm *et al.* (2008) studied the peptides in milk resulting at different levels of SCC and observed that different proteases dominated in activity at different levels of SCC.

The finding that 39 peptides were present in milk during infection as observed in this metabolomic study, confirms the high level of milk protein proteolysis that occurs during an IMI. Out of these peptides, 22 had completely disappeared again at the resolution (312 h). All these mastitis related peptides showed a trend C and declined or were absent at 312 h. When the entire experimental data was assessed, of all compounds which were not detected at 0h (absent from controls), the most significantly enhanced in intensity was the tri-peptide Leu-Ala-Gln. These findings corroborated the observation of Mansor (2012), where

metabolites showing the largest variation from control time were also found to be peptides.

Whilst there is a general increase in peptide levels in milk during mastitis due to the increased presence and activity of proteolytic enzymes, there was an observation, in this study, that some peptides increased with mastitis (trend C) while others decreased (trend A). This may be explained by the fact that after the rapid hydrolysis of milk proteins to yield small peptides, some of these peptides become used by the bacteria as a source of substrates for essential life processes. They undergo further hydrolysis to yield AAs that become incorporated into the bacteria's metabolic pathways (Kitt and Leigh, 1997), while other peptides are not. The choice of peptides removed by bacterial metabolism may be based on the specificity of the sequence of the peptides for the cleavage sites peculiar to proteases of the bacteria involved. Therefore, other peptides (products of milk proteins hydrolysis) which do not contain the specific cleavage sequences remain abundant in the mastitic milk.

In a study by Varhimo *et al.* (2011), mastitis causing *Strep Uberis* were shown to utilize proteolytic break down products of α-caseins and ß-caseins for biofilm formation. These biofilms were reported to enhance the survival of these pathogens, thus facilitating persistent infections by these bacteria. Proteolysis of the milk proteins were also described to be enhanced by the bacterial proteases.

25.2.3.3 Lipids and related compounds metabolism

Lipids are a very important constituent of bovine milk that play a crucial role of supplying the new-born with energy. The various classes of lipids that can be found in bovine milk include; triacylglycerols, diacylglycerols, monoacylglycerols, free (non-esterified) fatty acids, phospholipids and sterols (MacGibbon and Taylor, 2006). Mastitis is associated with increased lipolysis (Ma *et al.*, 2000) and hence an increased level of free fatty acids in milk (Hunt *et al.*, 2013). Increased fat content has been shown to be associated with mammary infections (Bruckmaier *et al.*, 2004; Ogola *et al.*, 2007; Shuster *et al.*, 1991). Different classes of lipids were identified in this experiment. They were classified as lipid metabolism compounds (14), fatty acyls (46), glycerolipids (2),

glycerophospholipids (19), flavonoids (4), prenols (2), sphingolipids (10, with more decreasing) and sterol lipids (7). Except for the flavonoid and glycerolipids subclass which showed a clear trend A, decreasing with infection, other classes of lipids did not show a clear course among the metabolites, although more sphingolipids tended to decrease with infection.

Chloroform-methanol sample extraction used is a standard method in lipid biochemistry called Folch's method which may significantly affect the lipid constituents of samples (Karl Burgess, pers. comm; Prasad and Ferenci, 2003), therefore the overall interpretation of results of this metabolomics study is subjective to the effects of this extraction. It is difficult to draw a conclusion on the general trend of fat content during this mastitis challenge; however more of the lipids were reduced with time from 0 h levels than increased. Notwithstanding the glycerophospholipids group had a slightly higher number of compounds with intensity increasing as infection progressed.

Based on the overall pattern observed for the lipid pathway, it can be suggested that mastitis causes a reduction in the fat content of milk, although variant views exist on the effect of mastitis on fat content of bovine milk (Malek dos Reis *et al.,* 2013).

Closely related to the controversy on the fat content of milk during mastitis, Hunt *et al*. (2013) observed an increase in the free fatty acid contents of milk from human mastitis compared to that of healthy ones, suggesting the effect of mastitis was only on lipolysis and not on lipid synthesis in milk.

Lipoprotein lipase (LPL) is the major lipase in milk that is involved in milk fat synthesis in the mammary gland, but may be transferred into milk where it causes the hydrolysis of milk fat, leading to the release of free fatty acids and triglycerides (Deeth, 2006). It has also been suggested that high NEFA that occur in mastitic milk samples are a result of disruption of the milk fat biosynthesis pathways due to inflammatory changes and not just as a result of increased spontaneous lipolysis (Deeth, 2006). Bacterial lipases have also been suggested to play a role in the increase of FA into milk.

Apart from fatty acyls, 46 lipid metabolites were found in the milk samples. Among these 6 were short-chain FA, 22 medium-chains, 15 long-chains and 3

very-long-chains FA. In the study of Mansor, 2012, high amounts of long-chain FA were observed, conflicting with the reports of Kisza & Batura (1959) and Randolph & Erwin (1974) that long chains C:18 were only present in early lactation milk and not in mastitis. In the present study, long-chain FA (of C:18, C:19, C:20 and C:21) as well as very-long-chain FA (lengths of C:22 and C:23) were identified. 10 out of the 46 FA were unsaturated. Out of these, 11 showed a distinguishable pattern of increase with infection (by 42 h), while the remainder had levels that varied from slight to profound reduction with infection time or minor irregular fluctuations around the basal levels.

There was not a clear trend of variation in levels, unique to the FA type (chain length or saturation number) with course of infection, although studies by Massart-Leën *et al*. (1994) showed a trend of the long-chain FA decreasing with mastitis. In the study of (Atroshi *et al*., 1989), both short and long chain FA were seen to be increased in milk during mastitis.

Furthermore reports by Murphy *et al*. (1989) and Mansor (2012), suggest that there is an increase in milk FA during mastitis. The significance of an increase in fatty acids in mastitis milk could be beneficial to the immunity of the host since fatty acids have an established role as precursors to eicosanoids that have significant roles in inflammatory response, thus it would be expected that their levels increase as infection progresses (Atroshi *et al*., 1989; Mansor, 2012).

Sources of increased FA during mastitis could be through the action of bile salts lipases as well as lipoprotein lipases (LPL) which are released from blood into milk (Jensen and Pitas, 1976). Fatty acyls are broken down to FAs and this can be reflected as increased FA in milk during infection. FA are also rapidly mobilized from tissues during episodes of negative energy balance (NEB) and the increase of non-esterified fatty acids (NEFA) in blood in late term has been associated with a greater incidence of developing mastitis at the periparturient period (Moyes *et al.,* 2009b). It has also been suggested that FA increase during inflammation as a result of lipid peroxidation by free radicals which are released to kill bacteria (Atroshi *et al*., 1989).

Three metabolites in the general lipid metabolism pathway were found to be totally absent at the 0 h, but appear and increase greatly in intensity as the

infection peaked, these were; Taurodeoxycholate (two isomers; Tauroursodeoxycholic acid and a sterol lipid N-(3alpha,7alpha-dihydroxy-5beta-cholan-24-oyl)-taurine) which plays a role in bile acid biosynthesis, the fatty acid ; O-Palmitoyl-R-carnitine and a phosphoethanolamine [PE (18:1/18:1)] 1-(1Z-octadecenyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine. All these complied with a trend C. While Taurodeoxycholate peaked at 57 h, the remaining two compounds did so at 81 h. Phosphoethanolamine (18:1/18:1); 1-(1Z-octadecenyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine was the most significantly increased newly appearing lipid metabolite at time (81 h) a glycerophospholipid involved in the glycerophospho-ethanolamine pathway, detected in negative ionisation mode.

This increase in the intermediates of bile acid biosynthesis is consistent with the expectation of the increase in the activity of bile salts leading to increases in lipolysis, as the presence of the bile salts is needed to activate the lipases (Wang *et al.*, 1999). Although it has been established that bile salt activated lipases are present in human milk as well as milk from a number of other mammals (Wang *et al.*, 1999), it has not been shown to be secreted into the colostrum and early post-partum milk of cows. Also taurine, which was present at 0 h, increased significantly with infection and peaked at 57 h.

At all the time points evaluated, the most significantly reduced compound in the lipid pathway was choline phosphate, its greatest reduction occurred at 81 h. It also showed a trend A, restoring back to normal by 312 h.

N-Heptanoylhomoserine lactone a fatty amine is another metabolite displaying this trend although its biological importance in milk during mastitis is not ascertained. What is known about it is that it can be produced by a bacterium *Erwinia psidii* and other gram negative endobacteria for bacterial cell-to-cell communication commonly referred to as quorum sensing (Kai *et al.*, 2012). The earliest (36 h) most significant changes in lipid metabolism occurred with [FA hydroxy (10:0)] N-(3S-hydroxydecanoyl)-L-serine-N-(3-Oxooctanoyl) homoserine lactone another compound which changed significantly early in the infection also followed a similar trend as the homoserine lactone above. This compound is also found in bacteria (*A. tumefaciens),* where it acts as a transcription promoter (Cayman Chemical, 2012). Phospholipids, regardless of the class, generally

showed a trend of decreasing with infection as would be expected since they are constituents of membranes which are destroyed during mastitis and acted upon by phospholipases and carboxylesterases. Sn-glycero-3-Phosphocholine, the lipid metabolite with the highest intensity, showed a decreasing trend with infection and rises almost back to control level at infection resolved. Hexadecanoic acid, the next highest intensity on the other hand showed a trend of increasing, peaking at 57 h and dropping to similar levels of 0 h by 312 h. In the study of Klein *et al*. (2012), the ratio of glycerophosphocholine to phosphocholine in the first few weeks of lactation was suggested to be indicative of metabolic status, while high glycerophosphocholine was said to be indicative of healthy animals at mid lactation. The cows used in this challenge study were mid-lactation cows and from the metabolomics experiment, glycerol- phosphocholine was found to have very high intensity at control (healthy) time. It decreased with progress of infection, and by resolution time, it had risen back almost to levels recorded at 0 h. This observation supports the finding that high glycerophosphocholine levels may be indicative of healthy animals.

In this study phosphocholine (identified as choline phosphate) was seen decreasing (by the most fold change) with mastitis and becoming totally absent at 57 h and 81 h, then showing reappearance of minimal relative amounts at 312h. Free choline itself, which is a precursor of most of the membrane phospholipids highlighted above, was observed with high levels at controls but reduced very significantly with infection, in the present study. It has previously been demonstrated that choline levels are fairly constant in milk throughout lactation (Zeisel *et al*., 1986).

On the other hand, a greater number of all (glycerol) phospholipids (PL) metabolites showed a tendency of decreasing with infection. This is an expected finding as damage to the cell membrane of mammary cells as well as milk fat globule membrane (MFGM) would cause a release of membrane structural constituents, chiefly phospholipids into milk during inflammation. Also the activity of phospholipases have been shown to increase during mastitis (Fitz-Gerald *et al*., 1981) however these findings did not agree with observations of Hunt *et al*. (2013), of no change in PL levels in mastitis as compared to non-mastitic human mammary glands.

In this study, eicosanoic acid (C: 20) was identified and observed to increase with infection, and this is the precursor of the eicosanoids that include thromboxanes (TXA), prostacyclins (PGI), prostaglandins (PG), leukotrienes (LT) and lipoxins (LX) which play role in signalling of inflammatory mediators, and may have arisen as a result of the increase in the activity of phospholipases that release arachidonic acids from acylglycerols. The increase in Eicosanoids in mastitis is generally accepted and regulation of this increase by Selenium has been demonstrated (Maddox *et al.*, 1991)

Leukotriene B4 (LTB4) was identified in the KEGG map under the arachidonic acid pathway with a formula of C20H32O5. This is similar to the chemical formula of many other metabolites under the eicosanoid group. The metabolites were identified with 48 different isomers all of which fall under the class of eicosanoids. These compounds had a trend of increasing in intensity from control levels as infection progressed (Trend B). The specific role of LTB4 in inflammation is the recruitment of leucocytes to sites of inflammation (Maddox *et al.*, 1991) and has been attributed the most potent endogenous chemoattractant (Bannerman, 2009). It also plays a significant role in activation and degranulation of PMN cells. The increased concentration of LTB4 is thus in accordance with the need for the influx into the mammary gland of PMN cells, where they are classified as somatic cells for the SCC, in order to kill invading bacteria during mastitis. In a closely related observation, metabolites of linoleic acid metabolism were increased during the peak periods of infection although not significantly, in this study.

From the studies of Sundekilde *et al.* (2013), other major metabolites that had significant correlations with high SCC included, lactate, acetate, butyrate, ß-hydroxybutyric acid (BHBA) and isoleucine. In the present study D-2-hydroxybutyrate was the closest compound in chemical formula to BHBA that was identified and it showed an increasing trend that was significant at 57 h ($P<0.01$) and 81 h ($P<0.05$). (s)-2-aminobutanoate (α-aminobutyric acid) showed a significant increase with infection with peak significant level at 57 h, and dropping back to normal at 312 h. Breed has been shown to be a possible cause of variation in its levels in milk (Klein *et al.*, 2012).

25.2.3.4 Nucleotide metabolism

Most of the nucleotide metabolites detected in this study showed trend A. Only 3 compounds that were not present in normal (control) samples appeared following infection, 7-Methyladenine, guanosine and inosine, reaching maximum levels at 42 h, 57 h and 57 h respectively. 7-Methyladenine is involved in the base excision repair pathway and its increase during mastitis may be related to the destruction of cellular and nuclear membranes with the release of intracellular and nuclear contents.

Uracil-5-carboxylate (orotate) was the nucleotide metabolite with the highest peak intensity and it showed a trend A (decrease with infection progress with lowest point at 81 h, and rising at 312 h). The presence of orotate in bovine milk at levels of 30-70 µg/ml has been previously reported (Indyk and Woollard, 2004), although it is absent in non-ruminant milk. In the study of Melzer *et al*. (2013), uracil, a related compound to orotate, was found to be highly correlated to the milk trait, SCC, the major indicator of mastitis in milk. In the present study, however, uracil was not identified as such, only the carboxylated form (orotate) was detected.

A closely related compound to uracil, cytosine changed the most of all nucleotides and was seen to follow a typical trend A also, with a lowest intensity at 57 h and was very significantly different from control levels at all time points except 312 h. It also showed the greatest change earliest in this metabolic pathway. Nucleotides are normal constituents of milk (Schlimme *et al*., 2000). Bovine milk typically has less nucleotides than that found in human milk hence the hence the practice of supplementing calf-infant formula with nucleotides and nucleosides (Gill *et al*., 2010; Ren *et al*., 2011). Therefore it can be said that mastitis further disrupts the nutritional value of bovine milk by causing a depletion of the major nucleotides.

Urate, a breakdown product of purine metabolism also involved in microbial metabolism and bile secretion was found showing a trend A during the challenge.

25.2.3.5 Vitamins and co-factors

Vitamins and co-factors play a very crucial role in mammalian metabolism. Nicotinamide followed close to a trend A, but still remained very low at 312 h compared to 0 h. The implication of this is that its response to resolution is not rapid enough for it to be a good marker of infection resolution. The decrease in nicotinamide levels in milk as the infection advanced may be due to the conversion of nicotinamide to nicotinic acid by activities of enzyme nicotinamidase. This enzyme is present in many bacteria including Streptococcus species and is a major regulator of intracellular nicotinamide concentrations in prokaryotic organisms (French *et al.*, 2010). Nicotinamide is a precursor to nicotinamide adenine dinucleotide (NAD^+) and its phosphorylated and reduced forms (NADP and NADH) which are central to energy production metabolic pathways.

Dethiobiotin, a high intensity metabolite, showed a trend B and peaked at 57 h, although increases were mostly not statistically significant. It is an intermediate in biotin metabolism, detected in the positive ionisation mode. Its increase during infection may relate to proteolysis of biotin synthetase which catalyses the conversion of dethiobiotin to biotin, resulting from bacterial damage to mammary gland cells and the action of neutrophilic proteases.

Pyridoxal, nicotinamide, 3-Hydroxy-2-methylpyridine-4,5-dicarboxylate and then thiamine were the most significantly changed (fold decrease) vitamin related metabolites after time 0 at 81 h. Thiamine is a water soluble vitamin obtained from grains and other plant sources of feed and involved in the catabolism of sugars and amino acids as well as the biosynthesis of neurotransmitters acetylcholine and gamma amino butyric acid (GABA).

Thiamine is normally secreted into milk as a means of excretion of excess quantities obtained from the cows' diet, explaining its high intensity in healthy milk samples; it is also found in high concentration in colostrum (Echols *et al.*, 1986; Kehoe *et al.*, 2007). The decreasing quantities observed during the peak of infection in this analysis, may be due to the presence of thiaminase factors associated with bacterial causing mastitis. The implication of this is that milk samples from mastitic udders are less able to supply nutritional thiamine. Rumen

bacteria have been suggested to be able to produce thiaminase factors that could affect the effects of dietary supplementation with thiamine on milk production (Shaver and Bal, 2000). 1-Methylpyrrolinium was the only significantly up regulated metabolite belonging to this pathway and the most fold increase occurred at 57 h. The majority of metabolites in the vitamin and co factors pathway displayed an overall D trend, fluctuating irregularly between control and resolution time, but with more tendency towards a trend A, implying that the vitamin nutritional composition of milk during mastitis is profoundly compromised (by their reduced presence in milk).

25.2.3.6 Minor pathways

Puromycin, Dihydroclavaminic acid and Taxa-4(20),11(12)-dien-5alpha-yl acetate were the only metabolites of biosynthesis of secondary metabolites that increased with time of infection peaking at 81 h (trend B) and dropping significantly by 312 h. Overall, in the category of all other metabolites, puromycin was the most increased in folds.

Furcatin was one of the metabolites that decreased significantly at 57 h, following a trend A and had the greatest level of significance in difference. Alpha-D-Galactosyl-1,3-beta-D-galactosyl-1,4-N-acetyl-D-glucosamine and 3-Dehydrocarnitine were the two most significantly up-regulated metabolites in terms of fold increase, both highest at 81 h. Phthalate was the only metabolite of xenobiotic degradation that was identified changing across time points. It is involved in Toluene degradation pathway.

25.3 Conclusion

In this chapter, the potentials of analysis of the milk metabolome as it changes during an experimental *S. uberis* mastitis challenge is investigated with the aim of recognizing new biomarker candidates for bovine mastitis and for understanding mastitis induced metabolic change. Numerous compounds were found to vary in milk in the presence of and during different time points of infection. While confirming previous reports on possible markers of mastitis such as lactose, lactate and hydroybutyrate, new metabolites showing a high correlation in trend with mastitis course and having high intensity such as

betaine, L-carnitine, sn-glycerophosphocholine and choline were identified and are suggested for further scrutiny and validation in relation to mastitis diagnosis. Others such as 7-methlyadenine and tauroxycholate, compounds otherwise not detected in normal milk, also project a possible potential (as newly appearing compounds) that could be used to detect and monitor the course of mastitis.

In addition, small peptides such as Leu-Ala-Gln, Ala-His and Ala-Leu-Ser-Ser may also be useful in understanding the proteolytic changes in milk during mastitis and show promise in being able to distinguish *S. uberis* mastitis from mastitis caused by other pathogens, thus such small peptides may be explored for ability to differentiate various causative organisms.

26 General Discussion

26.1 Milk acute phase proteins in the diagnosis of bovine mastitis

A major setback in the management of mastitis in dairy herds is the lack of adequate and sensitive measure(s) of the presence of mastitis in milk, which can be tested by a rapid on-farm format discussed previously (Chapter 1). Somatic cell counts remain the most widely used parameter for predicting the mastitis status of cows in dairy farms globally. However, pertinent problems continue to arise with the use of this measure and others such as the enzymes NAGase, LDH and electrical conductivity for diagnosis and monitoring of mastitis. The economic impact of bovine mastitis is growing, and with new technological innovations in dairy management, an added challenge is posed to the prompt recognition of IMIs by the increasingly popular use of automatic milking systems in dairy farming. There is a growing need for more sensitive and reliable markers of mastitis.

Since the first reports of the detection of the major bovine APP in milk over a decade ago (Eckersall *et al.*, 2001), a number of studies have come up to determine the potential for use of these APP for mastitis detection, not just as a laboratory measure, but also to have a on farm rapid format application. In the studies carried out in this PhD research, the usefulness of two major bovine APP (Hp and M-SAA3) and one other APP hitherto considered minor in bovines (CRP) was assessed in relation to diagnosing mastitis. Several types of samples ranging from quarter and composite milk from a herd of newly calved cows, natural infections of mastitis from several dairy farms and from experimental mastitis challenge on otherwise healthy udders were used in the assessment of these APP.

First, the development of an assay to measure APP was instigated and resulted in the successful development of an ELISA for measuring milk Hp. Several attempts were made to develop an ELISA for M-SAA3, these were however not successful, so a commercial kit was used for its measurement.

In order to further validate the Hp assay and determine the reference range of these APP in milk in a field condition, the profile of Hp, M-SAA3 and CRP in healthy and mastitis milk in relation to the SCC and in the periparturient period milk samples were explored in a dairy herd. The Cochno farm dairy herd (University of Glasgow) was used for this study. It was not however possible to obtain reference values for M-SAA3 due to many of the samples having undetectable amounts of M-SAA3 even at low dilutions (1:50) using the commercial assay available.

In addition, sets of samples submitted to a veterinary diagnostic laboratory for mastitis diagnosis, from several farms across Scotland and from an experimental model of mastitis were examined for APP. The results of these studies indicated that these APP in milk can reliably be used to discriminate healthy from clinical mastitis milk samples and subclinical mastitis from healthy. However some level of variability was observed between the three APP in bovine milk in relation to IMI, with Hp proving more specific to indicate mastitis from a wider variety of pathogens than M-SAA3. However, M-SAA3 showed higher response to a mammary inflammation (reaching a higher peak with peak of infection) during an experimental model mastitis study. Also it was observed that CRP tended to rise earlier than other APP during the course of the experimental mastitis, signifying a possible higher sensitivity of CRP than Hp and M-SAA3. This observation requires further validation using different models of mastitis and pathogens. It was also determined that Hp correlated more to SCC than M-SAA3 and CRP in samples of both healthy and mastitis milk, this presumably due to their (Hp and SCC) common source of origin in the neutrophils/somatic cells (Hiss *et al.*, 2004; Lai *et al.*, 2009).

Bovine CRP has been known as a minor APP due to its characteristics in serum of having very minimal variation in response to inflammation or infections (Eckersall and Conner, 1988), although, a few reports have pointed to a possible usefulness of CRP for mastitis recognition (Schrodl *et al.*, 1995; Kruger and Neumann, 1999 and Lee *et al.*, 2003). In this thesis, it was found that CRP concentration in milk varied considerably with presence of mastitis and even began to rise slightly earlier than the two major APP during the course of an experimental infection. Further studies are required to ascertain the origin and

detailed dynamics of CRP in bovine milk and the mammary gland under physiological and pathological conditions.

All three APP proved sensitive enough to indicate the presence of mammary infection but showed variable levels with SCC lower than 200,000 cells/ml and with some minor or mild mastitis causing pathogens such as CNS.

In further studies the properties of Hp including its localization in the bovine mammary gland was examined. Since no functional antibody for M-SAA3 was available for IHC or western blotting, similar studies were not possible for M-SAA3. However, results of the Hp characterization studies confirm that Hp is synthesized in neutrophils and MEC and possibly undergoes PTMs that gives Hp a range of pi.

From the findings in these studies, it is recommended that these APP should be assayed or assessed together to complement each other in making a diagnosis or prognosis for mastitis, as host response or pathogen virulence factors have the ability to alter trends in any specific APP response under different disease models. Reference values for Hp in milk using SCC cut off value of 200,000 cells/ml was established using the Hp profile of composite milk samples of a dairy herd (Chapter 2) and it was possible to use this reference cut off of Hp concentration to determine the acute phase response level/stage in the immediate post calving milk (Chapter 3), in natural mastitis milk samples (Chapter 4) and samples from an experimental mastitis challenge (Chapter 5). The results presented in this thesis have lent further credence to the potential usefulness of Hp and M-SAA3 in bovine mastitis diagnosis. It can be concluded that these APP have the specificity, sensitivity and precision to be used for diagnosis of bovine mastitis in milk, either alone or in combination with other traditional indicators of mastitis in milk.

26.2 Milk proteomics in the diagnosis of bovine mastitis

Proteomics has developed as a promising tool for the identification of disease biomarkers in recent years, thanks to advances in mass spectrometry and bioinformatics (reviewed in Chapter 1 and Chapter of 5). This necessitated the use of the proteomics platform in order to explore potential biomarkers of

mastitis in both natural and experimental models of the disease. Whilst gel based proteomics approaches (1DE, 2DE and DiGE) offered insight into quantitative variation of mostly high abundance proteins present in healthy and mastitis milk and also gave fractionation advantage to milk proteomics, characterization of mastitis discriminatory proteins was only possible using mass spectrometry.

Other fractionation methods employed in the study of milk proteins included a non-gel liquid phase isoelectric focusing which yielded a protein separation with an advantage of better resolution of lower molecular weight proteins away from the isoelectric point of the higher abundance milk proteins (Chapter 4). Overall, a few of the important proteins that became highlighted as potential biomarkers of mastitis from the proteomics studies of milk in this research included serpins, serotransferrin, NGAL, apolipoproteins and complement C3. Most of these proteins have already been identified in previous milk proteomic studies and would require further validation studies before becoming useful in detecting mastitis.

Going further into an area commonly considered a sub-field of proteomics, peptidomic analysis on bovine milk was able to offer more insight into the common proteolytic changes that occurs in milk, specifically during a *S. uberis* mastitis challenge model. Being an experimental model of mastitis, an added advantage of time point based changes in the milk peptidome was gained. Noteworthy peptidomic findings included the identification of 77 polypeptides which significantly (P=0.01) differentiated pre-infection samples from infected ones. Hence they represent a panel of peptides that can be considered as biomarkers of mastitis. Three to four of these polypeptides showed a possibility to be general polypeptide markers of mastitis, having been identified in previous peptidomic studies of milk from mastitis of different pathogen origin. It is possible that in the not too far distant future, the cost of MS could come down and mastitis diagnosis could use this technology on a routine basis especially if differing pathogen infection causes different patterns of peptides.

26.3 Milk metabolomics in the diagnosis of bovine mastitis

Metabolomics aims to quantitatively and qualitatively identify all small metabolites within a given biological sample. This was the aim in the study of milk metabolomics during the course of an *S. uberis* mastitis which was investigated in this research. As would be expected, a lot of significant differences in metabolite profiles were observed across the different time points of the mastitis challenge. More prominent changes were seen in the small peptides (up to four amino acid residues), carbohydrate, vitamins and co-factor metabolites composition across infection times. Numerous metabolites with unassigned metabolic pathways also showed wide deviations at the peak of mastitis, from pre-infection and resolution phase.

Numerous compounds were found to vary in milk in the presence of and during different time points of infection. While confirming previous reports on possible markers of mastitis such as lactose, lactate and hydroxybutyrate, new metabolites showed a high correlation with the course of mastitis and had high intensity. Thus betaine, L-carnitine, sn-glycerophosphocholine and choline were identified and are suggested for further scrutiny and validation in relation to mastitis diagnosis. Others such as 7-methlyadenine and tauroxycholate, which are compounds otherwise not detected in normal milk, also point to possible markers (as newly appearing compounds) that could be used to detect and monitor the course of mastitis. In addition, small peptides such as Leu-Ala-Gln, Ala-His and Ala-Leu-Ser-Ser may also be useful in understanding the proteolytic changes in milk during mastitis and show promise in being able to distinguish *S. uberis* mastitis from mastitis caused by other pathogens, thus such small peptides may be explored for ability to differentiate various causative organisms.

26.4 General conclusions and future direction

In this thesis, biomarker candidates for bovine mastitis in milk have been explored using the tools of immunoassays of acute phase proteins, proteomics, and metabolomics. The findings from this research form a platform from which future studies for potential biomarkers of bovine mastitis can be launched.

The use of APP as mastitis biomarkers would require their adaptation to rapid measurement formats. Other identified proteins, peptides and small metabolites need to be further experimentally and clinically validated as specific and sensitive markers of mastitis and would also require measurement (on farm) in rapid and reliable formats.

Appendices

Appendix Chapter 2

Table 1: M-SAA3 concentrations in two QC samples in 5 different assays.
This was used to determine the inter assay precision of the SAA ELISA; Mean % CV=33 %

	M-SAA3 (µg/ml)	
Assay	**High QC**	**Low QC**
1	1169.00	16.00
2	625.00	5.40
3	1122.00	6.20
4	1072.50	8.76
5	1101.65	9.34
Mean	**1018.03**	**9.14**
SD	**222.50**	**4.18**
CV	**0.22**	**0.46**

Table 2: CRP ELISA repeats of two quality control samples in 5 different assays.
This was used to determine the inter-assay precision (CV) to be 7 %.

Test	Low QC	High QC
Plate 1	1.90	6.80
Plate 2	2.00	8.50
Plate 3	2.18	8.66
Plate 4	2.04	8.33
Plate 5	2.20	7.13
Mean	2.06	7.88
SD	0.11	0.76
CV	0.05	0.09
	Mean CV	**0.07**

Appendix Chapter 3

Day 1-10 post calving milk 1DE MS analysis results (Henderson, 2013, MRes. Dissertation, University of Glasgow)

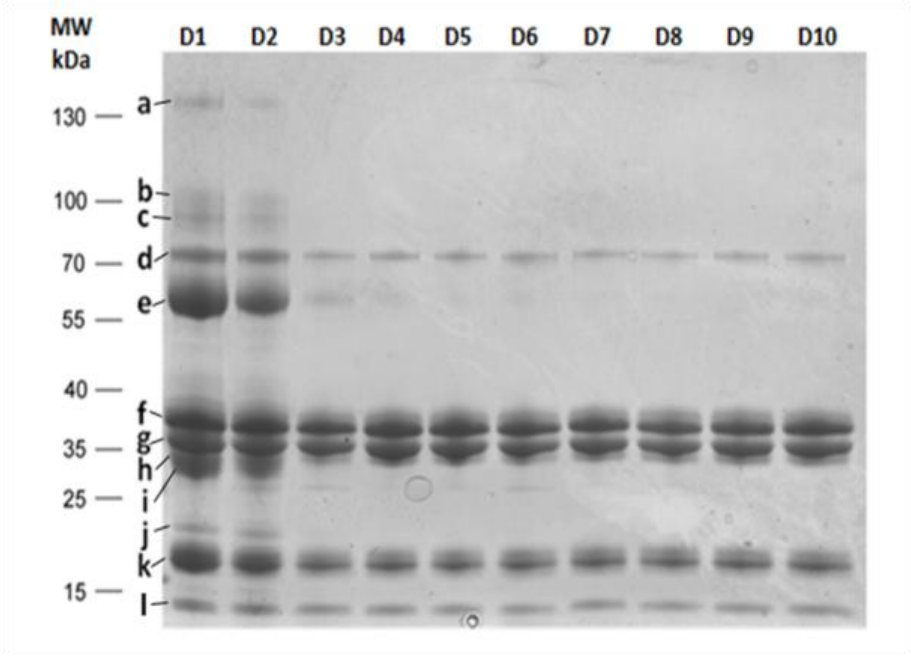

Figure 1: 1-D SDS PAGE Coomasie stained gel of bovine colostrum and milk for Cow 1, day 1-10.
Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity). Letters running horizontally across the top indicate the day number (Day 1 to Day 10), and letters running vertically label the specific bands that correspond with Table 3.

Table 3: Proteins found in bovine colostrum and milk in both healthy and unhealthy cows, with corresponding letters to those of Figures 1. Expected Molecular Weights (MW) are taken from Malcata, 2007. Observed MWs are measured from 1-D SDS PAGE gels. LC=Light Chain, HC = Heavy Chain.
Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity).

Band ID letter	Expected MW (kDa)	Observed MW (kDa)	Protein Name	Column1
a	25 (each LC) + 50-70 (each	130.0-150.0	Immunoglobulins (A, M and	
b	25 (each LC) + 50-70 (each	82	Immunoglobulins (A, M and	
c	80	78.0-80.0	Lactoferrin	
d	66.2	70.0 - 72.0	Bovine Serum Albumin	
e	25 (each LC) + 50-70 (each	55.0 - 57.0	Immunoglobulin (G)	
f	22.1 (α-s1) – 25.2 (α-s2)	35.0 - 37.0	Alpha-casein	
g	24	33.0 - 36.0	Beta-casein	
h	19	29.0 - 34.0	Kappa-casein	
i	25 (each LC) + 50-70 (each	25.0 -27.0	Immunoglobulins (G)	
j	25 (each LC) + 50-70 (each	20.0 - 22.0	Immunoglobulins (A, M and	
k	18.2	18	Beta-lactoglobulin	
l	14.2	12.0 - 14.0	Alpha-lactalbumin	

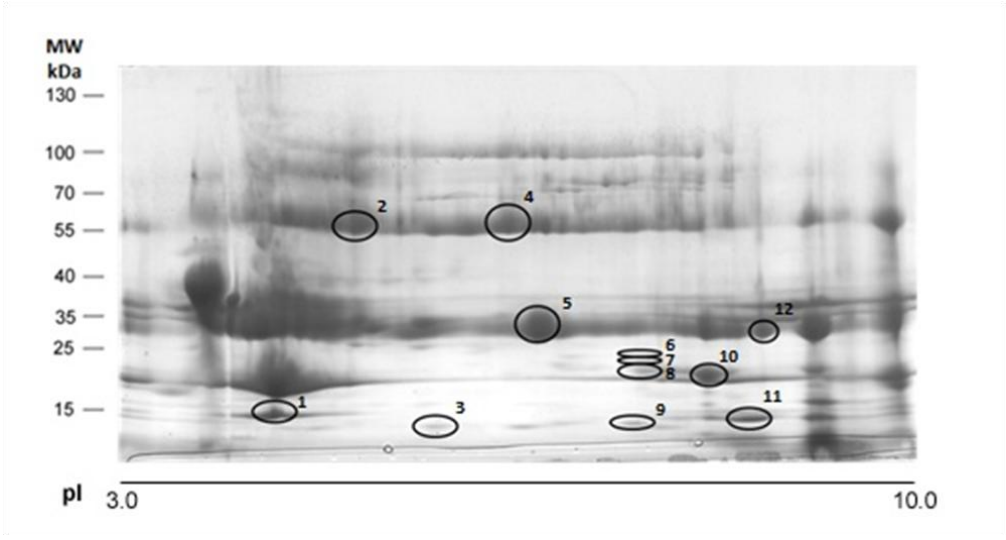

Figure 2: 2 dimensional electrophoresis gel for Day 1 (colostrum) sample.
Protein spots chosen for LC-MS/MS are circled and numbered, with numbers corresponding to Table 4. Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity).

Table 4: LC-MS/MS results for Day 1 bovine colostrum protein spots 2DE gel, with spot number corresponding to Figure 2.
Spot number, protein name from Mascot database, Mass, isoelectric point (pi), score, sequence (Sqnce), protein sequence coverage (PSC) and emPAT are listed. Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity).

Spot	Protein Name	Mass	pI	Score	Sqnce	PSC	emPAT
1	Alpha-lactalbumin	14603	4.8	580	11(11)	0.6	41.91
	Chain A, 12-Bromododecanoic Acid	18641	4.76	371	13(5)	-	6.35
2	Immunoglobulin heavy chain	51391	6.1	643	12(9)	0.24	1.39
	Alpha-S1-casein precursor	24570	4.98	229	8(5)	0.39	2.16
	Immunoglobulin gamma-2 chain C	36590	8.04	191	10(2)	0.33	1
	Alpha-1-antiproteinase precursor	46417	6.05	190	9(4)	0.19	0.51
3	Alpha-S1-casein precursor	24570	4.98	124	8(4)	-	0.89
	Immunoglobulin lambda locus	24910	5.84	116	5(4)	0.22	0.88
	Immunoglobulin light chain, lambda	24863	7.53	103	4(3)	0.2	0.66
4	Immunoglobulin heavy chain	51391	6.1	631	14(9)	0.34	1.54
	Alpha-S1-casein precursor	24570	4.98	182	8(4)	-	1.45
	Glycoprotein antigen MGP57/53,	45704	7.1	140	5(3)	0.13	0.32
5	Immunoglobulin lambda light chain	25032	5.84	2020	9(7)	0.31	2.49
	Unknown (protein for MGC:159378)	25059	7.52	1999	13(9)	0.53	4.08
	Immunoglobulin light chain, lambda	24863	7.53	1986	11(8)	0.46	3
6	Chain A, Bovine Beta-Lactoglobulin,	18583	4.83	180	7(5)	0.38	3.5
	Beta-lactoglobulin	18641	4.76	168	7(5)	0.38	3.46
7	Chain A, 12-Bromododecanoic Acid	18641	4.76	501	14(8)	-	9.24
	Chain A, Bovine Beta-Lactoglobulin,	18583	4.83	501	13(8)	0.64	7.78
	Beta-lactoglobulin	18641	4.76	457	14(8)	0.64	9.24
8	Chain A, 12-Bromododecanoic Acid	18641	4.76	485	13(9)	-	7.67
	Beta-lactoglobulin	18641	4.76	470	13(9)	0.61	7.67
9	Chain A, Bovine Beta-Lactoglobulin,	18583	4.83	276	11(5)	0.54	3.5

Spot	Protein Name	Mass	pI	Score	Sqnce	PSC	emPAT
	Beta-lactoglobulin	18641	4.76	265	11(5)	0.54	3.46
10	Chain A, 12-Bromododecanoic Acid	18641	4.76	772	16(10)	-	18.91
	Chain A, Bovine Beta-Lactoglobulin,	18583	4.83	757	16(9)	0.69	13.5
	Chain A, Structural And Functional	18555	4.83	742	16(8)	-	13.5
11	Chain A, Crystal Structure Of Bovine	11742	7.96	969	9(7)	0.42	9.05
	Anti-testosterone antibody	24786	7.53	501	6(3)	0.31	0.88
	Immunoglobulin lambda locus	24910	5.84	487	7(3)	0.33	1.13
12	Immunoglobulin light chain, lambda	24863	7.53	1117	10(6)	0.36	2.11
	Immunoglobulin lambda light chain	11464	8.49	1104	8(5)	0.51	7.22
	Immunoglobulin lambda light chain	25032	5.84	1019	10(6)	0.36	2.49

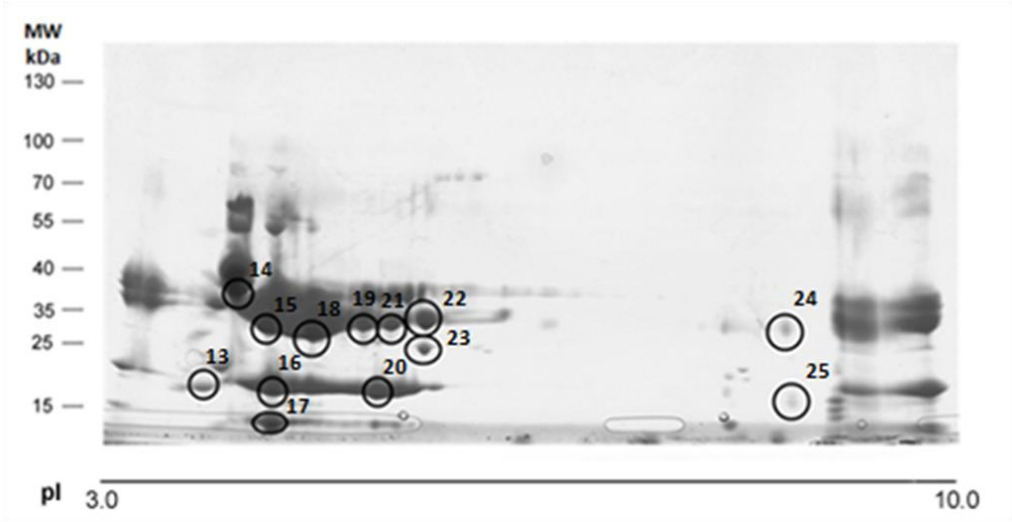

Figure 3: 2 DE reducing gel for bovine milk from Day 10.
Protein spots chosen for LC-MS/MS are circled and numbered, with numbers corresponding to Table 5. Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity).

Table 5: LC-MS/MS results for Day 10 bovine milk protein spots from a 2DE gel, with spot number corresponding to Figure 3.
Spot number, protein name from Mascot database, Mass, isoelectric point (pI), score, sequence (Sqnce), protein sequence coverage (PSC) and emPAT are listed. Adapted from Henderson C.L (2013). University of Glasgow MRes Infection and Immunobiology Project (Title; Proteomics investigation of bovine colostrum for molecules of innate immunity).

Spot	Protein Name	Mass	pI	Score	Sqnce	PSC	emPAT
13	Alpha lactalbumin	14603	4.8	1019	12(10)	0.62	51.88
	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	348	10(6)	0.46	5.29
	Beta-lactoglobulin	18641	4.76	346	10(5)	0.46	4.27
14	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	233	7(4)	0.42	2.81
	Beta-lactoglobulin	18641	4.76	207	7(4)	0.42	2.78
	Alpha-S1 casein	24477	4.85	189	4(2)	0.18	0.29
15	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	464	12(8)	0.6	5.29
	Beta-lactoglobulin	18641	4.76	452	12(8)	0.6	5.22
	Alpha S1 casein	22442	4.71	449	9(4)	0.45	3.63
16	Kappa-casein precursor	21326	6.82	933	7(6)	0.34	6.75
17	Chain A, 12-Bromododecanoic Acid Binds Inside The Calyx Of Bovine Beta-Lactoglobulin	18641	4.76	2783	15(13)	-	37.7
	Chain A, Structural And Functional Consequences Of Point Mutations Of Variants A And B Of Bovine Beta-Lactoglobulin	18555	4.83	2773	14(13)	-	32.44
18	Chain A, 12-Bromododecanoic Acid Binds Inside The Calyx Of Bovine Beta-Lactoglobulin	18641	4.76	2943	15(13)	-	26.76
	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	2869	14(11)	0.78	22.94
19	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	442	12(9)	0.58	7.78
	Beta-lactoglobulin	18641	4.76	386	12(9)	0.58	7.67
20	Kappa-casein precursor	21326	6.82	1743	8(7)	0.34	9.38
	Kappa-casein precursor	10620	5.16	1323	4(4)	0.42	28.13
	Kappa-casein	12393	8.92	701	7(5)	0.55	6.02
21	Serum albumin precursor	71274	5.82	2253	64(42)	0.85	16.07
	Albumin	68083	5.76	2073	60(38)	0.81	14.4
22	Chain A, Bovine Beta-Lactoglobulin, Lattice X	18583	4.83	125	9(3)	0.58	1.73
	Beta-lactoglobulin	18641	4.76	114	9(3)	0.58	1.71
23	Beta-casein	25148	5.26	136	7(3)	0.21	0.86
24	Immunoglobulin light chain, lambda gene cluster	24863	7.53	486	8(4)	0.33	1.42
	Immunoglobulin lambda light chain	25032	5.84	470	9(3)	0.39	1.72
	Immunoglobulin lambda light chain constant region 3	11464	8.49	414	7(4)	0.61	5.32
	Anti-testosterone antibody	24786	7.53	386	8(3)	0.34	1.43
	Immunoglobulin lambda locus	24910	5.84	384	9(4)	0.36	1.74
25	Chain A, Crystal Structure Of Bovine Beta2-Microglobulin	11742	7.96	482	9(6)	0.42	6.78
	Immunoglobulin lambda light chain constant region 3	11464	8.49	113	2(1)	0.29	0.69

Appendix Chapter 4

1) Bradford Assay:
   a) Bradford reagent; Brilliant blue G, phosphoric acid and methanol

   b) Protein standard; Bovine serum albumin at 2 mg/ml (Sigma-Aldrich, Dorset, UK), double diluted from 2 mg/ml to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml to make standards.

   c) Assay Procedure: Milk samples were diluted to 1:20 or 1:40 in milli Q water. 5 µl of diluted samples and standards were added in duplicates into wells of a Costar™ 96-well ELISA plate (Fisher scientific, UK) and 250 µl of Bradford reagent added per well. The mixture was left to stand for few minutes at RT after which absorbance were read at 595 nm using a FLUOstar Optima plate reader. A linear regression standard curve was plotted from value of standards and concentrations of proteins in each well were interpolated from the curve.

2) 1DE
   a) Laemmli Buffer (2x); was made up of 65.8 mM Tris-HCl, pH 6.8, 2.1 % SDS, 26.3 % (w/v) glycerol, 0.01 % bromophenol blue (Bio-Rad, Hemel Hempstead, UK). 2x Sample buffer was prepared by addition of 50 µl of ß-mercaptoethanol (BME) to 950 µl of 2x Laemmli buffer. Pre-diluted protein samples were further diluted 1:1 in sample buffer and heated at 95°C for 4 min on a heating block before loading onto precast gel wells.

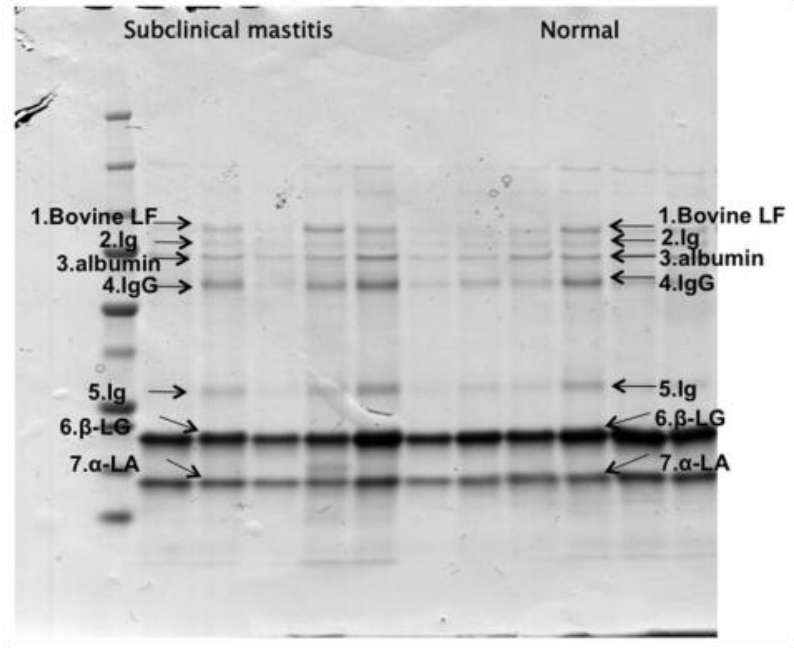

Figure 4: 1D gel electrophoresis of a subclinical mastitic and a healthy milk sample. Showing all the proteins identified by the MS based on the Mascot database
Adapted from Mansor, R (2012), University of Glasgow PhD thesis (Titled; Proteomic and Metabolomic Studies on Milk during Bovine Mastitis)

Appendix Chapter 5

1. DiGE and 2DE
    a) Lysis buffer; made up of 6 M urea, 2 M thiourea 4 % CHAPS and 25 mM Tris base.
    a) DiGE lysis buffer; 7 M urea, 2 M Thiourea, 4 % CHAPS, 25 mM Tris HCl
    b) Equilibration buffer I; pH8.8, 1.5M Tris HCl, 216.21 g urea ,180 ml glycerol 12 g SDS and 100 mg DTT
    c) Equilibration buffer II; pH8.8, 1.5 M Tris HCl, 216.21 g urea, 180 ml glycerol, 12 g SDS 280 and 250 mg iodoacetamide

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