Proteomic and Metabolomic Studies on Milk during Bovine Mastitis

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Abstract

The principal objectives of the study presented in this thesis were to study the changes of milk proteomes, peptidomes and metabolomes during the course of bovine mastitis in comparison with normal milk samples and to discover new bovine mastitis biomarkers using various modern and up-to-date methodologies such as proteomics, peptidomics and metabolomics.

Bovine mastitis caused by bacterial infection of the mammary gland of dairy cows is often associated with loss of milk production due to a reduction in milk composition and quality which in turns, lead to negative economic impact on dairy industry.

Two important acute phase proteins (APPs) which serve as valuable biomarkers in bovine mastitis were investigated in every chapter using developed and validated enzyme linked immunosorbent assay (ELISA) for bovine milk haptoglobin and commercially available ELISA for bovine milk serum amyloid A3 (M-SAA3). These APPs were quantified alongside somatic cell counts (SCC) and California Mastitis Test (CMT) to confirm the disease status of each animal used in this study.

Proteomic methodologies were applied including 1D gel electrophoresis, 2D gel electrophoresis, MALDI-TOF analysis and difference gel electrophoresis to investigate the changes of milk proteome in both subclinical and clinical mastitic milk samples in comparison with healthy milk samples. However these investigations did not reveal novel biomarkers for mastitis.

Next, peptidomic methodologies were used to study the changes in milk peptidome and to detect the presence of any significant disease biomarkers in the presence of bovine mastitis by using CE-MS and LC-MS/MS. A total of 31 and 14 polypeptides can be used to discriminate control from infected groups and *E. coli* from *S. aureus* infected groups respectively.

Lastly, metabolomic methodology was applied with an intention to study the changes in milk metabolome and ultimately to detect the presence of novel biomarkers in bovine mastitis. Di- and tri-peptides were found higher in *S. aureus* than in *E. coli* infected groups and based on metabolic pathways, arachidonic, arginine and galactose metabolites were seen increased in mastitic milk samples in comparison to healthy milk samples.

Overall, the findings detailed in this thesis indicate that the use of advanced proteomic and metabolomic methodologies could deliver on their promise of the discovery of potential significant bovine mastitis biomarkers. Further studies are needed for validation of these proposed biomarkers and it was hoped that better prevention and treatment methods for bovine mastitis can be achieved in the future.

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List of Abbreviations

%	percentage
~	approximately
£	Pound sterling
+	plus
<	less than
>	more than
°C	degree Celcius
15d-PGJ_2	15-Deoxy-Delta-12,14-prostaglandin J_2
2D	two dimensional
2DE	two dimensional electrophoresis
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
4CN	4-chloro-1-naphthol
AA	amyloid A
ABP	activity-based probe
ABS	antibody binding solution
ACE	angiotensin converting enzyme
ACN	acetonitrile
AcP	acid phosphatase
ADP	adenosine diphosphate
AGP	acid glycoprotein
Ala	alanine
AMP	adenosine monophosphate
amu	atomic mass unit
AP	alkaline phosphatase
APP	acute phase protein
APR	acute phase reaction/response
AQUA	absolute quantification
Arg	arginine

ASAT	aspartate aminotransferase
ASG	acid-soluble glycoprotein
Asp	aspartic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AUC	area under curve
BCA	bicinchoninic acid
bcSCC	Box-Cox transformed somatic cell count
BH	Benjamini-Hochberg
BHB	beta-OH butyrate
BHBA	beta hydroxybutyrate
bHp	bovine haptoglobin
BMC	buttermilk concentrate
BMSCC	bulk milk somatic cell counts
BSA	bovine serum albumin
C	complement
CATHL1	cathelicidin 1
CD	cluster of differentiation
CE	capillary electrophoresis
cells/ml	cells per millilitre
CE-MS	capillary electrophoresis mass spectrometry
CFB	complement factor B
CHAPS	3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate
CHCA	cyano-4-hydroxy cinnamic acid
Chem	chemical
cIEF	capillary isoelectric focusing
Cl^-	chloride ion
CL-43	collectin-43
CLU	Clusterin
CM	clinical mastitis

CMT	California Mastitis Test
CN	Casein
co.	corporation
ConA-	Concanavalin A
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CPLL	combinatorial peptide ligand library
Cu	copper
CV	Coefficient of variance
Cy	cyanine
CyDye	cyanine dye
Cys	cysteine
Da	Dalton
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
EC	electrical conductivity
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
ESI-MS	electrospray ionization mass spectrometry
EU	European Union
FA	fatty acid
Fe	iron
FFFA	free fatty acid
FGF	fibroblast growth factor

FGFBP	fibroblast growth factor binding protein
FTICR	fourier transform ion-cyclotron resonance
FTMS	Fourier transform mass analyzer
g/litre	gram per litre
GC-MS	gas chromatography mass spectrometry
Gly	glycine
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
Glu	glutamic acid
GSH	glutathione
GSHPx	glutathione peroxidase
H	histidine
H_2O_2	hydrogen peroxide
Hb	haemoglobin
HbCN	cyanmethaemoglobin
HDL	high density lipoprotein
Hb-Hp	haemoglobin-haptoglobin
HCl	acid hydrochloride
His	histidine
HMW	high molecular weight
HNMR	proton nuclear magnetic resonance
Hp	haptoglobin
Hp-HbCN	haptoglobin-cyanmethaemoglobin
HPLC	high-performance liquid chromatography
Hp-MMP9	haptoglobin-matrix metalloproteinase
HSA	human serum albumin
ICAT	Isotope-coded affinity tag
ID	identification
IEF	isoelectric focusing
IEX	ion exchange chromatography
Ig	immunoglobulin

Ile	isoleucine
IL-1	interleukin-1
IL-10	interleukin-10
IL-12	interleukin-12
IL-19	interleukin-19
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	interleukin-8
IMI	intramammary infection
Inc.	incorporation
iNOS	inducible nitric oxide synthase
IPG	immobilized pH gradient
IQR	inter-quarter ratio
IRT	infrared thermography
iTRAQ	isobaric tags for relative and absolute quantitation
K	lysine
K^+	potassium ion
kDa	kilo Dalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kV	kilo volt
LAB	lactic acid bacteria
lb	pound
LBP	lipopolysaccharide binding protein
LC	liquid chromatography
LC MS	liquid chromatography mass spectrometry
LC-HSRM-MS	liquid chromatography-high-resolution selected reaction monitoring- 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
LKA_4	leukotriene A_4
LKB_4	leukotriene B_4
LMW	low molecular weight
logSCC	logarithmic somatic cell count
logNAGase	log N-acetyl-beta-D-glucosaminidase
Lp	lactoperoxidase
L-PGDS	lipocalin-type prostaglandin D synthase
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LTA	lipotechoic acid
LTB_4	Leukotriene B_4
Lys	lysine
m or M	methionine
M	molar
MALDI	matrix-assisted laser desorption ionization
MALDI TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectrometry
MALDI-TOF MS/MS	matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry
Met	methionine
MFGE8	milk fat globule EGF factor 8
MFGM	milk fat globule membrane
mg/ml	milligram per millilitre
MH+	protonated mass of peptide
MHC	major histocompatibility complex
MHp	milk haptoglobin
min	minute
ml	millilitre
mM	millimolar
MPO	myeloperoxidase

Mr	relative molecular mass
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS	mass spectrometry
mS/cm	milliSiemens per centimetre
MS/MS	tandem mass spectrometry
M-SAA3	mammary-associated serum amyloid A3
Mud-PIT	multidimensional protein identification
MUFA	monounsaturated fatty acid
MWCO	molecular weight cut off
m/z	mass to charge ratio
n	total
Na^+	sodium ion
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NAGase	N-acetyl-β-D-glucosaminidase
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
NEB	negative energy balance
NEFA	non-esterified fatty acid
nESI-MS/MS	nanoelectrospray-tandem mass spectrometry
NET	neutrophil killing mechanism
ng/ml	nanogram per millilitre
NH_4	ammonium
NH_4OH	ammonium hydroxide
NH_4SO_4	ammonium sulphate
NHS	N-hydroxysuccinimidyl
NHS-PEO4	N-Hydroxysuccinimide polyethylene oxide 4

NK-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometre
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
NPN	non-protein nitrogen
O_2^-	superoxide anion
OD	optical density
OH	hydroxyl radical
P or p	proline
P4	progesterone
PAGE	polyacrylamide gel electrophoresis
para-κ-CN	para-kappa casein
PBS	phosphate buffered saline
PCM	milk prostacyclin
PCR	polymerase chain reaction
PGD_2	prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF1α	prostaglandin F1 alpha
PGF2α	prostaglandin F2 alpha
PGI_2	prostacyclin
PGJ_2	prostaglandin J_2
PGLYRP1	peptidoglycan recognition protein 1
pH	power of hydrogen
PHA-	phytohaemagglutinin
pI	isoelectric point
Pm	probability of misclassifying
PMF	peptide mass fingerprinting
PMN	polymorphonuclear
PMNL	polymorphonuclear leukocyte

pmol/µl	picomol per microlitre
ppm	parts per million
PPV	positive predictive value
Pro	proline
PSD	post-source decay
psi	pounds per square inch
PTM	post transitional modification
PUFA	polyunsaturated fatty acid
p-value	probability that null hypothesis is true
Px	peroxidase
Q	quadrupole
Q-TOF MS/MS	quadruopole-time of flight tandem mass spectrometry
r	correlation
R	arginine
RAM	restricted access media
RP	reversed phase
RP-HPLC	reversed phase-high performance liquid chromatography
RSNO	S-nitrosothiols
RT	rectal temperature
RT-PCR	reversed transcription polymerase chain reaction
RBP	retinol binding protein
S. agalactiae	*Staphylococcus agalactiae*
S. aurues	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. uberis	*Streptococcus uberis*
S. dysgalactiae	*Streptococcus dysgalactiae*
SAA	serum amyloid A
SCC	somatic cell counts
SCX	strong cation resin
SD	Standard deviation

SDPP	short-day photo period
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	selenium
SELDI-TOF	Surface-enhanced laser desorption ionization-time of flight
Ser	serine
SFA	saturated fatty acid
SHp	serum haptoglobin
SILAC	Stable isotope labelling with amino acids in cell culture
SNF	solid non-fat
SOD	Superoxide dismutase
spp	species
SPE	solid phase extraction
SPME	solid phase microextraction
SPR	surface plasmon resonance
SRID	single radial immunodiffusion
SST	skin surface temperature
SVM	support vector machine
T	trace
TBS	Tris-buffered saline
TCA	citric acid cycle
TFA	trifluoroacetic acid
TLR	toll-like receptor
TMB	Tetra methyl benzidine
TNF-α	tumour necrosis factor-alpha
TOF	time-of-flight
Tris-HCl	Tris-hydrochloride
TrpRS	tryptophanyl-tRNA-synthetase
TTR	transthyretin
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2

UFA	unsaturated fatty acid
UK	United Kingdom
UPLC	ultra-high pressure liquid chromatography
USA	United States of America
utlIEF	ultra-thin layer isoelectric focusing
UV	ultra violet
UV/VIS	ultra violet visible
V	voltage
Val	valine
vs	versus
v/v	volume over volume
w/v	weight over volume
WPC	whey protein concentrate
WST	Whiteside test
X	times
XCorr	cross correlation value from the search
x g	centrifugal force in gravity
Zn	zinc
z-value	standard score
α1-AGP	alpha 1 acid glycoprotein
α_1-PI	alpha 1-acid proteinase
α-CN	alpha casein
α-LG	alpha lactoglobulin
αS1-CN	alpha S1 casein
αS2-CN	alpha S2 casein
β-CN	beta casein
β-Gase	β-glucuronidase
β-LA	beta lactalbumin
γ	gamma
γ_1-CN	gamma 1 casein

γ_2-CN	gamma 2 casein
γ_3-CN	gamma 3 casein
δ-12-PGJ_2	delta-12-prostaglandin J_2
δ-CN	delta casein
κ-CN	kappa casein
µg	microgram
µg/ml	microgram per litre
µl	microlitre

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Rozaihan Mansor, February 2012

Author's Declaration

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

Rozaihan Mansor, February 2012

Dedication

To my daughter, Nur Adaira Safiyyah Mohd Hasan

Chapter I. Introduction

1.1 Bovine Mastitis and its Impact on the Dairy Industry

Mastitis is an inflammation of mammary gland parenchyma which is characterized by a range of physical and chemical changes of the milk and pathological changes in the udder tissues (Radostits *et al.* 2000). Significant milk changes that can be observed in bovine mastitis are the presence of clots in milk, milk discolouration and high numbers of leukocytes in affected milk. Furthermore, apparent clinical signs in bovine mastitis comprise swelling, heat and pain in the udder.

Mastitis is usually caused by bacterial pathogens which can be classified into two groups; the contagious pathogens which include *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma bovis* as well as environmental pathogens which include *Streptococcus* species (*Streptococcus uberis* and *Streptococcus dysgalactiae*) and environmental coliforms (Gram negative bacteria *Escherichia coli*, *Klebsiella* spp., *Citrobacte*r spp., *Enterobacter* spp., *Enterobacter faecalis* and *Enterobacter faecium*., and other gram negative bacteria such as *Serratia, Pseudomonas* and *Proteus* (Radostits *et al.* 2000)

The bovine mammary gland is protected by innate and specific immune responses (Sordillo & Streicher 2002). However, factors such as environmental and physiological could compromise the defence mechanism of the mammary gland (Hopster *et al.* 1998; Waller 2000). Milking by using machines can also contribute to teat damage which in turn increases the susceptibility of mastitis-causing pathogen colonization. Poor housing environment, increased cow densities per unit and low ventilation can also increase the susceptibility to bovine mastitis. Nevertheless, the most important factor in contributing to increase bovine mastitis susceptibility is the lactation stage of a cow. It is known that during the periparturient period, the immunity of the udder is compromised due to physiological changes that happen in preparation for lactation (Oliver & Sordillo 1988).

Mastitis is one of the most prevalent diseases in dairy cows. It is an endemic disease and happens to be the most frequent and most costly disease affecting dairy herds worldwide (Halasa *et al*. 2007; Miller *et al.* 1993). It affects the quality of the milk through changes in milk composition and in return, affects the economy of dairy industry. The United Kingdom (UK) is the third largest milk producer in the EU after Germany and France and

ninth largest producer in the world (Defra, *Agriculture in the United Kingdom 2009,* April 2010, Table 9.1). Around one million cases of bovine mastitis occur each year in the UK with £200 million of losses in production and treatments every year (Science Daily 2008).

Various papers have discussed the economic impact of bovine mastitis. To simplify their conclusions, economic losses due to mastitis can be caused by milk production losses, use of treatments, discarded milk, veterinary services, labour, product quality, materials and investments, diagnostics, other diseases and culling (Halasa *et al.* 2007). Persistent decrease in milk production is the main detrimental effect that contributes to the economic impact of mastitis (Seegers *et al.* 2003). However, it is quite difficult to estimate the economic losses caused by bovine mastitis as they are influenced by various factors.

According to Bhikane & Kawitkar (2000), this most important disease in dairy cattle can cause up to 70% of reduced milk production, 9% of milk discard after treatment, 7% of the cost of veterinary services and 14% of premature culling.

Traditionally, the control of mastitis has involved the use of chemical disinfectants, antiseptic or herbal teat dips (Maiti *et al.* 2004) and antibiotic therapy. Antibiotic therapy has been used in mastitis control for about 50 years (Sharma 2007). However, it was suggested that antibiotic therapy was not actually helping to reduce the incidence of mastitis. In fact, several problems arise from the use of antibiotics for example, developing resistance to antibiotic, questionable drug efficacy and presence of antibiotic residues in the milk. Thus, Sharma (2003) suggested the use of alternative approaches to control mastitis that enhance the immunity of mammary gland by using immunoregulatory micronutrients (Vitamin E and selenium), vaccines and cytokines.

1.1.1 Bovine Milk

Milk is one of the important biological fluids and contains considerable amounts of nutrients. All types of milk contain proteins in colloidal dispersion as micelles, fats in emulsified globules coated with a membrane and easily digested, minerals, vitamins and other components (Jensen *et al.* 1991, 1995). Each of these components plays beneficial roles to the host.

Colostrum is another type of milk that is produced by cows during the first several days postpartum. It is important for the growth and health of the neonates as it contains antibody (immunoglobulins) and complement that provide antimicrobial properties against different types of pathogens which can be detrimental to the newborn especially during early age

(Korhonen *et al.* 2000). Thus, colostrum provides nutrients to the newborn as well as conferring passive immunity to them. However, in this thesis, we will focus only on milk protein of bovine.

1.1.2 Milk Protein

Proteins are arguably the most important nutritional factors in the milk. In bovine, six major gene products of the mammary epithelial cells have been identified which include α_{S1}-, α_{S2}-, β-, κ-caseins, β-lactoglobulin and α-lactalbumin (Swaisgood 1982, 1993). Apart from the presence of blood plasmin and plasminogen in the milk, small peptides (e.g; proteose-peptone which is largely derive from the N-terminus of β-casein and from fat globule membrane glycoproteins) and large peptides (e.g; γ-caseins are derived from the C-terminus of β-casein) are also present in milk with varying concentrations (Swaisgood 1993) as well as a number of low abundance minor proteins and enzymes (plasmin and lipoprotein lipase).

Protein is one of the milk compositions known to affect more of the properties of milk and most dairy products than any other constituent, thus considerable amounts of research have been performed to investigate their unique properties and technological importance.

Milk contains acid-precipitated proteins known as casein (CN) which are converted to curds by rennin (Kinsella *et al.* 1984). The liquid left after casein precipitation of skim or whole milk is called whey proteins. Another two groups of proteinaceous materials are proteose peptones and non-protein nitrogen (NPN) which was discovered by Rowland (1938).

Pedersen and Mellander (McMeekin 1970) successfully resolved isoelectric casein into three proteins namely α-, β-, γ- caseins depending on their electrophoretic mobility. Alpha-casein was further resolved by Waugh and van Hippel (1956) into α_s-casein which contains two types of proteins α_{s1}- and α_{s2}- caseins as well as κ-casein. According to Fox & Brodkorb (2008), α_{s1}-casein, α_{s2}-casein, β-casein and κ -casein represent approximately 38, 10, 35 and 12% respectively of whole bovine casein.

As for the whey proteins, they are globular molecules in which the acidic/basic and hydrophobic/hydrophilic amino acids are evenly distributed along their polypeptide chains (Evans 1982). Whey proteins comprise of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (Igs), lactoferrin and lactoperoxidase, together with other minor components (Madureira *et al.* 2007).

Other proteins that are present in bovine milk comprise metal-binding proteins (glutathione peroxidase), β-microglobulin, osteopontin and vitamin-binding proteins, angiogenins, kininogen, glycoproteins, proteins in the milk fat globule membrane and growth factors.

Milk Proteins	**Examples**
Caseins	α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein
Whey proteins	β-lactoglobulin, α-lactalbumin, immunoglobulins, lactoperoxidase
Others	metal-binding proteins, β-microglobulin, osteopontin, vitamin-binding proteins, angiogenins, kininogen, glycoproteins, proteins of milk fat globule membrane and growth factors

Table 1-1: Examples of caseins, whey and other proteins present in bovine milk composition

1.2 Diagnostic Methods for Bovine Mastitis

These traditional diagnostic methods discussed below are the most common ways of diagnosing bovine mastitis at the farm level worldwide. They are known to be used widely in detecting bovine mastitis. Despite of several major drawbacks in these methods, they still are widely used in diagnosing bovine mastitis in dairy herds.

1.2.1 Somatic Cell Counts

Somatic cell count (SCC) has proved to be one of the most useful diagnostic techniques to detect the presence and occurrence of bovine mastitis and especially for bovine subclinical mastitis (Dohoo & Meek 1982; Schukken *et al.* 2003). According to Shook (1989), currently, the dairy industry relies on this method for milk quality payment schemes (Viguier *et al.* 2009) where elevated levels of somatic cells result in low payments. Automated devices have been developed recently so that rapid determination of somatic cell counts can be performed by using the Coulter Milk Cell Counter; where the particles are counted through an electric field and the Fossomatic; where the cells are stained using fluorescent dyes before counting the fluorescent particles (Dohoo & Meek 1982).

Normally, in a non-infected mammary gland, the SCC is low and macrophages are the predominant cells in the milk, which account for 60% of the total somatic cells with 12%

consisting of neutrophils and 28% of lymphocytes (Burvenich *et al*. 1995; Napel *et al.* 2009). However, following microbial infection, approximately within 6 hours after the release of bacterial endotoxin, the somatic cells increase dramatically from fewer than 10^5 cells/ml of milk to an excess of 10^6 cells/ml (Kehrli and Shuster 1994). At this stage, the majority of cell populations are neutrophils (>90%), which remain the main cell type up to 59 hours following infusion (Saad & Ostensson 1990). However, according to Van Werven *et al.* (1997), it may take about 3 weeks for the SCC to return to its normal concentration following successful bacterial elimination. Thus, the levels of neutrophils as the percentage of the SCC can be used as a mastitis indicator (Hamann and Kromker 1997). Leukocytes, primarily neutrophils are recruited from the blood in order to fight against bacterial colonization and thus making neutrophils the cells predominant in milk somatic cells during mastitis (Leitner *et al.* 2000).

The presence of increased somatic cell counts can be explained by the pathogenesis of bovine mastitis. Following microbial establishment in the mammary gland, the bacteria will stimulate both the cellular and humoral defence mechanisms of the udder and will further deteriorate its condition by multiplying and liberating toxins. Endotoxin released by the bacteria induces leukocytes and epithelial cells to release chemoattractants such as cytokines, interleukins (IL-1, IL-8), eicosanoids (PGF2α), oxygen radicals and acute phase proteins (APPs). Polymorphonuclear cells (PMNs) are attracted to the site of inflammation to engulf and destroy the invading bacteria and in turn, release intracellular granules which contain bactericidal peptides, proteins, enzymes, and neutral and acidic proteases. The apoptotic PMNs are later engulfed and ingested by macrophages and the dead and sloughed off mammary epithelial cells together with dead leukocytes give rise to high somatic cell counts in the milk

To correctly interpret the SCC, several factors need to be considered. First, is the infection status of the quarter of the mammary gland. The SCC depends on the causal organisms (commensal organisms; *Corynebacterium bovis* or coagulase negative streptococci or major pathogens; Streptococci species, *Staphylococcus aureus* and coliforms) as different types of pathogens, result in different ranges of SCC. Second, the number of quarters infected is particularly important in composite (cow) SCC as the high cell count milk from infected quarters is diluted with low cell count milk from uninfected quarters. Increased age of a cow has been reported to be associated with an increase of cellular content of milk due to increased prevalence of disease in older cows (Marshall & Edmondson 1962;

Reichmuth 1975; Dohoo & Meek 1982). After calving, there is a possibility of the somatic cell counts rising in uninfected quarters and then decreasing to the normal level 4-5 days postpartum (Dohoo & Meek 1982; Barkema *et al.* 1999). Some authors have reported that the somatic cell counts increase as the lactation progresses (Schultz 1977). Towards the end of lactation, SCC is seen to be elevated (Brolund 1985; Miller & Paape 1988) but return to a low level if the quarter is uninfected. However, if there is a presence of infection, the SCC remains high and this can be used to detect a new intramammary infection postpartum (Barkema 1999). Thus, stage of lactation seems to affect the interpretation of SCC.

Milking frequency also seems to affect the SCC. Increasing the frequency of milking (from two times a day to three times a day) decreased the SCC of the bulk milk (Hogeveen *et al.* 2001). Cell counts were seen to be elevated in the afternoon rather than in the morning (Kukovics 1996) and also they appeared to be highest in the milk strippings. These cell count concentrations could persist for up to 4 hours before return to low levels (Smith & Schultze 1967). Other factors that should be considered include season, stress, day to day variation, technical aspects and management (Dohoo & Meek 1982).

The type of sample taken for SCC analysis (quarter, composite or bulk tank) is an important factor when interpreting the results. One should remember that there are possibilities of having false-positive or false-negative results. Infected cows sometimes have low cell counts and vice versa. Second, cell counts are only an indication of the presence of infection within the mammary gland, rather than indicating the presence of pathogen. Finally, the evaluation of herd averages is better than the evaluation of individual quarter, cow or bulk tank level (Dohoo & Meek 1982).

With quarter milk samples, the threshold of 200 000 cells/ml has been recommended to classify the quarter as being infected (with a major pathogen) (Hillerton 1999a). When interpreting the composite SCC, the dilution effect of milk from uninfected quarters on the elevated counts from infected quarters should be taken into consideration. It has been suggested that the logarithmic transformation of the SCC can be done before the average of cell count is calculated (Dijkman 1975). Bulk tank SCCs seem to be a good indicator of the general state of udder health and levels below 250 000 cells/ml indicate a good state of udder health and over 500 000 cells/ml indicate the presence of severe mastitis in a herd (Dohoo & Meek 1982).

High somatic cell counts affect the milk quality and milk production as well as milk composition. Due to high SCC, the quality of milk is altered as it causes deterioration in the flavour quality and the shelf life of the milk. These effects result from the breakdown of milk protein and fat and an increase in acid degree value (Ma *et al.* 2000). High levels of whey protein and low casein levels lead to lower cheese yields (Sharif & Muhammad 2008) because an elevation of rennet to cutting time and reduced curd firmness are found. In turn, severity of mastitis affects the value of food in terms of low protein and fat contents in milk (Ullah *et al.* 2005). Thus, an increase in SCC is an indicator of losses in milk production due to subclinical mastitis. However, it must be remembered that other factors associated with mastitis could also lead to production losses such as discarded milk, drug costs, veterinary fees, extra labour, increased cow replacement costs and loss of genetic potential (Dijkman 1975). An intramammary infection during mastitis can cause considerable impact on the mammary gland of a cow. Permeability of the blood-milk barrier is disrupted and results in increased leakage of blood components into milk such as serum albumin and immunoglobulins (Haenlein *et al.* 1973). Furthermore due to infection, the synthetic activity of mammary tissue seems to be affected, thus decreasing lactose fat and α-lactalbumin level in milk (Harmon 1994).

1.2.2 California Mastitis Test (CMT)

A rapid, simple test to detect mastitis called the California Mastitis Test (CMT) was developed by Schalm and Noorlander in 1957. In this test, a mixture of the reagent/detergent and milk caused precipitation and gel formation which reflected the cell count of the milk (Barnum & Newbould 1961). According to Carroll and Schalm (1966), deoxyribonucleic acid (DNA) originating from the nuclei of somatic cells constituting the inflammatory exudates cause this CMT reaction. Jensen (1957) reported that this CMT reaction was due to the formation of a gel of leukocyte proteins and the reaction was negative until the level of cell count exceeded 500 000 cells/ml. Thus, CMT is one of the popular cow-side mastitis tests to indicate the presence of mastitis based on the leukocyte count in the infected milk.

Detergents used in the CMT decrease the surface tension, change the structure and conductivity of cell membranes and the nucleus of the cells. Other than that, it disturbs the osmotic balance, blocks oxidizing reactions and stimulates proteolytic enzymes and increases milk viscosity, leading to gel formation (Sargeant *et al.* 2001; Ruegg & Reinemann 2002; Middleton *et al.* 2004). According to Barnum & Newbould (1961), the test was performed by pouring the well-mixed milk to a volume of 3ml in one part of a

partitioned plastic paddle before adding approximately 3ml of the commercial CMT reagent. The paddle can hold 4 samples at one time and is rotated quickly by hand 10 times and graded as either negative, trace (T), 1+, 2+ and 3+. The description is as follows:

CMT reactions	**Description**
Negative	No change in consistency.
T-Trace	No visible change in consistency, but when paddle is tipped a slime is momentarily seen on the bottom.
1+	A gel or thick slime forms, but when the paddle is swirled the solution does not move into the centre.
2+	A thick lumpy gel forms, which, when swirled, quickly moves toward the centre
3+	A distinct gel forms which tends to adhere to the paddle, and during swirling a distinct central peak forms.

Table 1-2: Description on the changes of milk after adding the CMT reagent and their CMT scores. (*Barnum & Newbould 1961*)

Although SCC estimation is a reliable tool to be used to screen and identify intramammary infection (IMI) (Sargeant *et al.* 2001), determining SCC requires sample submission to a laboratory and is not readily accessible to on-farm use. Thus, CMT serves as an important method of diagnosis that is readily accessible to on-farm use and its scores correlate well with the SCC (Hogan *et al.* 1999). The CMT and Modified Whiteside (Murphy 1942; Murphy *et al.* 1941) tests are useful diagnostic tests to identify subclinical mastitis and evaluate its severity (Forster *et al.* 1966). According to Middleton *et al.* (2004), if CMT is a reliable test to identify subclinical infected mammary quarters, then it could be used in selecting quarters for cessation of lactation. This therapeutic cessation of lactation in infected mammary quarters can help in decreasing individual cow SCC and thereby bulk tank SCC (Middleton *et al.* 2004; Middleton & Fox 2001).

Because the CMT reaction is an indication of leukocyte counts, various studies have been performed to correlate the relationship between CMT and leukocyte content of milk.

Barnum & Newbould (1961) reported that CMT showed close association with the leukocyte count as 71.7% of samples with positive CMT were from quarters classified as infected mammary quarters. More samples with less than 500 000 cells/ml gave a trace reaction of CMT. Furthermore, only 2.7% of samples showed a false positive result but indicated that the paddle method of CMT needed experience in both the method of testing and interpretation of the reactions to achieve uniformity and accuracy of the results.

The understanding on the effects of milk composition and yield caused by the varying levels of subclinical mastitis is important in dairy industry. Thus a study by Daniel *et al.* (1966) determined the effects of subclinical mastitis (as determined by CMT on samples of 24-hour milk production) on milk composition and yield. This study indicated that by one unit of difference in CMT value, the average changes were a decrease in lactose concentration by 0.1%, increased protein level by 0.042%, reduced solid non-fat (SNF) levels by 0.046% and most importantly, reduced milk production by 4.9lb per cow.

One study on the relationship between CMT reaction and milk production and composition of milk from opposite quarters were done by Forster *et al.* (1966). Before CMT became available, the opposite-quarter comparisons of milk production was established by Crossman *et al.* (1950) based on that subclinical mastitis was actually associated with decreased milk production. Based on comparisons made from 1258 opposite-quarter milkings from 763 cows, average decreases in milk production were seen according to the CMT reactions (on total quarter milk). The biggest changes of milk production were seen at test reaction of 3 with 43.4% decrease in milk production.

The relationship between CMT reaction and bacteriological analyses has also been studied by Wesen *et al.* (1967) as it seems probable that both the type of pathogens involved and leukocyte numbers should be considered in diagnosing mastitis. It appeared that there was a relationship between CMT and the type of pathogens identified in a particular quarter as there was at least one type of pathogen found in quarters with different type of CMT reactions. The non-pathogenic pathogens were identified more frequently in quarters with low mastitis test reactions (negative, trace and 1) than in quarters with high mastitis test reactions (2 and 3).

Miller & Kearns (1966) studied the effectiveness of CMT to measure the leukocyte content of quarter milk samples and found that the CMT was reliable for the low leukocyte count milk when the CMT score is 0 with the percent of correct was 90.7. They concluded that as the CMT score increased, the leukocyte content can be expected to increase materially and

that CMT was a reliable tool to estimate leukocyte content from a herd of cows under normal management practices.

To determine the effectiveness of CMT in identifying subclinical mastitis, Middleton *et al.* (2004) found that the sensitivity of the CMT with a trace result as the cut off for a positive result, was low (<0.61) which indicated CMT was not useful as a screening test as only 50% of infected mammary quarters would yield a positive test result. An ideal screening test should have maximal sensitivity to minimize the false-negative results. Differences on specificity and sensitivity between this study and previous studies are due to the characteristics of the individual pathogens and the prevalence of these pathogens in the herds studied. *Staphylococcus aureus* for example has different pathogenicity from other bacterial infection which lead to variation in the severity of mastitis they induced (Brookbanks 1966).

Dingwell e*t al.* (2003) indicated that early detection of IMI during early lactation may have significant economic benefits. This author agreed on the idea that a screening test with a high sensitivity (no false-negative CMT reactions) would be ideal to discriminate between non-infected and infected quarters. Although the highest calculated sensitivity and positive predictive value (PPV) were on days in milk (DIM) 4 at a cut-off point of >0, it cannot be concluded that DIM 4 is the best day to perform the CMT because samples were not taken from every cow on every day in this study. Nevertheless, CMT can still be a rapid, accurate and economically feasible test for fresh cows to identify specific pathogens.

Reports by Sharma *et al.* (2010) were in accordance with other previous studies (Reddy *et al.* 1998; Tanwar *et al.* 2001; Goswani *et al.* 2003) that SCC was the most accurate test to diagnose subclinical mastitis followed by the modified CMT and the modified Whiteside Test (WST) based on the sensitivity and specificity values. They suggest that CMT could be useful as a regular mastitis screening test in dairy herd monitoring programmes even by less trained dairymen as supported by Sargeant *et al.* (2001).

1.2.3 Enzyme levels

One enzyme that is known to be a reliable and sensitive mastitis indicator is N-acetyl-β-D-glucosaminidase (NAGase) (Kitchen 1976) which has been shown to originate from the cytosol of mammary gland secretory cells (Kitchen *et al.* 1978). It was first reported by Mellor (1968) that bovine NAGase is derived wholly from leucocytes and its concentration might be convenient to indicate the presence of bovine mastitis. During mastitis, an

increase in cytoplasmic material being shed into milk happens as a result of permeability change in the plasma membranes of the secretory cells together with extensive tissue damage (Chandler *et al.* 1974).

It has been found that the level of NAGase in milk can be used to estimate the SCC in milk using spectrophotometric measurement (Kitchen & Middleton 1976; Kitchen 1976). However, an improved assay procedure using a fluorimetric substrate has been proposed to facilitate automation of the method (Kitchen *et al.* 1978). NAGase is one of the indigenous enzymes which found was to be increased in milk during inflammation which originates from phagocytes (Pyorala 2003).

According to Kitchen *et al.* (1978), the fluorimetric procedure used to analyse NAGase is superior to the previously spectrophotometric assay due to greater sample throughput and ability to get an absolute measure of the level of the product released without problem of sample turbidity problem. Besides, this procedure is suitable for an automated method. They also found good correlation coefficient value between the SCC of 243 quarter foremilk samples determined by the Fossomatic instrument and the fluorimetric assay (r=0.86). This study also concluded that the level of NAGase in milk is significantly influenced by the integrity of the mammary gland secretory cells as the level of NAGase from the mammary gland tissue was the highest compared with other sources listed. Thus, NAGase level in milk could serve as a sensitive indicator of pathological and physiological changes in the mammary gland.

Fitz-Gerald *et al.* (1981) reported that both NAGase and SCC increased markedly in the milk of first milking after infusion of endotoxin (9-hour interval) in line with a similar increase in the blood component blood serum albumin (BSA) and in Na^+/K^+. Furthermore, NAGase levels took longer to return to normal than did SCC and BSA and this can be explained due to a healing of injured tissue is a slower process compared with the reversal of other changes.

However, Obara and Komatsu (1984) found that NAGase activity in milk is a better indicator to grade the severity of inflammation than estimating the level of SCC based on the higher correlation between NAGase and chloride (r=.88) and lactose (r = -.80) than correlation between NAGase and SCC (r=.72). In the healthy udders, low levels of NAGase are secreted from the cytosol of secretory cells but during IMI, NAGase is

released from the cells into milk according to intensity of the inflammation. Thus, NAGase provides a useful indicator of the severity of udder inflammation and the injury implicated on the mammary gland. They also found close correlation (r=.91) between NAGase and lactoferrin which is also known to increase in milk from injured epithelial cells after initiation of udder inflammation.

Although the range of NAGase activities were found to be low in bulk milk samples compared with the composite and quarter milk samples (Kitchen *et al.* 1984a), there was a significant variation of NAGase activities in the bulk milk samples. The NAGase test is more versatile than other alternative mastitis diagnostic methods (BSA, conductivity) as it can be applied to all levels of milk sampling (quarter, composite and bulk), it is rapid and has a simple procedure. The combined use of SCC and NAGase provides a promising and meaningful interpretation of mastitis diagnosis so that herds could be classify according to their level and extent of infection (Pyörälä & Pyörälä 1997).

Previous studies have observed the significance relationship between NAGase and its reference test SCC to assess the inflammatory responses in the mammary gland. However further research work by Kitchen *et al.* (1984b) has observed the relationship between NAGase and the presence of isolated pathogenic bacteria causing mastitis as the growth of bacterial pathogen in the udder caused secretory disturbances as a results from tissue damage (Schalm *et al.* 1971; Giesecki 1975). The presence of udder pathogens during mastitis was seen to have a definite relationship with NAGase activities in the infected quarters (Kitchen *et al.* 1984b). Udders that were infected with minor pathogens had only a slight increased in NAGase activity compared to major pathogens causing mastitis which caused marked increased in NAGase activity. Furthermore, little variation in NAGase activity in the infected milk made it more meaningful in assessing the infection severity than SCC. NAGase levels are also useful in diagnosing bovine subclinical mastitis by applying the composite NAGase analyses on regular monthly basis. However, as previously mentioned, the combination between NAGase and SCC provide a more definitive interpretation on secretory disorders in affected quarters.

Nagahata *et al.* (1987) have studied the activities of two different enzymes in severe mastitic milk samples NAGase and β-glucuronidase (β-Gase). Beta-glucuronidase was also derived from leukocytes and seems to be a further useful marker in bovine mastitis (Kiermeier & Güll 1966). From their study (Nagahata *et al.* 1987), they have found

remarkable increases in both NAGase and β-Gase levels in milk samples from acute clinical mastitis cases but only a slight increase in these enzyme levels was observed in milk samples from chronic mastitis. They also assumed that most of NAGase and β-Gase activities found in mastitic milk derived from blood serum, neutrophils and macrophages associated with inflammatory process apart from mammary secretory cells.

Sandholm *et al.* (1984) proposed the use of milk antitrypsin as a sensitive indicator of bovine mastitis because its activity has shown to be rapidly increased during mastitis. Matilla *et al.* (1986) indicated that NAGase level was superior to any other tests (BSA, antitrypsin, SCC) when single threshold values were applied. However, when inter-quarter evaluation was applied to avoid background 'noises', the differentiating abilities of antitrypsin were improved equalling that of NAGase. NAGase also showed highest deflection which is important in monitoring composite and bulk milk samples to minimize the dilution factor in mastitic quarters. The authors concluded that the use of combination between NAGase and antitrypsin to achieve optimal diagnostic potential as mastitis involves in different types of inflammatory reactions (increased leakage of plasma proteins such as antitrypsin and BSA and cell destruction as shown by NAGase).

Teat canal infections can predispose the mammary gland to mastitis as the mammary gland infection is thought to be ascending infections started through the teat canals (Forbes & Herbert 1968). To differentiate between the teat canal infections and mastitic infections, analyses of both cultures of swabs of teat canals and milk samples drawn from lactiferous sinus by teat puncture were done (Murphy & Stuart 1954; Forbes & Hebert 1968; Giesecke & Viljoen 1974). However, Kaartinen & Jensen (1988) claimed that release of NAGase into teat canal secretions could be an indicator of teat canal infections. They found that in *S. uberis* infected quarter of mammary gland, the NAGase levels in teat sample fractions were significantly lower than in respective milk samples showing that the invasion of *S. uberis* through teat canal did not cause inflammation or colonization. However, if the quarters were infected with either *S. aureus* or *Micrococcus*, NAGase levels in teat sample and in the respective milk samples were at the same level. Thus, these pathogens initially colonize the teat canal before attacking the upper part of milk compartment with the constant bacterial irritation within the streak canal. Analysing the NAGase gradients between teat canals and milk showed a promising evaluation on the inflammatory condition of the teat canal.

Chagunda *et al.* (2006) found an increased activity of NAGase in clinically mastitic cows as compared with healthy cows but that other factors should be taken into consideration before interpreting the level of this enzyme. The origins of NAGase have been discussed as some authors claimed that the contribution of NAGase from plasma into milk was not important (Timms & Schultz 1985). However, there has been a report on the increased of NAGase level during dry period in the mammary gland compared with plasma indicating that the enzymes originated from mammary gland (Timms & Schultz 1985).

The presence of PMN leucocytes and macrophages in the mammary gland during subclinical and clinical mastitis induced the phagocytic process with secretion of hydrolytic enzymes and as the consequence, tissue degradation occurred. Among the enzymes found are both nonlysosomals (lactate dehydrogenase and 2-naphthylamilase) and lysosomals (NAGase, β-Gase, β-galactosidase and α-manosidase) (Perdigon *et al.* 1985). However, β-Gase proved to be the most significant selectively released enzyme in the inflammatory process (Schnyder, & Bagglioni. 1978; Schorlemrner 1977). Studies performed by Perdigon *et al.* (1985) indicated a high correlation between the SCC and the presence of enzymes β-Gase (76%). This enzyme was also related to the presence of both major pathogens such as *S. agalactiae, E. coli, Klebsiella pneumonia, S. aureus* as well as minor pathogens (e.g; *S. epidermidis).* The reaction of β-Gase is very sensitive and because of its high specificity and simplicity, the detection of this enzyme could be applied for mastitis detection.

L-lactate dehydrogenase (LDH) is another type of enzyme that can be used as mastitis indicator. It is involved in the glycolytic pathway and found in the cytoplasm of all cells and tissues in the body (Chagunda *et al.* 2006). It has 5 isotypes (LDH1 to LDH5) and in milk is known to increase in quantity with mastitis (Bogin et al 1977; Harmon 1994). Chagunda *et al.* (2006) found that the activity of LDH increased in milk during clinical mastitis compared with healthy cows. However, the enzyme level was influenced by parity, stage of lactation and month of production. Thus, it is important to take these factors into consideration before classifying cows as sick. The association between LDH levels and SCC was stronger in clinically mastitic cows than in healthy cows. It was thought that the origin of elevated LDH was from leukocytes (Bogin 1977) but Kato *et al.* (1989) found that granulocytes and lymphocytes in mastitic milk might partly contribute to the displacement of LDH isoenzymes into milk. However, Chagunda *et al.* (2006) indicated that the somatic cells (neutrophils) might contribute to the presence of this

enzyme. When comparing between NAGase and LDH, LDH seem to be better in classifying mastitic cows than NAGase as LDH is a more sensitive indicator in predicting the clinical mastitis.

Another type of enzymes that can be used to detect bovine mastitis is myeloperoxidase (MPO). This enzyme is a lysosomal enzyme which is found in the primary granules of neutrophils (Cooray 1994). Due to its important role in the oxygen-dependent antimicrobial system of neutrophils (Nauseef *et al.* 1988; Klebanoff, 1991and Cooray *et al.* 1993), it can be used to estimate neutrophil number as one of the indirect estimator in bovine mastitis. Cooray (1994) found a high correlation between MPO levels in elevated numbers of neutrophils with SCC (r=0.91). Due to its presence in high quantities in mastitic milk, this enzyme has the potential to be an important indicator of bovine mastitis.

1.2.4 Electrical Conductivity

Electrical conductivity (EC) is a measure of the resistance of a particular material to an electric current (Nielen *et al.* 1992). Normally, milk has a resistance of between 4.0 and 5.5 mS/cm at 22ºC (Wong 1988) and is measured in log normal (Knudsen & Jensen 1990). The concentration of sodium chloride (NaCl) is often expressed as milk electrical conductivity (Kitchen *et al.* 1980; Linzell & Peaker 1972; Linzell *et al.* 1974; Peaker 1978).

Conductivity is thus used in diagnosing bovine mastitis, and cow-side tests and on-line measurements have been developed (Nielen *et al.* 1992). As for on-line measurements, the EC was measured using an electrical cell placed in the milking equipment (Maatje *et al.* 1983; Puckett *et al*. 1984). Another system has been developed without electrodes being in contact with the milk but using two toroidal ferromagnetic cores and a milk loop in the system (Onyango *et al.* 1988).

During mastitis, the destruction of blood capillary permeability, the destruction of tight junctions as well as the failure of the active ion-pumping system causes changes in ion composition in milk. Na^+ and Cl^- which have high concentrations in extracellular fluid pour into the lumen of mammary gland. In contrast, lactose and K^+ leaked into the extracellular fluid and blood (Kitchen 1981; Oshima 1977). These changes caused an increased of EC in mastitic milk (Kitchen 1981). However, several other factors also

influenced the ionic content of milk such as temperature, fat concentration, milk solids and milk fraction (Woolford *et al*. 1998). These are important factors that should be taken into consideration when interpreting the EC in the diagnosis of bovine mastitis as intramammary infection induces a 15-50% of elevation in EC (Greatrix *et al*. 1968; Wolfe *et al*. 1972; Linzell *et al*. 1974; Fernando *et al*. 1982).

Electrical conductivity was once assumed to be a difficult method as there was no established value for normal milk making it impossible to interpret the results directly (Little *et al*. 1968). However, this problem can be overcome if simultaneous comparison was made for all 4 quarters (Greatrix *et al.* 1968; Wolfe *et al.*1972) as first proposed by Davis in 1949.

It is thought to be less sensitive than the measurement of sodium (Linzell & Parker 1971, 1972), through daily measurement of foremilk conductivities. Furthermore, it is more practical to automate reading than milk sodium concentrations (Linzell *et al.* 1974) and it was hoped that daily monitoring of milk conductivity, would allow subclinical mastitis to be detected using this method.

A study by Linzell *et al.* (1974) indeed indicated that under normal herd management, the use of electrical conductivity can be used as indicator in subclinical mastitis. This study also made it possible to distinguish a value of milk conductivity (56.5mM) above which it referred to severely infected quarters.

Fernando *et al.* (1982) found that in mastitis caused by major pathogens (*S. aureus*, coliforms), the conductivity of stripping milk was higher than foremilk conductivity. Furthermore, conductivity of post-milking strippings was more sensitive indicator in intramammary infection than those of foremilks with 3.8% false negative value. In identifying infected and uninfected quarters, a combination of both differential and absolute conductivity provides the most accurate methods. Electrical conductivity (EC) with an in-line system in milking parlour is known to be a rapid and easily obtained measurement.

Further study by Fernando *et al.* (1985) confirmed that increases of EC, Cl^-, Na^+, BSA and logSCC were related to intramammary infection. Marked changes were more pronounced in infection caused by major pathogens. It was also confirmed that the EC were higher in

strippings milk than in foremilks. Furthermore, the accuracy of EC was found to be higher in strippings milk than in foremilks. Their results however were in contrast with results of Linzell *et al.* (1974) as they found accurate results from foremilks. This difference was thought to be due to variation in sampling techniques. Tests for EC, Na^+ and Cl^- contents were more accurate in detection of infection variables than other predictors. However, their results were in accordance with Woolford & Williamson (1982) and Yamamoto (1982) who found increased sensitivity of strippings to udder infection. Due to increased intramammary pressure from accumulation of milk, there is a possibility of higher concentration of ions in the mastitic milk. Thus it was suggested that EC has the potential as a screening test in bovine mastitis as its accuracy to detect mastitis compared favourably with other indirect mastitis tests (SCC, BSA, Na^+, Cl^-, etc)

However, EC also showed to be influenced by herd variation. Sheldrake *et al.* (1981) claimed that the accuracy of EC varied considerably between herds. Because SCC in this study showed similar sensitivity and specificity between herds, SCC appeared to be a more accurate method at detecting infected quarters than EC. It was then suggested that overall, EC is unsuitable cow-side test to detect the presence of intramammary infection.

EC was also influenced by stage of lactation as well as the number of lactations (Sheldrake *et al.* 1983a) as the concentration of chloride increased during lactation (Caufield & Riddell 1935) and changes in EC were seen due to physiological factors in all four quarters. However, in mastitis, changes of EC occurred only in infected gland (Linzell *et al*. 1974). Sheldrake *et al.* (1983a) observed that as lactation advanced, there was little effect of EC seen in uninfected mammary quarters whereas gradual increase of EC was seen in quarters infected with minor or major pathogens.

Sheldrake *et al.* (1983b) found that the probability of misclassifying (Pm) quarters for EC ranged between 22 to 32%. Pm reduced the apparent distortion of percentages caused by unequal numbers of infected and uninfected quarters as it is the point at which false negative and false positive diagnoses are equal. However, this study was in conflict with Davis (1947) in which the comparison within cow by differences and ratios from different quarters yielded little advantage over absolute estimations. However, the finding was in accordance with studies by Linzell *et al.* (1974) and Fernando *et al.* (1982).

EC has been proposed to detect the presence of clinical mastitis earlier than by inspection by herdsmen to detect the physical changes of the milk (Milner *et al.* 1996). In 55% of cases, the diagnosis of mastitis by EC can be made at an average 2 milkings before any visible clinical changes occurred. In about 34% of cases, diagnosis was made at the same time as clots appeared in the milk. Thus, changes in EC as detected using automated sensors could be applied as early as or earlier than visible changes in milk detection to diagnose clinical mastitis.

Differences in the EC values between milk fractions in infected quarters have been studied by Woolford *et al.* (1998). In infected quarters, decreased EC values ($p<0.05$) were seen from first foremilk to main flow milk fractions. Meanwhile, in quarters infected with *S. aureus*, conductivity was increased ($p<0.05$) from main flow milk to stripping milk fraction. In contrast, in uninfected quarters, the conductivity decreased with successive milk fractions due to elevated fat levels. However, variation in electrical conductivity values in infected quarters was not due to fat levels but may be the result from the mixing of milk from infected to uninfected areas of the gland. Localized infection and differential drainage from the infected area of secretory cells were the factors which caused the variation change in EC from first foremilk to stripping milk fraction. The authors suggested that using the foremilk fraction provided the highest sensitivity when EC was used to diagnose subclinical intramammary infection.

Various EC traits have been used to study its association with the udder health (Norberg *et al.* 2003). Four EC traits defined as the inter-quarter ratio (IQR) between the highest and lowest quarter EC values, the maximum EC level for a cow, IQR between the highest and lowest quarter EC variation, and the maximum EC variation for a cow were calculated for every milking throughout lactation. All EC traits were increased markedly ($P<0.05$) in subclinical and clinical mastitis. The IQR performed best in classifying cows correctly with 80.6% of clinical and 45% of subclinical cases were classified correctly. About 74.8% were correctly classified as healthy. This is due to the fact that this trait reflected the level of EC rather than variation of EC.

Biggadike *et al.* (2002) suggested that an assessment of changes in EC should be made on an individual quarter basis to minimise other factors such as stage in lactation that influenced the changes in a whole udder. However, the authors found that the conductivity of individual quarter milk samples is not as suitable to be used as a reliable early mastitis

predictor due to low sensitivity (54%) and positive predictor value (55%). It was suggested that cows with high EC values in their individual quarters, additional evidences are needed before initiating any treatment such as NAGase test or ATPase test.

1.2.5 Infrared Thermography (IRT)

Infrared thermography (IRT) has been employed as a diagnostic method in bovine mastitis based on heat detection generated from the udder. The thermal camera used in this method absorbs infrared radiation and based on the amount of heat generated, the images are produced and generated (Eddy *et al.* 2001; Mazur & Eugeniusz-Herbut 2006). The ability of IRT in detecting increased body temperature has made it possible to assess many aspects in animal industry. In animal production, it has been used to assess meat quality (Schaefer *et al.* 1989) and detecting oestrus in animals (Hurnik *et al.* 1984). Furthermore, it also can be used to assess animal welfare (Stewart *et al.* 2005) that is associated with stress (climate, managerial practices) (Kimmel *et al.* 1992; Eicher *et al.* 2006). Lastly, it has shown to have an advantage over other methods of diagnosis in diseases such as bovine viral diarrhoea in calves (Schaefer *et al.* 2004, 2007) and bovine lameness in dairy cows (Head & Dyson 2001; Nikkhah *et al.* 2005).

In bovine mastitis, inflammation induced by the pathogens increased the temperature of the animal. Hurnik *et al.* (1984) identified 4 out of 6 cases of mastitis using IRT. Endotoxin infusion into the mammary gland caused an increase of 2.3ºC of temperature as measured by the IRT (Scott *et al.* 2000).

Colak *et al.* (2008) studied the relationship between CMT score and skin surface temperature (SST) of the udder as well as the rectal temperature (RT). During mastitis, increased vascularisation at the infected site and tissue metabolism increased the SST (Berry *et al.* 2003) which can be detected by IRT. At cow average, strong correlation ($r=0.93$; $P<0.0001$) was seen between CMT score and udder SST but weak correlations between CMT score and RT ($r=0.27$; $P<0.01$) and between udder SST and RT ($r=0.24$; $P<0.02$). Nevertheless, IRT has shown that it was sensitive enough to detect an increase in udder temperature.

Although Barth (2000) claimed that IRT was not suitable to detect early stage of mastitis, Berry *et al.* (2003) developed a predictive model for the temperature of the udder surfaces and suggested that IRT has the potential to diagnose early stage of mastitis.

Furthermore, Hovinen *et al.* (2008) found an increase in udder temperature of both experimental and control quarters 4 hours post-inoculation which was in line with a rise in rectal temperature. In contrast, mild physical changes in udder and milk which occurred 2 hours post-inoculation were not detected by IRT. Swelling of the udder caused by the plasma leakage into the interstitium (McGavin & Zachary 2007) may result in undetected increases in udder surface temperature. The equal temperatures seen in both experimental and control quarters were caused by the systemic effects of LPS (Hovinen *et al.* 2008). Although this method might bring additional value in detecting mastitis with continuous monitoring of a herd, the authors concluded that more studies should be conducted in naturally occurring mastitis in field conditions where the systemic inflammatory reactions might be less pronounced.

Polat *et al.* (2010) however successfully found high predictive diagnostic ability (positive predictive value of 95.0) of IRT similar to CMT (positive predictive value of 99.2) thus making it a sensitive tool in detecting early onset of subclinical mastitis. However, several cow factors such as parity, milk production and time relative to feeding and milking cows under different environmental conditions (air temperature, humidity and velocity) should be taken into account before analysing the reliability of IRT in diagnosing early onset of subclinical mastitis.

1.3 Biomarkers in bovine mastitis

The discovery of disease biomarkers has become a major research interest in recent years especially with the development of new technologies. Biomarkers are indicators of biological processes and pathological state that can reveal a variety of health and disease traits (Biomarkers Definitions Working Group, 2001). They are important analytes that can be used to predict the progress and outcome of a disease. In bovine mastitis, the search for biomarkers has been underway since the late 1990's. Traditional methods that are still used to diagnose bovine mastitis may have their technical drawbacks that affect their efficiency (Chagunda *et al.* 2006). For example, somatic cell counts (SCC) as described above appears to be a less sensitive and less specific indicator when assessing milk quality in the bulk tank milk level (Le Roux *et al.* 2003) than for individual cows. In contrast,

bacteriology using culturing techniques to detect the micro-organisms that cause mastitis has disadvantages of lacking in speed, is very labour-intensive and expensive (Labohm *et al.* 1998; Hillerton 2000; Viguier *et al.* 2009). Detection of mastitis using electrical conductivity or pH is inexpensive and easy to monitor but has been shown to be relatively insensitive (Nielen *et al.* 1995; De Mol & Ouweltjes, 2001, Viguier *et al.* 2009). Furthermore, results from California Mastitis Test (CMT) can often be mis-interpreted especially when diagnosing a subclinical mastitis case that can lead to false positive or negative (Chagunda *et al.* 2006; Viguier *et al.* 2009). Thus, the discovery on specific mastitis biomarkers is needed for an early diagnosis of bovine mastitis so an early therapeutic intervention can be applied to reduce the severity of the disease.

Following mammary gland infection, various chemoattractants are released which are induced by liberation of toxins from the bacteria. These mediators include various types of cytokines attract circulating immune effector cells mainly PMN on the site of infection. The proteins of PMN have been studied for biomarker potential. The PMN cells are involved in destroying invading bacteria by releasing their intracellular granules which contain bactericidal peptides, proteins, enzymes and neutral and acidic proteases such as elastase, cathepsin G, cathepsin B and cathepsin D.

A reliable indicator has become an important interest and several criteria have been set so that a serious and prolonged period of subclinical and clinical mastitis can be avoided. A mastitic biomarker should have a fast response to infection so that the treatment course can be initiated as soon as possible. The response towards infection should also be distinguishable from the basic milk values as it is an important factor in early warning procedure. The presence of indicator only in the infected quarters and not spreading in other neighbouring quarters is essential if the intended mastitis parameter is adapted to be used at the single quarter level or composite milk level (Larsen *et al.* 2010).

An alteration in the permeability of the microvasculature due to inflammatory responses has led to the identification of blood proteins leaking from the intravascular compartments into milk through the separated tight junctions between endothelial and epithelium cells and have been examined as potential mastitis biomarkers. One of the better indicators is bovine serum albumin (BSA) which is one of the smaller molecular weight of protein of plasma (Giesecke & Viljoen 1974). However the determination of BSA concentrations in

milk requires immunochemical procedures, it is difficult to automate and thus has not gained popularity.

Barta *et al.* (1990) investigating blastogenesis inhibitory factors suggested that mastitis might be also associated with the presence of these inhibitory factors in milk whey. Bovine lymphocyte cultures were used to detect DNA synthesis inhibitory factors in whey and showed that these factors may be released from somatic cells or other tissue cells during inflammation. In this study, clinical mastitis was associated with inhibition of Concanavalin A (ConA-) and phytohaemagglutinin P (PHA-) mitogen induced DNA synthesis by more than 95 and 86%, respectively. A positive correlation was shown between such inhibitory activity, SCC and with electrical conductivity in samples from quarters with mastitis indicating that inhibitory factors in whey may be used as a novel indicator of subclinical mastitis. However, this is relatively complicated and cannot be applied in daily practice although it can be simplified by isolation of the molecules responsible for DNA synthesis inhibition.

A study by Baeker *et al.* (2002) using a proteomic approach revealed the upregulation of lipocalin-type prostaglandin D synthase (L-PGDS) in quarters affected with subclinical mastitis which could be used as a marker in bovine mastitis. Prostanoids are known to have a role as inflammatory mediators via anti-inflammatory effects of this enzyme and its metabolites. Pro-inflammatory mediators which are induced by bacteria or their cell wall compartments as well as lipopolysaccharide (LPS) can activate several signalling pathways that leads to an expression of cyclooxygenase-2 (COX-2) and finally results in increased production of prostanoids. Mammary gland and mammary epithelial cells are able to produce prostanoids and leukotrienes. The upregulation of L-PGDS suggested that there is a probability of coupling of this enzyme to COX-2 induction. As a result, increases in production of prostaglandin D_2 (PGD_2) and its metabolites such as PGJ2, δ-12-PGJ_2 and 15d-PGJ_2 could be seen in the subclinically mastitic cows on this study.

A study by Zeng *et al.* (2009) involved determining the level of lipopolysaccharide binding protein (LBP) in naturally acquired subclinical and clinical mastitic cows in comparison with the level of acute phase protein; haptoglobin (Hp) which is becoming accepted as a diagnostic marker in bovine mastitis. However, milk and blood concentrations of LBP could not be differentiated between subclinical intramammary infection with clinically healthy cows. In contrast to LBP, blood and milk concentration of haptoglobin were

significantly different between cows with clinical mastitis and subclinically infected as well as clinically healthy cows. Thus, LBP was not a suitable candidate in bovine mastitis and haptoglobin appeared to be a better biomarker in differentiating healthy and subclinical mastitic cows.

Another type of biomarker which has been studied to differentiate between acute and chronic inflammatory diseases is Hp-matrix metalloproteinase 9 (Hp-MMP9) complexes. Although a study by Bannikov *et al.* (2010) did not focus specifically on bovine mastitis, it showed that this complex which was released from neutrophils upon degranulation might serve as an important indicator during acute inflammation. This complex has shown significant difference in the serum of cattle with acute septic disease compared to those with chronic/metabolic inflammatory disease or healthy animals (Bannikov *et al.* 2010). However, neither individual Hp nor MMP-9 was suitable marker to differentiate between acute and chronic inflammatory diseases for example mastitis. Furthermore, it is common to find MMP-9 alone in the sera of clinically healthy animals while these proteins might have dual roles in inflammatory process either pro-inflammatory or anti-inflammatory activity. Thus, the use of Hp-MMP 9 complexes to diagnose acute septic inflammation in cattle was more reliable when compared to free Hp or MMP-9.

1.4 Acute Phase Proteins in Bovine Mastitis

The measurement of acute phase proteins following infection has been used extensively in human patients as a part of monitoring disease progression and to evaluate the successful of treatment given. Following infection, a number of systemic events take place which include the acute phase response. The release of various inflammatory mediators and cytokines stimulates the hepatocytes in the liver to produce and secrete acute phase proteins (APP). The released cytokines also able to produce a wide range of host responses including pyrexia, leucocytosis, hormone alterations such as an increase in circulating cortisol and a reduction in thyroid hormone levels and trace element disturbances with reductions in the serum iron and zinc concentrations (Eckersall 1995). This is part of the innate immune response in the defence against pathogens prior to full activation of immune response.

In cattle, Hp and serum amyloid A (SAA) are the most sensitive APP during inflammatory or infectious conditions (Conner *et al.* 1986; Eckersall *et al.* 2001). Thus, evaluation of these proteins using specific assays has become one of the most promising methods to

detect inflammation. Haptoglobin is known to be one of the most reactive acute phase proteins in bovine and exhibits high relative elevated level during acute infection. It has been shown to protect the host from oxidative activity of haemoglobin by binding to the free haemoglobin. Thus, decreased levels of haptoglobin in any sample indicate the presence of haemolysis of red blood cells. Serum amyloid A, another major APP in cattle is synthesized by the liver in different isoforms and released into the plasma following stimulation of inflammatory mediators such as interleukin-1(IL-1), interleukin-6(IL-6) and tumour necrosis factor (TNF-α).

It has been demonstrated that the concentration of Hp is low and often undetected in normal and healthy animals but can increase 50-100 times during inflammation. Although SAA is also known to be increased during the acute phase response, it seems to react faster during inflammation and produce higher differences between healthy and diseased animals compared to Hp. The use of SAA (which is a precursor of amyloid A protein) as a marker of tissue injury in cattle was originally limited due to its difficulties in purifying and quantifying as it is an apo-lipoprotein complexed in the high density lipoprotein fraction (Horadagoda *et al.* 1999), but commercial assays have been available for several years.

The value of using Hp and SAA as important markers in detecting inflammation has proved to be greater than the use of haematological analyses. Following on the successful measurement of the relative concentration of serum amyloid A in cattle using ELISA by Boosman *et al.* (1989) and purification of amyloid A from acute phase bovine serum by Horadagoda *et al.* (1993), the measurement of serum amyloid A (SAA) in the serum can be quantified using ELISA. Assessment of acute phase proteins is recommended compared to haematological analyses as these proteins are quite stable and the measurement can be performed on frozen samples. Haptoglobin for example, has proven to be a sensitive inflammatory indicator in both sheep and dogs (Skinner and Roberts 1994; Solter *et al.* 1991). These findings were supported by work in cattle by Horadagoda *et al.* (1999) where it was found that acute phase proteins were able to discriminate between acute and chronic inflammation. However, due to differences in the release of cytokines following infection, variations in the responses of acute phase proteins might occur. For example, the concentrations of SAA increased significantly during the early course of disease compared to haptoglobin which indicated that SAA is more sensitive to stimulation than haptoglobin. Measurements of both proteins helped to determine to which extent the inflammatory responses has occurred.

Although these two APP are known to be elevated in bovine serum during either spontaneous or induced mastitis, studies by Eckersall *et al.* (2001, 2006) have shown that serum amyloid A can be produced locally in the mammary gland making it a more reliable indicator of bovine mastitis. This has suggested that measurement of acute phase proteins is more sensitive and less influenced by the physiological state of the cow. In order for the SAA to be increased in the milk during mastitis, an intramammary inflammatory stimulus is required.

One study which confirmed the presence of mammary-associated serum amyloid A3 (M-SAA3) was done by McDonald *et al.* (2001) in which they found high levels of this extrahepatically produced SAA3 isoform in the colostrums of healthy cows. It may have a role in providing immunological benefits to the neonates. This is further confirmed by Molenaar *et al.* (2009) in which they found that bovine mammary gland epithelium was capable of expressing M-SAA3 and had a role in developing mammary gland immunity.

Despite showing production of SAA in the mammary gland during mastitis, disruption to the permeability of blood-milk barrier is observed which could lead to leakage of serum proteins in milk (Eckersall *et al.* 2006). However, because no correlation was found between concentrations of SAA in milk and serum, they suggested that SAA might be produced locally in the inflamed mammary gland. It has been be concluded the discrepancy that exists in the levels of acute phase proteins found in serum and milk is caused by a variation in the duration and severity of the udder infection (Nielsen *et al.* 2004)

In one study of APP in mastitis by Kovac *et al.* (2007), acute phase proteins were found to be more accurately detectable in milk rather in serum and thus may be a useful tool in diagnosing mastitis and a useful marker of milk quality. Hp and SAA milk levels were significantly higher in cows with the somatic cell counts exceeded 400 000 cells/ml than in cows with SCC less than 400 000 cells/ml. Thus, it was concluded that due to high correlation of acute phase proteins levels in milk and SCC, the analysis of APP in the milk may be useful in monitoring the health of the udder.

1.4.1 Naturally occurring mastitis

The dynamics of the acute phase response has been studied in naturally occurring coliform mastitis (Ohtsuka *et al.* 2001; Wenz *et al.* 2010). The changes in the level of both haptoglobin (Hp) and α1-acid glycoprotein (AGP) in serum were different in two groups of coliform mastitis; severe and moderate respectively. Serum α1-acid glycoprotein (AGP)

was shown to be increased later in the course of disease in severe coliform mastitis cows whilst the concentration of Hp levels only peaked at 3 days after the onset of the disease (acute inflammatory condition). Thus, the severity of the inflammatory processes can affect the regulation of different APP.

A comparison study on acute phase proteins; haptoglobin, SAA and α_1-acid glycoprotein (α_1-AGP) was done to evaluate the diagnostic potential of these proteins as mastitis indicators in both milk and serum (Eckersall *et al.* 2001). The result of this study revealed that there was no significant difference in terms of APP levels in the serum established between mild and moderate inflammation. Thus, acute phase proteins in the serum are not suitable indicator to discriminate between these groups. However, these APPs in the serum are able to differentiate between acute and chronic inflammatory condition in which Hp and SAA were both affected by acute conditions while AGP is a better indicator in chronic inflammation. AGP was not found in the milk as it is only a moderate acute phase protein in cows and that SAA and Hp might have an inherent advantage over AGP as well as α1 antitrypsin. In this study, the specificity of both SAA and Hp were 100% with no false positive results. Only SAA in milk showed significant differences between mild and moderate inflammatory responses which suggest the production of SAA in milk was under different control mechanisms from that of Hp in the milk or the SAA in serum.

Grönlund *et al.* (2004) also studied a naturally occurring chronic subclinical mastitis to evaluate the usefulness of acute phase proteins such as haptoglobin, serum amyloid A and adenosine triphosphate (ATP) enzyme in milk. The diagnosis of subclinical mastitis has always been challenging to diagnose due to an absence of observable clinical signs of mastitis. Although cow-side test such as CMT, measuring adenosine triphosphate or NAGase as well as electrical conductivity can be used to diagnose subclinical mastitis, these analyses are less efficient to detect subclinical mastitis and thus new disease markers and its methods of detection are needed. In this study, they found that APP level varied markedly in chronic subclinical mastitic cows but there were significant correlations between Hp, SAA and ATP. As compared to the previous study by the same author, Hp was found to be often detected than SAA. Different amount and/or combination of cytokines involved and also variation in host response towards inflammation might contribute to this result. The duration and different phases of inflammation also seem to affect the type of APP being produced. Lastly, it can be concluded that Hp and SAA analysed in udder quarter sample are preferable to evaluate the usefulness of these APP to determine the health status of udder.

Safi *et al.* (2009) has proposed that M-SAA3 was the most accurate test for the diagnosis of subclinical mastitis followed by CMT, SCC, MHp, SAA and SHp. Tests on APP in milk generally were more accurate than tests on serum in the diagnosis of subclinical mastitis due to the significantly larger area under curves (AUCs) of the proteins in milk than those in serum.

Another study on naturally occurring clinical mastitis (CM) investigated the factors that influenced the concentrations of selected acute phase proteins and cytokines on mastitis caused by different pathogens (Wenz *et al.* 2010). They found that the level of Hp, IL-19, IL-12, TNF-α and IL-8 were greater in cows with moderate to severe versus mild systemic diseases. When analysing the season of the year when CM occurred, it appears that some proteins (APP and cytokines) increased during summer and others increased during winter. Thus, it possible that season does affect the immune response of the cows during mastitis. This result showed that with higher amount of proteins being expressed during winter, it was associated with the fact that the immunity is enhanced during short-day photo period (SDPP). Lower levels of IL-10, IL-12 and lactate dehydrogenase (LDH) in the first 60 days of milking are associated with low immunity in periparturient cows. Cows infected with gram-negative bacteria seemed to have higher level of IL-10 and IL-1β as compared to cows infected with gram-positive bacteria. Finally, this study provides a comprehensive background on the factors affecting the innate immune response of cows with naturally occurring clinical mastitis and their influences on proteins associated with infection.

1.4.2 Experimental Induced Mastitis

When evaluating the acute phase response in experimental induced mastitis, it was found in an early study that serum Hp was a good prognostic indicator based on its higher level in severely affected heifers inoculated with *Actinomyces pyogenes*, *Fusobacterium necrophorum* and *Peptostreptococcus indolicus* (Hirvonen *et al.* 1996). Although acid-soluble glycoproteins (ASG) were associated with severely affected heifers, it was less sensitive than Hp as ASG is comprised of several acute phase reactants (e.g: α_1-acid glycoprotein and some proteinase inhibitors). Meanwhile, the use of fibrinogen as a reliable marker was not found to be useful due to the insignificant differences between severe and moderately affected animals. As for α_1-acid proteinase (α_1-PI) activity, it was found not to be a suitable marker to predict the severity and outcome of the disease. Although the activity level was higher in severely affected animals, the differences are not

significant. Fibrinogen and α_1-PI however, can be used as markers to detect the presence of inflammatory condition (Hirvonen *et al.* 1996).

Following the revelation by Eckersall *et al.* (2001, 2006) on the discovery of APP in milk, a number of experimental studies have looked at the responses of the APP in milk. A study by Suojala *et al.* (2008) used two consecutive experimentally induced mastitis by *E. coli* studies to evaluate the possible carry-over effects of the previous intramammary infection. The result showed that the cows had a moderate systemic immune response towards *E. coli* inoculation in the first challenge but the clinical signs were milder and disappeared faster in the second challenge. The level of inflammation indicators (Hp and SAA) followed the same pattern producing significant difference in both milk and serum. Faster response towards infection and milder condition of the disease might be due to effective bacterial elimination by neutrophils through LPS recognition. Concentration of SAA in milk was higher as compared to the serum and this rapid response is due to local immune response in the mammary gland itself. However, the milk Hp concentration was only half of those in the serum and the response was less pronounced during the infection. As for the LBP, it is involved in the formation of LPS-CD14-complex which is known to increase the sensitivity of the host immune response towards gram-negative bacteria.

One experimental study on Staphylococcal causing mastitis has been performed to describe the local and systemic acute phase response during acute and chronic phases of mastitis (Grönlund *et al.* 2003). *Staphylococcus aureus* is one of major pathogen causing bovine mastitis ranging from peracute to chronic form. However, the peracute form is rare but if the acute form is not successfully treated, it can progress into chronic, subclinical form. Chronically infected cows become source of infection where the bacteria are intermittently being shed. During acute clinical mastitis, APP were increased in infected quarters suggesting the local production of proteins particularly SAA in the mammary gland. The rise of APP was correlated with the increase of SCC and the onset of clinical signs. The problem arises when diagnosing subclinical chronic form of mastitis. SAA has shown significantly higher level in infected quarters than pre-infection and healthy control quarters. Also, the increase of APP in the serum might suggest a non-specific response due to other acute inflammatory response but chronic form of mastitis was found to be able to initiate the systemic acute phase response. Thus, SAA is an excellent indicator to diagnose chronic subclinical mastitis (Grönlund *et al.* 2003).

One experimentally induced subclinical mastitis study (Eckersall *et al.* 2006) showed that the occurrence of increased APP in milk happened prior to the detection of increased

serum concentrations of both of these proteins. This demonstrated that blood was unlikely to be the source of the immediate increase in milk. A moderate increase of both APP, particularly the Hp indicate that this experimental model was successful to induce subclinical mastitis in which only mild or moderate APR was stimulated. The authors also suggested that these APP can be use to estimate the severity of the mastitis condition because it showed a significant increase in APP level after second infusion of bacteria as compared to the first infusion. Results from immunocytochemistry suggested that the origin of M-SAA3 in milk may be synthesis within secretory epithelial cells and cells lining the gland cistern of the mammary gland. Although Hp synthesis was not detected by immunocytochemistry in the mammary gland following *S. aureus* infusion, quantitative RT-PCR has supported the probability of local production of both M-SAA3 and Hp mRNA in the mammary gland. M-SAA3 and Hp were thought to have similar activities as serum Hp and SAA and these could be useful to provide resistance to diseases as well as passive transfer of innate immunity to neonate and calf.

Another study on cows following inoculation of LPS by Larsen *et al.* (2010) showed that Hp level is more reliable indicator to distinguish between moderate and severe cases of mastitis. However, the acute phase reactants (Hp and SAA) have their own drawbacks for having prolonged phase of decline and that the declining phase may last for several days. The use of ELISA-based principle to analyse the milk Hp is not suitable for in-line system but the application of biosensor to determine the Hp level might give a better value on practical purposes (Åkerstedt *et al.* 2006). As for the concentration of enzymes involved in mastitis, only LDH, NAGase and AP are known to increase in a faster rate upon LPS infusion. They also showed clear distinct between inflamed quarters with those placebo-treated neighbouring quarters. This study also discussed the possible origin of some of the enzymes during mastitis. For example, although acid proteinase (AcP) and alkaline phosphatase (AP) are native constituents in bovine milk and the mammary gland is the source of these enzymes, enhanced expression of leukocytes lead to upregulation of these enzymes which can be seen during mastitis. Furthermore, LDH activity has shown not only originate from plasma but also from granulocytes and lymphocytes in mastitic milk. Thus, it can be concluded that AP, LDH and NAGase are the sensitive markers in mastitis as they were increased significantly as Hp, M-SAA3 and SCC during early phase of inflammation.

1.4.3 APP and Milk Quality

One study that investigated (Åkerstedt *et al.* 2007) the relationship between APP and SCC in all clinically healthy animals found that a large proportion of animals (53%) had detectable levels of Hp and/or SAA. Although the presence of both APP was significantly more common in milk at higher SCC, SAA was found more frequently in the milk regardless of the sample type (quarter, cow composite and bulk tank) and had a significant relationship with SCC in quarter and composite milk samples. The reason why there was no relationship between Hp and SCC in bulk tank milk might be due to the dilution effect from quarter to bulk tank level. However, although the SCC of the cow composite sample was normal, SAA seemed to be detected in all four quarters in the cow composite sample. During certain physiological conditions (dry-off and oestrus), the permeability of blood-milk barrier is altered which leads to leakage of blood proteins into milk. Thus, the presence of APP in milk suggests the independent relationship between APP and SCC.

Hp and SAA have been studied on their use to assess milk quality. Several parameters concerning milk quality such as total protein, whey protein and casein levels, casein number, proteolysis and levels of fat and lactose in cow composite sample were compared with the level of both APP (Åkerstedt *et al.* 2009). Healthy milk samples with detectable level of Hp showed lower total protein and casein levels but higher SCC. Healthy milk samples with detectable levels of SAA showed lower casein number, lower lactose level but also higher SCC. Although total protein level in the milk is commonly used as a milk quality parameter, casein level is a more specific quality trait since total protein may include less valuable serum proteins that may increase during mastitis. It is said that measuring casein instead of total protein level in the milk is more relevant because the degree of casein degradation also affects the processing properties of the milk other than yield and quality of the dairy products. No relationship was observed between APP and proteolysis found in this study might be due to insensitive fluorescamine method to detect small differences in proteolysis. Proteolysis might also cause decreased casein levels and lower casein numbers as proteases particularly plasmin, bind to casein and hydrolyse the proteins to soluble peptides. Lower lactose level with detectable SAA is due to the release of specific peptides during hydrolysis of casein by plasmin that may serve as regulators in lactose secretion. However, lactose is not sensitive and specific enough to be detected in the bulk tank milk and thus, not suitable to be used as milk quality parameter. Furthermore, in samples with both APP detected, reduced whey protein level was observed which might be due to reduced synthesis of the milk proteins, including the whey proteins. And lastly,

no relationship was found between total proteins and casein levels/casein number with SCC but high SCC was found together with decreased lactose level and increased whey proteins (Åkerstedt *et al.* 2008).

The assessment on the composition of bulk tank milk is done to determine the quality of raw milk sample (Åkerstedt *et al.* 2009). Subclinical mastitis often constitutes a problem as the absence of macroscopic changes in the milk goes unnoticed and the milk from unaffected glands mixes with the milk from infected quarters. Bulk tank milk somatic cell count was reported as not a suitable predictor of raw milk quality especially for cheese production. Thus, investigation on Hp and SAA and their relationships with various raw milk quality parameters in bulk tank milk could be important to determine if the levels of APP can be related to the milk quality traits. In bulk tank milk samples with detectable Hp, lower casein content as well as lower casein number were found due to higher proteolytic activity in these samples. Proteolysis is seen in bulk tank milk but not in cow composite milk samples because of different factors. The fact that bulk tank milk consists of commingled milk from different milkings as well as milk from non-infected and subclinically infected quarters contribute to presence of proteolytic activity in bulk tank milk. Furthermore, the longer storage time before collection and freezing allows proteolysis to proceed for a longer time. Decreased levels of both APP were related to reduce lactose level as damaged epithelial cells contribute to decreased lactose synthesis. Proteolysis of β-casein released the peptides with a regulatory effect on lactose secretion (Silanikove *et al.* 2000). Thus, it can be concluded that APP can be serve as potential candidates to predict the raw bulk tank milk quality in relation to protein quality because the presence of APP in milk is related to disadvantageous changes in milk composition.

1.5 General Proteomics Application

Proteomics has become one of the most significant ‘post-genomic era’ tools due to advances in proteomic technologies which allowed an extended experimental approach to investigation of biological systems. Originally, the initial proteomic experiments which rely on two-dimensional gel electrophoresis (O’Farrell 1975) and application of chromatography techniques (Neverova & Van Eyk 2002) could not be used for broad proteomic application due to difficulty in identifying proteins (Graham *et al.* 2005). However, this problem is overcome by an introduction of mass spectrometry (MS) in combination with developing genomics databases (Graham *et al.* 2005).

Proteomics is defined as the study of the proteome; a full complement of expressed proteins from the genome of a given tissue, cell or biological fluid in all isoforms, polymorphisms and post-translational modifications (PTMs) at a particular point of time. (Wilkins *et al.* 1995; Graham *et al.* 2005; Manso *et al.* 2005; Hiendleder *et al.* 2005; Mitton & Kranias 2003). Proteomics provides a holistic and global approach to separate, identify, characterize and quantify proteins and their PTMs to provide information on protein abundance, location, modification and protein-protein interaction in a proteome of a given biological system (He & Chiu 2003; Stoughton & Friend 2005). Principally, proteomics offers an inventory of the different proteins and their modifications within a defined biological state and comparison of proteomes derived from different biological states, e.g. normal in comparison to diseased states or with treatment to non-treatment (Hiendleder *et al.* 2005).

According to Mitton & Kranias (2003), proteomics has advantages over genomics and transcriptomics because cellular protein composition and activity are positively correlated with changes in overall cellular function. Furthermore, proteomic methods have the potential to generate a bulk of data quickly and they are able to generate high-throughput systems which are useful to analyze a complex biological system. Furthermore, proteomics is able to determine the time course of protein behaviour and to characterize the presence and relevance of PTMs to a given process.

While its potential usage is outstanding, proteomics faces three major challenges (Mitton & Kranias 2003). First, cellular proteomes are highly dynamic with changes in the proteome occur at the same time as changes in cellular functions. Thus, great care needs to be taken to standardize and interpret the results. Second, proteomics has to deal with a variety of proteins in terms of its molecular weights, partition coefficients (hydrophobicity/hydrophilicity), electrostatic charges, ion chelation properties and PTMs (glycosylation, lipid conjugation and phosphorylation). This also complicates the detection of low-abundance proteins (Hiendleder *et al.* 2005). Finally, technically difficult and time-consuming proteomics methods have been considered as a major hurdle although vast improvements in the last decade have improved its reliability and efficiency.

1.5.1 Proteomics in Animal Sciences

The use of proteomic methods in animal sciences can help researchers to identify key proteins and their changed expression in response to different experimental conditions. Furthermore, it will provide data libraries of the abundant proteins expressed in a single

cell, cell compartments, tissues and body fluids (Lippolis & Reinhardt 2005; Cho *et al.* 2006; Reinhardt & Lippolis 2006; Radosevich *et al.* 2007). These proteins derived from pathogens or hosts are important to examine the status of disease progression and recovery, organ development or host responses to stress. Furthermore, it also helps with the intervention of diagnostic tests, development of new therapies or even on a discovery of disease biomarkers (Lippolis & Reinhardt 2008).

With the technologies of proteomics promised to provide the identification of proteins that are differentially expressed in normal and disease conditions, naturally occurring animal diseases can be studied to improve the health of animals which in turn may lead to improvements in the characterization, clinical management and treatment of human diseases through comparative medicines (Doherty *et al.* 2008).

For example, dilated cardiomyopathy which occurs in the Simmental-Red Holstein crossbreed of cattle has been studied to investigate its molecular pathogenesis (Weekes *et al.* 1999). This study revealed that poly-ubiquitination of proteins was increased in this disease and may play a role in heart disease. Paratuberculosis or Johne's disease has been implicated as a possible factor in the development of Crohn's disease in humans (Grant 2005). Ten proteins have been identified as up-regulated proteins in naturally occurring Johne's disease which provide an important fact for the host-parasite relationship which can act as model system for the parallel Crohn's disease in human (Hughes *et al.* 2007).

Proteomics has been applied to study the innate immunology pathways in stress-induced immunosuppression (Lippolis *et al.* 2006a; Mitchell *et al.* 2007). One of the bovine innate immunity proteins discovered was histone H2A.1 in bovine milk (Lippolis *et al.* 2006b) which involved in neutrophil killing mechanism (NET) (Brinkmann *et al.* 2004). Additionally, proteomic investigation on milk fat globule membrane proteome (MFGM) revealed that mammary epithelial cells may be directly involved in signalling the early recruitment of innate immune cells due to the presence of innate immune signalling molecules, TLR2, TLR4 and CD 14 (Reinhardt & Lippolis 2006). This study on MFGM also provides new data on mammary development in early lactation.

Proteomic analysis on bacterial-causing diseases helps in developing new diagnostic test from new protein markers and also development of vaccine from newly identified candidate proteins. Examples of animal diseases which used similar proteomic approaches are Johne's disease (Radosevich *et al.* 2007*), Brucella abortus* (Connolly *et al.* 2006),

Pasteurella multocida (Boyce *et al.* 2006) and *Mycobacterium bovis* (Mollenkopf *et al.* 2004).

The proteomes from 3 important bacteria used in the production of dairy products; *Lactobacillus bulgaricus, Lactococcus lactis* and *Streptococcus thermophiles* have been studied for any protein changes resulting from growth in laboratory medium or in milk (Rechinger *et al.* 2000; Derzelle *et al.* 2005; Gitton *et al.* 2005). This data provides new information to food scientists in modifying these bacteria to achieve good food production practices.

Proteomic approaches also been widely used to examine post mortem changes in slaughtered beef and pork that affect the quality of the meat (Jia *et al.* 2006a,b; Sayd *et al.* 2006; Laville *et al.* 2007; Suman *et al.* 2007). In addition, the variation in types and amounts or proteins in seminal fluid of bull has been studied using proteomic methods (Kelly *et al.* 2006). Although it is not known yet if these findings will lead to improvement of bull fertility, it is likely that such methods will have a good future in animal production profitability.

Because proteomics is able to screen large numbers of proteins simultaneously, it has become an important role at identifying disease biomarkers from the thousands of molecules present in a tissue of body fluid (Ilyin *et al.* 2004; Seo & Ginsberg 2005). In order for proteomics to be used effectively in biomarker discovery, a combination of efficient and stringent separation technologies and high-resolution mass spectrometry (Aebersold & Mann 2003; Rifai *et al.* 2006) are needed. Cairoli *et al.* (2006) applied proteomics to study the changes in protein expression in pregnant cows and in the peripartum periods in cows with or without postpartum uterine infections. They found that both haptoglobin and orosomucoid/α1-acid glycoprotein fluctuated at the time of calving. Orosomucoid is also known to significantly decrease in cows with endometritis compared with healthy cows which hold promise as a prognostic biomarker in cattle health post-partum. Proteomics has also identified PR-39 in pigs with respiratory disease as a potential disease biomarker at day 21 of infection (Hennig-Pauka *et al.* 2006).

1.5.2 Milk Proteomics

Although milk proteins have been studied for well over 50 years, only recently have proteomic techniques been widely used to study milk and milk products by separation of major milk proteins including caseins (αs_1-, αs_2-, β- and κ-casein) and whey proteins (α-

lactalbumin, β-lactoglobulin and bovine serum albumin) (O'Donnell *et al.* 2004; Manso *et al.* 2005).

The milk proteome is an extremely complex entity due to the presence of several molecular forms of many of the proteins as a consequence of different genetic variants, changes in the level of phosphorylation or glycosylation as well as the localization of PTMs of milk proteins (Eigel *et al.* 1984). Furthermore, there are a number of lower abundance proteins such as lactoferrin, immunoglobulins, glycoproteins, hormones and endogenous enzymes (Fox & Kelly 2003) present in the milk which may also be studied by means of proteomic approaches.

An early observation on bovine and human milk proteins was first reported by Anderson *et al.* (1982) using high resolution 2 DE. Major milk proteins were demonstrated in this 2D mapping study which included α-LA, β_2-LG, α- and β-caseins, 4 post-transitionally modified κ-chains, para κ-casein, Lf and albumin. Studies by Galvani *et al.* (2000) performed on powdered and fluid milk samples using MALDI analyses revealed the electrophoretic position of common high abundance proteins (caseins, β-lactoglobulin, etc) that can be found in milk and with modifications such as proteolysis, glycosylation and phosphorylation.

Normally, low-abundance proteins in milk are rarely seen on traditional 2DE maps due to high amount of major proteins in milk which obscure the detection of low-abundance proteins. Incomplete solubilization during sample preparation can also reduce the detection of low abundance proteins (Manso *et al.* 2005). However, low-abundance milk proteins account for approximately 5% of the total milk proteins and a number of studies have been applied on these proteins to aid in the search of their biological role and impacts on animal and human health (Lippolis & Reinhardt 2005; Reinhardt & Lippolis 2006; Fong *et al.* 2008; Smolenski *et al.* 2007; Reinhardt & Lippolis 2008). Low abundance proteins are frequently thought to have an important role as regulatory proteins, signal transduction proteins, receptors and enzymes (Fox & Kelly 2003).

An early study of low-abundance milk proteins was done using human colostrum and mature milk samples (Murakami *et al.* 1998). In total, 22 low-abundance proteins were identified using 2DE followed by microsequencing in both human colostrum and mature milk samples. Interestingly, no significant difference was found on 2DE patterns of low-abundance human milk proteins between colostrum and mature milk samples.

Further study on milk proteins was carried out by Yamada *et al.* (2002) using the same proteomic methods (2DE and microsequencing) but with bovine colostrum and mature milk. In this study, 29 low-abundance proteins were identified with several proteins being present only in bovine colostrum such as gelsolin, chitinase3-like 1, apolipoprotein H, complement C3 and fibrinogen β-chain. There may be important in the health and development of calves during early lactation. They also observed significant difference in 2DE patterns of low-abundance proteins between bovine colostrum and mature milk samples.

Another study by Baeker *et al.* (2002) demonstrated the expression of a group of proteins from the whey from inflamed mammary quarters. These proteins were identified by MALDI-TOF as isoforms of lipocalin-type prostaglandin D synthase and suggested to be a new biomarker of bovine mastitis.

Low-abundance proteins in human colostrum were studied by Palmer *et al.* (2006) using 2D-LC MS/MS, who found around 151 proteins of which 83 proteins were not previously reported. In this study, immunoabsorbents were applied to remove the major proteins: secretory IgA, lactoferrin, alpha-lactalbumin and human serum albumin (HSA). Most of the newly identified proteins exhibited many important functions for the newborn's immunity, growth and nutrition such as complement C1r-like proteinase, complement factor I, calgranulin A, calgranulin B, cysteine-rich secretory protein-3, mucin 5B, neutrophil gelatinase-associated lipocalin, and uteroglobinrelated protein 2.

The whey proteins are the soluble part of protein fractions. Major components of whey proteins include Igs, α-LA, β-LG, lactoferrin (Lf), serum albumin and lactoperoxidase (Lp) (Severin & Wenshui 2005). According to Casado *et al.* (2009), whey proteins can be further separated using ultrafiltration to concentrate the proteins or generates whey protein concentrates (WPC) respectively.

Proteomic approaches have been used to study whey proteins particularly in relation to the beneficial effects of these whey proteins and their bioactive peptides as functional ingredients in food (Michaelidou & Steijns 2006; Toba *et al.* 2000). Furthermore, many low-abundance proteins which arise from proteolytic activity from the major milk proteins, extrusion or leakage from the plasma or mammary epithelia (Hogarth *et al.* 2004; Larsen *et al.* 2006) or are products of the somatic cells (Kelly & McSweeney, 2003 ; Paape *et al.* 2003) might contribute to the health benefits of whey proteins.

Various fractionation methods have been employed to increase the resolution of such low-abundance proteins in whey fraction such as isoelectric focusing (IEF) (Zuo & Speicher 2002), affinity tagging (Holland *et al.* 2006) and immunoabsorption (Yamada *et al.* 2002). In one study by Fong *et al.* (2008), the use of semi-coupled anion and cation exchange chromatography to fractionate whey from late-lactation milk samples into acidic, basic and non-bound fractions has revealed a large number of minor whey proteins which have not been found previously in bovine milk such as factor XIIa inhibitor, tetranectin, CD5 antigen-like, complement components C4, C8, C9, factors I and D as well as nucleobindins.

In a recent work by D'Amato *et al.* (2009), the use of a combinatorial peptide ligand library (CPLL) has been shown to enhance the low-abundance protein species in milk. This work which comprised of IEF in gel followed by immunoblotting with unambiguous detection of polymorphic IgG revealed 100 unique gene products in milk which were not previously mentioned. In total, about 149 proteins were presented in a comprehensive whey proteins list under a combination of untreated and treated colostrum and mature milk with CPLL1 and CPLL2 (library 1 and 2 respectively).

However, Le *et al.* (2011) demonstrated that by using ion exchange to fractionate milk proteins, higher sample load was allowed which in turn, allowed more whey proteins to be identified. The total of whey proteins found was double the number of proteins identified using CPLL with 293 proteins found in bovine colostrum and mature milk. About 217 proteins were found in both bovine colostrum and mature milk whilst 36 proteins were only found in colostrum and 40 proteins came only from mature milk. Semi-quantitative analysis also revealed several lower-abundance proteins being up-regulated in colostrum such as CLU (complement system), MFGE8 (antiviral activity), CATHL1 (antibacterial activity), SAA3 (acute-phase protein), CD5L (immune system), PGLYRP1 (innate immunity and antimicrobial) and CFB (complement system) that function as natural defence mechanism.

A study on human whey proteome was performed by Liao *et al.* (2011) using LC-MS/MS with enrichment methods using Proteominer Protein Enrichment Technology. Major whey proteins such as Lf and α-LA or even casein subunits were still detected when using this approach but a total of 115 proteins have been identified of which 38 proteins not being mentioned before in human milk. These proteins were known to perform a wide variety of physiological functions in milk. Relative quantification of these minor proteins also showed that α -1-antitrypsin, α –lactalbumin, carbonic anhydrase 6, chordin-like protein 2,

galectin-3-binding protein, lactadherin, lactoferrin, prolactin-inducible protein, and tenascin were significantly expressed in higher levels in both colostrum and 1-month milk whey with immune modulating functions.

Proteomic studies on milk fat globule membrane (MFGM) provide an understanding on lipid synthesis and secretion in the milk. By characterizing its proteins associated with this membrane, one may be provided with information on signalling and secretory pathways of the mammary gland and eventually the cellular origin of the membrane (Casado *et al.* 2009). However, MFGM is known to be highly hydrophobic and thus, solubilization is difficult. To overcome such problems, various solubilization strategies (Quaranta *et al.* 2001; Cavaletto *et al.* 2002) have been employed including the use of detergent or reducing agent solution as well as by extraction using organic solvent that may help to improve the recovery of MFGM proteome (O'Donnell *et al.* 2004).

In early studies, MFGM proteins were identified as mucins, xanthine oxidase and butyrophilin) by immunological methods and N-terminal sequencing (Cavaletto *et al.* 2004). Mather *et al.* (2000) also identified MFGM proteins using traditional biochemical methods which were slow, laborious and addressed only one protein at one particular time. In humans, multiple forms of both butyrophilin and lactadherin were found after combining SDS-PAGE with sequence analysis (Cavaletto *et al.* 1999). Meanwhile, in bovine, major proteins associated with MFGM were demonstrated by Mather (2000) which includes mucin 1, xanthine oxidase, CD36, butyrophilin, adipophilin, lactadeherin and fatty acid binding protein.

Several studies on human MFGM proteins have been investigated. According to Quaranta *et al.* (2001), about 23 proteins identified by microsequencing and MS analysis confirming the presence of lactadherin and butyrophilin as the major glycoproteins in human MFGM. Other proteins that have been revealed include adipophilin, disulfide isomerase ER-60, apolipoprotein J as well as carbonic anhydrase which were not previously mentioned in human milk.

Charlwood *et al.* (2002) further demonstrated the presence of previously resolved MFGM proteins which were α-LA, Lf, lysozyme precursor, β-CN, clusterin, milk fat globule EGF-factor 8 protein and polymeric IG receptor precursor. By characterizing the N-linked glycans found in MFGM proteins, multiple fucosylation products can be studied which give rise to maternal preparation to the infant's immune system.

The development of 2-DE database for human MFGM proteins has led to the characterization and identification of 107 protein spots with multiple PTMs on lactadherin, butyrophilin, Lf, adipophilin and carbonic anhydrase. Of these proteins, 46% were known previously as MFGM proteins composition, 10% involved in folding and protein destination and another 9% involved in intracellular transport and/or receptor activity. Also, the discovery has been made of low-abundance proteins in MFGM which involved in cell development and differentiation, cell motility and signal transduction.

Reinhardt & Lippolis (2006) first reported the proteomic study on bovine MFGM which aimed to provide a good research foundation on the secretory mechanism of mammary gland in this species. They applied an extraction of MFGM using carbonate to enrich the intrinsic membrane proteins of MFGM and found 120 proteins from bovine MFGM. Most of these proteins were associated with membrane/protein trafficking or cell signalling. The most significant findings of this study were the presence of CD14, TLR2, and TLR4 on MFGM suggesting a role of protecting the mammary gland against infection.

Later, Reinhardt & Lippolis (2008) used a more advanced proteomic approach to identify and measure the level of protein expression changes in MFGM from colostrum to milk on day 7 of lactation. An amine-reactive isobaric tag (iTRAQ) was used to quantify protein changes in MFGM between colostrum and day 7 milk and revealed 138 MFGM proteins in which 26 proteins were upregulated whilst 19 proteins downregulated in d 7 MFGM compared with colostrum MFGM. Amongst proteins that were significantly upregulated individually in d 7 MFGM were mucin 1 and 15, the tripartite complex of proteins of adipophilin, butyrophilin, and xanthine dehydrogenase as well as proteins involved in lipid transport synthesis and secretion (acyl-CoA synthetase, lanosterol synthase, lysophosphatidic acid acyltransferase, and fatty acid binding protein).

Vanderghem *et al.* (2008) have established a simple and fast method to purify the MFGM by using Zwitterionic detergents (4% (w/v) CHAPS). This method proved to remove the majority of skim milk proteins as no caseins were found on their 2-DE gel. A total of 230 proteins spots were excised and analysed by MALDI-TOF/TOF and further characterized by PMF and MS/MS. Amongst the proteins identified were butyrophilin, lactadherin, adipophilin, xanthine dehydrogenase/oxidase and fatty acid binding proteins as previously mentioned.

Over the past few years, an increasing interest in quantification of proteins in MFGM has been seen. This is due to the important bioactivity of MFGM proteins in different health

benefits in human such as anti-cancer effects (Spitsberg & Gorewit 2002), anti-bacterial effects (Schroten *et al.* 1992; Wang *et al.* 2001) and anti-viral effects (Murgiano *et al.* 2009). Thus, Fong & Norris (2009) have used the absolute quantification (AQUA) technique to quantify the 6 major MFGM proteins in butter milk protein concentrate. The released peptides were measured at the same time using liquid chromatography-high-resolution selected reaction monitoring-mass spectrometry (LC-HSRM-MS).

Due to the importance of the MFGM efforts have been made to increase the efficiency of sample preparation in the protein extraction and solubilization prior to proteomic analysis of MFGM protein, Bianchi *et al.* (2009) compared 4 different types of lysis buffers with different detergents and chaotropes together with delipidation to maximize the visualization of MFG protein expression profile. In conclusion, delipidation, urea/thiourea combination and CHAPS/TritonX100 application as surfactants resulted in a proper MFG protein pattern visualization. In all, 82 protein spots with 42 different polypeptides were identified by MS and a reference 2-D map of cow MFGM was produced in this study. The majority of proteins identified were involved in lipid synthesis or in fat globule secretion.

Affolter *et al.* (2010) have used 3 different qualitative and quantitative analytical profiling on two MFGM enriched milk fractions (whey protein concentrate; WPC and buttermilk concentrate; BMC). An LC-MS/MS-based shotgun approach identified 244 proteins in WPC and 133 in BMP with 4 main functional categories of proteins (signal transduction, proteolysis, immunity and defence and lipid metabolism). A second approach using label-free profiling gave a rapid and efficient semi-quantitative comparison of the top 34 proteins present in both fractions. Lastly, the application of stable isotope dilution combined with LC-MRM-MS/MS technology to quantify proteins gave a highly flexible and adaptable analysis for a large number of proteins in a complex mixture.

The complexity of milk in its protein heterogeneity arises from significant levels of phosphorylation and glycosylation together with genetic variation, proteomic analyses have been used for the discovery, detection and characterization of these post translational modifications (Baeker *et al.* 2002; Charlwood *et al.* 2002). The majority of caseins exist as mixture of phosphoproteins. Mamone *et al.* (2003) identified more than 30 phosphorylated caseins using combined polyacrylamide gel electrophoresis with MALDI-TOF and nanoelectrospray-tandem mass spectrometry (nESI-MS/MS). Holland *et al. (*2004) in other hand identified 10 forms of κ-casein at different isoelectric point (pI) values as well as two phosphorylated and glycosylated forms.

Glycosylation also occurs in numerous milk proteins particularly on both *N*- and *O*-linked glycoproteins. As described by Holland *et al.* (2004), The *O*-glycosylation sites were identified by tandem MS with 10 different glycoforms of κ-CN were resolved and identified. Charlwood *et al.* (2002) on the other hand studied the N-linked glycosylation on four major proteins of the human MFGM namely clusterin, lactotransferrin, polymeric immunoglobulin receptor and lactadherin which possess a wide range of different sugar motifs. Proteins were separated and identified by MALDI-TOF analysis while Tandem MS and nESI source were used to identify the sugar constituents of isolated glycans.

1.5.3 Proteomics on Bovine Mastitis

Changes in the milk proteome during bovine mastitis could provide a significant insight on variation in protein composition that could lead to the discovery of protein biomarkers for bovine mastitis. Furthermore, by applying proteomic analyses, it may be possible to identify specific and potential protein target for therapies and immune manipulations against bovine mastitis (Yong *et al.* 2009).

Hogarth *et al.* (2004) applied pre-fractionation to remove abundant caseins from milk so that proteomic analyses of mastitic whey in comparison with normal whey could be investigated. However, despite an attempt to remove caseins, many other higher abundance proteins were seen from the results of this study and few low abundance proteins were apparent suggesting an alternative approach to remove these high abundance proteins. Serum albumin and serotransferrin were among the proteins found to be present or increased in mastitic whey. It is interesting to see that normal major whey proteins such as β-LG, α-LA and the casein remnants were absent from whey samples from cases of clinical mastitis.

Smolenski *et al.* (2007) analyzed mastitic milk for the presence of minor milk proteins involved in host defence mechanism. By using direct liquid chromatography-tandem mass spectrometry (LC-MS/MS), and two-dimensional electrophoresis (2-DE) followed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS), several proteins involved in host defence were identified in bovine milk such as chaparonins, proteins secreted from neutrophils, lymphocyte proteins, DNA-binding proteins, enzymes, transport proteins and structural proteins probably present as a consequence of the shedding and subsequent lysis of somatic cells in milk. A number of host defence proteins were also found in MFGM and there were significant differences in the MFGM proteins between mastitic and healthy milk.

Furthermore, Boehmer *et al.* (2008) successfully identified complement factors C3 and C4 and the apolipoproteins A-IT and C-Ill in mastitic whey samples inoculated with *E. coli*. It has proved that by using 2-DE and MALDI-TOF PSD, the identification of low-abundance proteins can be achieved without prior sample clean-up. In addition, a wide range of differentially expressed proteins were identified in mastitis whey compared with normal whey due to larger sample size which improve the discovery of protein biomarkers in coliform mastitis.

Proteomic approaches have also been applied to study protein expression of the mammary gland from both normal and clinical mastitic dairy cows as done by Yang *et al.* (2009). Six differentially expressed proteins were identified by tandem MS such as haemoglobin-beta, κ-CN and Tryptophanyl-tRNA-synthetase (TrpRS) which can be found from healthy mammary gland tissues as well as cytochrome C oxidase and annexin V which were identified from mastitic mammary gland tissues.

Over the years, an interest on quantification of the abundance of proteins exists in a complex biological fluid has grown tremendously. In milk, Boehmer *et al.* (2010) have applied an LC-MS/MS label-free approach to quantify relative changes in identified proteins over a time course following *E. coli* infection of the udder. A number of medium- to high-abundance proteins such as α_{S1}-, α_{S2}- β-, and κ-caseins and whey proteins serum albumin, β-lactoglobulin, and α-lactalbumin were found in all whey samples at all time points. In addition to that, lower abundance proteins of potential use as inflammation markers were also characterized such as lactoferrin, transferrin, apolipoprotein AI, fibrinogen, glycosylation-dependent cell adhesion molecule-1, peptidoglycan recognition receptor protein and cyclic dodecapeptide-1.

Danielsen *et al.* (2010) have also applied quantitative proteomic approaches to assess the protein differential expression in the milk proteome of individual cows before and immediately after LPS challenge. In all, 49 proteins were found to be significantly up-regulated during LPS challenge including apolipoproteins, complement C3 and C4 as well as other anti-inflammatory proteins.

Pathway analysis can be incorporated when studying the enriched proteome that emerged during *E. coli* and *S. aureus* responses as done by Ibeagha-Awemu *et al.* (2010). The proteomic profiles of whey milk from naturally infected *E. coli* and *S. aureus* in comparison to whey from healthy cows were revealed by one-dimensional, liquid chromatography tandem MS (LC-MS/MS). A total of 73 proteins were found to be

differentially expressed between normal and whey from quarters infected with *E. coli* appeared to cause more significant alteration in the concentration of the affected proteins than *S. aureus* itself. The acute phase response signalling pathway was the most significant enriched canonical pathway in this study. In addition, more lower abundance proteins were identified as compared to other previous studies such as the serpins and proteins with binding activities (glucose phosphate isomerizes and PFN1)

Proteolysis of casein which is known to increase in bovine milk during mastitis has been studied by Larsen *et al.* (2010) after infusion of lipoteichoic acid from *S. aureus*. According to 2-D gel electrophoretograms, hydrolysis of β- and α_{S1}-caseins occurred in milk 6 hours after infusion with the toxin. Those peptides were subsequently separated by reversed-phase HPLC and C18 columns and further identified by MALDI-TOF MS/MS. About 20 different peptides were successfully characterized and confirmed to originate from α_{S1}- and β-CN.

Proteomic study on different pathogens causing bovine mastitis could also provide important data on the proteome of bacteria isolated from mastitis cases. This could aid in defining specific vaccine and therapeutic targets as suggested by Taverna *et al.* (2007). They produced a (2-DE) reference map of surface associated proteins of isolated *S. aureus* from a case of bovine mastitis with protein identification using MALDI-TOF MS.

1.6 General Peptidomics

Peptidomics is regarded as the sequel to the proteomic technologies and is one of the new branches of chemical biology based on post-genomic technological platforms. The term peptidome refers to the whole set of endogenous peptides present in a given biological sample (Ivanov *et al.* 2009) and peptidomics is a comprehensive study of all natural peptides in an organism, a tissue or a body fluid (Clynen *et al.* 2001; Schrader & Schultz-Knappe 2001). In the previous discussion on proteomics, 2D-PAGE is often utilized to separate and visualize the protein complement present in any cells, tissues and fluids followed by identification of expressed proteins by the MS (Baggerman *et al.* 2004). However, due to major limitation of 2D-PAGE in detecting molecules between 10kDa and 200kDa, these standard proteomics approaches are unsuitable for the analysis of peptidomes (Ramström & Bergquist 2004). It is thought that the very important groups of peptides and neuropeptides are outside the range of this mass region (Baggerman *et al.* 2004). Furthermore, according to Schulte *et al.* (2005), due to high complexity and concentration of other proteins in a sample, 2D-PAGE is not an appropriate method to

detect these important endogenous peptides especially as they are often present at very low concentrations.

Peptides are referred to as low-molecular weight polypeptides molecules with the mass of <20kDa (Ramström & Bergquist 2004, Hu *et al.* 2009). They are often the products of proteins after proteolytic cleavage of the protein precursor by active proteases that have very specific functions as mediators and regulators of many physiological processes in nervous, endocrine, immune and other systems (Ivanov *et al.* 2009, Hu *et al.* 2009). Peptides just like proteins change dynamically according to different physiological and pathological conditions and are influenced by metabolism, disease states, stress, drug intervention, genetic modifications and other external factors (Ivanov *et al.* 2009; Ramström & Bergquist 2004). Although the circulating peptides in the body fluid or tissue may reflect the biological events, the real function of specific peptides is frequently unknown and further studies were needed (Petricoin *et al.* 2006). According to Hu *et al.* (2009), peptides in the peptidomes are divided into two groups; first, the bioactive peptides that have important roles in biological processes such as cytokines, hormones, growth factors and MHC class 1 peptides; and second, the degraded peptides arising from proteolytic cleavage.

Peptidomics was only introduced at early 2000s after several advances in the development of MS techniques coupled with the completion of the genome sequences in majority of living species (Ivanov *et al.* 2009). Subsequently, several fields of studies have successfully employed peptidomic approach such as neuroendocrine studies (Baggerman *et al.* 2002; Clynen *et al.* 2003), biomarker discovery (Yuan & Desiderio 2005; Schrader & Salle 2006) and therapeutic development against oncology, neurodegeneration, osteoporosis and others (Clynen *et al.* 2008).

In earlier years, the study of peptides involved the purification of peptides from tissue extracts with the extracted peptides being characterized by Edman sequencing (Ramström & Bergquist 2004). As a result of the introduction of MS, hundreds of newly identified peptides were discovered and sequenced (Ivanov *et al.* 2009; Ivanov *et al.* 2005) and termed as peptide profiles (Slemmon *et al.* 1997) or peptide pools (Karelin *et al.* 1998). It is not an easy task to analyse peptides in a real sample due to their low concentrations, the complexity of the sample together with the presence of other components in the sample in a wide range of concentration and the availability of the analytes at different chemical properties (hydrophobicity, size, pI) (Ramström & Bergquist 2004). Thus, a very sensitive, unbiased method should be applied with short running time, and which is easily automated

and able to handle a great abundance of data. The different peptidomics approaches will be discussed in the final chapter.

1.6.1 The presence of peptides in bovine milk and dairy products

Milk or dairy products possess an important role in providing the main nitrogenous nutrients in human food as it has a high content of proteins. Many breakdown products of proteins upon ingestion of milk proteins are called bioactive peptides (Srinivas & Prakash 2010; Schmelzer *et al.* 2004). The hydrolytic reactions of digestive enzymes present in the gastrointestinal system as well as in vitro proteolysis by proteolytic enzymes such as plasmin, (Bastian *et al.* 1991), cysteine proteases or acid proteases (Wedholm *et al.* 2008) lead to the production of these bioactive peptides. Bioactive peptides in milk or dairy products such as cheese and yoghurt provide a health enhancing factor that can be incorporated into functional foods and /or used as nutraceuticals (Meisel & Fitzgerald 2003). Most of the bioactivities of these peptides remain in a latent state within the protein sequence and to exert their functions, proteolytic processes must be initiated (Smacchi & Gobbetti 2000; Srivinas & Prakash 2010). However, some of bioactive substances in milk are fully active as proteins (Smacchi & Gobbetti 2000). These bioactive peptides in dairy products serve numerous biological and physiological functions.

The functions of bioactive peptides in dairy products have been reviewed by Smacchi & Gobbetti (2000) which include opioid characteristics, antihypertensive, antimicrobial and antithrombotic effects. Examples of these peptides include peptides from α_{S1}- and β-CN (Maruyama & Suzuki 1982), β-lactoglobulin (Mullaly *et al.* 1996), α-lactalbumin (Parker *et al.* 1984), and lactoferricin (Yamauchi *et al.* 1993). Some of these milk-derived peptides exert multi-functional activities such as β-casomorphins and casokinins; both are Angiotensin-1 Converting Enzyme (ACE)-inhibitory and immunostimulatory (Grant *et al.* 1993; Meisel & Schlimme 1994).

Smacchi & Gobbetti (2000) have also produced lists of bioactive peptides present in different types of dairy products such as milk and milk hydrolysates, fermented milks and cheese. Proteolytic bioactivities depended upon the differentiation, functional states as well as the intramammary inflammation of mammary gland (Schanbacher *et al.* 1997). Various peptides were also found in fermented milk which influenced by the type of bacteria starter used. The production of bioactive and non-bioactive peptides during proteolytic event of cheese ripening may inhibit the lactic acid bacteria (LAB) peptidases in many ways.

In another study, Srivinas & Prakash (2010) have isolated and characterized the multifunctional properties of bioactive peptides from α-CN from bovine milk. To generate peptides from α-CN, several proteolytic enzymes were employed such as aminopeptidase, carboxypeptidase, fungal and bacterial proteases and chymotrypsin. However, chymotrypsin hydrolysates were chosen as they released smaller size of peptides. Peptides identified in this study showed multifunctional properties such as ACE inhibition, antioxidant activity, propyl endopeptidase inhibitory activities and antimicrobial properties.

In terms of technical determination and characterization of proteolytic products of dairy products, numerous methods have been employed. In early years, these include polyacrylamide gels with urea (urea-PAGE), denaturing agent (SDS-PAGE) or IEF techniques (Molina *et al.* 2000). With time, the separation and identification of peptides has been successfully performed by coupling these previous analytical techniques with the MS. Details on the various analytical tools on peptide studies will be discussed in the final chapter.

Most of the studies on bovine milk peptides revolved around proteolytic activities on casein, the major milk protein. Plasmin has been regarded as the main indigenous proteinase in bovine milk (Bastian *et al.* 1991; Somma *et al.* 2008) and it preferentially cleaves Lys-X and Arg-X residues (Le Bars & Gripon 1989). This enzyme activity increases with the stage of lactation, lactation number and severity of bovine mastitis (Politis *et al.* 1989; Schar & Funke 1986) and it leads to detrimental effects on milk quality due to decreased amount of intact CN.

Recio *et al.* (1997) have employed capillary electrophoresis (CE) with hydrophilically coated capillary and low pH buffer containing urea to separate proteolytic activites of plasmin and chymosin on caseins. They identified several casein breakdown products which include γ_1-CN A1, γ_1-CN A2, γ_1-CN B, γ_1-CN C, γ_2- CN A, γ_1- CN A3, γ_2- CN B, γ_3- CN A and γ_3- CN B including proteose peptones derived from different genetic variants of β-CN. Breakdown products of αS1-CN and κ-CN were also found which were produced by chymosin proteolysis.

Molina *et al.* (2000) employed CE to study the fractions of casein and its breakdown products treated with plasmin and chymosin from three different species of animals (cow, ewe and goat). It showed that hydrolysis of β-casein by plasmin gives rise to γ_1-CN, γ_2-CN and γ_3-CN. Additionally, they found a decrease in the peaks corresponding to α_{S2}-CN as it

was also very susceptible to hydrolyse by plasmin due to its high lysine residues (Le Bars & Gripon 1989). Hydrolysis of casein in cheese by chymosin and rennet was also studied with its main degradation products include para-κ-CN and α_{S1}-I-CN.

Another study on peptides derived from bovine β-CN by peptic cleavage was studied by Schmelzer *et al.* (2004). By using two types of MS analysis; MALDI-TOF MS with post-decay source and LC-MS/MS, they successfully identified 41 peptides with a high sequence coverage (75%). The peptides identified had lengths of between 2 and 36 amino acids which originated from the C-terminus of the protein.

Somma *et al.* (2008) compared two different approaches between capillary isoelectric focusing (cIEF) and ultra-thin layer isoelectric focusing (ultIEF) in combination with MS to characterize the products of β-CN digestion by plasmin from both bovine and water buffalo milk samples. The presence of a novel CN breakdown product was found from the hydrolysis of water buffalo β-CN which was not present in hydrolysis of bovine β-CN. They concluded that cIEF is faster, requires shorter processing times and does not involve staining when compared with ultIEF technique.

Apart from proteolytic activities, studies of peptides have also been employed to investigate the presence of adulteration in milk or other dairy products. The most common fraud observed was the addition of cow's or sheep's milk to goat's cheese due to seasonal fluctuations, availability and price (Guarino *et al.* 2010). Thus, the need of rapid, fast and reliable control methods to accurately determine the level of adulteration in milk or dairy products is increasing. Previously, the use of immunological technique (Levieux & Venien 1994), PCR-techniques (Bottero *et al.* 2003) or slab gel electrophoresis (Addeo *et al.* 1990) have been used to detect the presence of bovine milk proteins in dairy products from other species. However, all these techniques only provide semi-quantitative results and failed to detect small amounts of bovine milk proteins in ovine or caprine products (Muller *et al.* 2008). Other techniques include analysis of whey proteins, chromatographic techniques (De Frutos *et al.* 1991) and UV/VIS spectrophotometric detection (Cifuentes 2006) which allow quantitative analysis of milk proteins.

The fraudulent addition of rennet whey solids to dairy products can be determined by the detection and quantification of caseinomacropeptide (CMP), the fragment of κ-CN released by chymosin during milk clotting. It should be absent in normal milk but certain bacterial proteases that degrade κ-CN milk could give rise to the similar degradation products as CMP (Recio *et al.* 1997). Van Riel & Olieman (1995) successfully separated

the different forms (non-glycosylated forms) of CMP in their studies. It can be concluded that adulterations of dairy products with soya or milk from different species can be detected based on CE patterns.

Muller *et al.* (2008) demonstrated the use of CE coupled with ESI-MS to determine the adulteration of ovine/caprine milk by bovine milk. This technique was chosen due to electrophoretic clean-up of the milk samples and subsequent identification and quantification of latoglobulins. It was concluded that CE-MS analysis was a rapid, simple and accurate technique to determine and quantify amounts of bovine milk in milk of other species.

Another study on adulteration of dairy products was performed by Guarino *et al.* (2010) in which LC-MS/MS has been employed to identify and quantify the percentage of sheep's milk in cow's and goat's cheeses. Plasmin was chosen over other proteolytic enzymes for proteolysis due to simple chromatographic separation, mass spectra analysis and simple, fast and widely used protocol. This method appeared to be suitable to monitor adulteration as it allows quantitative detection of the use of sheep's milk in goat's or cow's cheeses.

1.6.2 Peptidomics in Bovine Milk/Bovine Mastitis

Due to the importance of peptides in animal physiology, various studies have dealt with this approach using a wide range of samples (cells, tissues or body fluids). In total, a very limited number of peptidomic studies were done to analyze the direct effect of intramammary inflammation on the changes of levels of peptides in bovine milk. However, an application of various techniques of protein/peptide separation coupling with MS (as described above) can be used to study the proteolytic activity in milk samples as proteolysis is increased during mastitis (Verdi *et al.* 1987).

Apart from plasmin, a principal milk enzyme which hydrolyses β-CN into γ-CN and proteose peptones and to a smaller extent hydrolyses α_{S1}- and α_{S2}-CNs; there are a number of native proteinases in milk derived from somatic cells or leaked out through the damaged somatic cells which also contributes to proteolytic activity in bovine milk (Larsen *et al.* 2006). PMN which are the main cellular immune components that increase during infection (Azzara & Dimick 1985) contain several enzymes including serine proteases, cathepsin G and elastase (Kirschke & Barrett 1987; Travis 1988; Owen & Campbell 1999). The lysosomes of somatic cells are also thought to contain cysteine proteases (cathepsin B, H, L and S) as well as acid proteases (cathepsin D) (Owen & Campbell 1999).

During mastitis, the increase of somatic cell counts is one of the important disease manifestations. With increasing cell counts, it was estimated that the peptide count in milk was also increased by a factor of 100 (0.03 to 2.71mg/ml) (Lindmark-Mansson *et al.* 2006). It is also thought that in high SCC milk, proteolysis is caused more by proteases apart from plasmin (Larsen *et al.* 2004; Kelly *et al.* 2006). In cheese production, higher SCC contribute to lower yield and poorer quality of cheese (Grandison & Ford 1986), increased moisture content of cheese and the activity of lysosomal enzymes in cheese (Marino *et al.* 2005) and less elastic with soft cheddar cheese due to degradation of α_{S1}-CN (Creamer & Olson 1982).

Wedholm *et al.* (2008) have studied the presence of peptides produced from proteolytic activities of different level of SCC in milk and demonstrated the responsible proteases based on their cleavage sites. Using RP-HPLC to separate the peptides and further characterized by MALDI-TOF MS/MS, peptides that successfully identified were in the range of mass from 1023 to 2000 Da which originated from α_{S1}-, α_{S2}- or β-CN. It was concluded that plasmin, cathepsin B, D and leucocyte elastase were responsible for these proteolytic activities.

Larsen *et al.* (2010) studied the proteolytic cascade in a model system which used the bacterial lipotechoic acid (LTA) or gram-positive *S. aureus* to mimic inflammation of the mammary gland. They have also employed RP-HPLC and MALDI-TOF MS/MS in this study and concluded that approximately 20 different peptides were identified originating from α_{S1}- and β-CN. The responsible proteases were plasmin, cathepsin B and D, elastase and amino- and carboxypeptidases.

In summary, by employing various methods of peptidomic analyses, the presence of peptides in mastitic bovine milk can be studied and characterized. These biological peptides may contribute to the discovery of important and novel mastitis biomarkers. Furthermore, identification of these peptides may be applied in the development of therapeutic interventions of bovine mastitis.

1.7 Animal-applied Metabolomics

Metabolomics is one of the new technologies that has gained interest and can be considered as important as other "-omics" studies such as genomics, transcriptomics and proteomics. The metabolome is a set of low-molecular weight metabolites that are present in a biological fluid, cell or organisms under different physiological states (Alvarez-

Sanchez *et al.* 2010). According to Kell (2006), metabolomic analysis is more promising for several reasons. Metabolites appear to be in smaller sizes, thus the sample processing can be reduced as compared to genes and proteins. It is also evident that preparation for metabolic profiling is less expensive than proteomics and more samples can be analysed to provide a good and reliable results and finally metabolomic technology is more generic than any other analysis.

A problem that the metabolomic analysis faced is lack of comprehensive and standardized methods. In order to make sure a generalized detection and identification of metabolites, an integration of various analytical approaches is being made [e.g., gas chromatography combined with mass spectrometry (GC-MS), liquid chromatography combined with mass spectrometry (LC-MS) and nuclear magnetic resonance(NMR)].

The implementation of metabolomics in milk and investigations of the pathophysiology of animal diseases has not been the objective of much research. To date, there is no major study on the metabolomics in bovine mastitis but there are relevant publications.

Metabolomic analysis has been used in milk in order to monitor a whole range of metabolic processes. One classical metabolomic measurement approach is by using chromatography (Martens *et al.* 2007) to analyse the fatty acid profile in bovine milk sample where it was used to quantify with variation in their chain lengths and unsaturation properties.

Another study by Boudonck *et al.* (2009) revealed the use of liquid and gas chromatography that successfully identified over 223 metabolites between 10 different milk varieties. Metabolic profiling was shown to possibly provide a distinct biochemical signature in different types of milk and a specific marker could be assigned which could describe specifically for particular milk.

Apart from examining milk metabolomics, the method has been applied to a few investigations of animal models. Metabolomic analyses have been used to study the metabolic changes in canine liver disease (Whitfield *et al.* 2005). Plasma samples were analysed using LC-MS and metabolites that were identified to increase significantly with liver disease were the taurine conjugates of the bile acids, cholic and chenodeoxycholic acids whilst 16:0-, 18:2- and 18:0-lysophosphatidylcholine were decreased in diseased animals. This analysis was able to distinguish animals with congenital portovascular anomalies from those with acquired syndromes (Moore *et al.* 2007).

Bertram *et al.* (2004) have studied splanchnic metabolism in cattle using proton nuclear magnetic resonance (H NMR) from arterial, portal and hepatic plasma. It was concluded that the methyl groups of propionate, 3-β-hydroxybutyrate and lactate were increased in spectral intensities in the H NMR portal plasma samples. As for hepatic plasma samples, due to hepatic uptake of short fatty acids (n-butyrate, propionate, iso-butyrate, valerate and iso-valerate), there was reduced in spectral intensities of these regions. In addition, a hepatic excretion of β-hydroxybutyrate and glucose resulted in increased in spectral intensities of these regions. These spectral differences can be used to describe changes in metabolites which could be supported by biochemical analyses.

In another study by Bertram *et al.* (2009), they have used the similar approach to investigate propylene glycol toxicosis in a Holstein cow. However, no significant metabolite differences could be detected in the biofluids of the cow which developed toxicosis. It could be due to the vitality of the metabolites or they might be present below the detection limit of NMR. Although there was a decreased in content of isopropanol,isobutyrate, β-hydroxybutyrate, acetate, acetone and acetoacetate shortly after feeding with propylene glycol, it might be due to reduce in fermentation activity in the rumen as well as reduce absorption in ruminal atony. It can be concluded that shallow breathing manifested by the affected animal was due to pulmonary vasoconstriction from the sulphur-containing compounds produced during fermentation of propylene glycol. However, no sulphur-containing compounds were observed in the NMR spectra.

The influence of different lactation stages on physiological and metabolic variables has been studied by Klein *et al.* (2010) using high resolution nuclear magnetic resonance (NMR) and mass spectrometry (MS). They have identified 44 milk metabolites in milk samples from individual cows during early and late lactation with an emphasis on metabolites related to energy metabolism. There was a large difference in the levels of acetone and β-hydroxybutyrate (BHBA) during early lactation. These two compounds are important biomarkers for subclinical ketotic conditions. The large discrepancies values suggest that different individual animals cope very differently with the metabolic stress during this period and it is highly true for high-producing animals.

In conclusion, the new technology of metabolomics has made it possible for researchers to study the effect of disease progression on the metabolome and could provide new, low molecular weight biomarkers to assess the health condition of animals. However, due to high diversity and variability of metabolome, there are no available analytical platforms that can define metabolites simultaneously (Dettmer *et al.* 2007). Thus, an efficient

sampling and separation of metabolites should be achieved so that low-abundance metabolites can be detected with the use of small sample volumes. Furthermore, data analysis and visualization tools, libraries, and databases for metabolomics need to be developed so that standardization in data analysis can be achieved.

1.7.1 Metabolites found in bovine milk

A number of studies have been performed to evaluate the presence of metabolites in bovine milk with earlier, non-metabolomic methods. Investigations which compared the presence of metabolites between mastitic milk samples and healthy milk samples (assuming low somatic cell counts and negative microbiological culturing) will be discussed further in Chapter 5, Section 5.1.2.

Amongst many metabolites found in bovine milk, fatty acids are one of the important molecules in milk. About 96% of lipid in bovine milk are triacylglycerols and were comprised of a molecule of glycerol and 3 esterified fatty acids (Jensen 1995). Fatty acids on the other hand are composed of 3 different groups based on the number of double bonds present in their chemical structure. They are saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). MUFA and PUFA form the unsaturated group of FA (UFA) (Soyeurt *et al.* 2011). Previously, the level of FA in milk was determined by GC after an extraction, followed by saponification and transmethylation but this method was expensive and time consuming. Thus, Soyeurt *et al.* (2011) have proposed to use mid-infrared spectrometry to quantify the individual content of FA in bovine milk. Ferrand *et al.* (2011) have also performed a mid-infrared spectrometry to estimate the main fatty acids in bovine milk by applying the genetic algorithm method to improve the quality of estimation. The variation observed in the milk fatty acid composition was determined by several factors such as diet; forage to concentrate ratio (Griinari *et al.* 1998) and intake of unsaturated fatty acids (Dhiman *et al.* 1999) as well as seasonal which consequently affected the dietary intake of the cows (Lock and Garnsworthy 2003).

An earlier study on the metabolite levels and enzyme activities in bovine mammary gland has been studied by Waldschimdt and Rilling (1973) at different stages of lactation. Previously, studies have been focussed on the mammary gland of laboratory animals for example rats (Wang 1960; Baldwin & Cheng 1969). This study however determined the level of metabolites in the udders of heifers and cows at peak and near the end of lactation as well as during dry period. Pyruvate, lactate and AMP were not significantly different at

different stages of lactation but quantities of malate, ATP and ADP were higher in cows with high milk yields. Additionally, pyruvate levels showed variations with season whilst the NAD: NADH ratios in mammary cytoplasm showed no significant differences between different groups of cattle. ATP: AMP ratios in mammary gland tissues were highest in lactating animals.

Hollis *et al.* (1981) studied the presence of vitamin D and its metabolites which include 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, 25,26-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in both human and bovine milk using chromatographic purification method coupled with ligand-binding assay. It is known that these antirachitic sterols in milk were the same as those in plasma though the concentration in milk is much lower. They are secreted into the milk, bound to plasma and/or cytosol binding proteins (e.g. vitamin D binding protein). Eventually these sterols migrate from the aqueous portion to the fat portion of the milk. Furthermore the levels of anticharitic sterols appear to be linked with dietary intake of these sterols.

With the use of reverse-phase high-performance liquid chromatography (RP-HPLC) Tiemeyer *et al.* (1984) determined all pyrimidine and purine compounds in milk to relate the nucleic acid metabolites to different rates of protein biosynthesis. Biosynthesis of proteins in the mammary gland is often accompanied by nucleic acids biosynthesis and degradation and consequently a turnover of pyrimidine and purine compounds. In this study, it was established that bovine milk contains nucleosides (uridine, cytidine, pseudouridine) and free bases (hypoxanthine) as well as ortic and uric acids. There are mainly two sources of metabolites in the milk; 1) derived from mammary cell and leukocytes or 2) possibilities of 5'-nucelotidase activity on the cell surface as described for blood cells. There are also two groups of metabolites based on the influence of milk production; 1) metabolites which are not affected by milk yield (hypoxanthine,uridine, cytidine and pseudoridine) and 2) decrease in amounts with increase in the rate of milk production (orotic acid, uric acid and allantoin). The close correlation between the milk yield and protein secretion showed that high milk production were also accompanied by increase rates of nucleic acid turnover which eventually lead to accumulation of nucleic acid metabolites in both mammary cells and in milk. This study showed rate-limiting steps in the catabolic pathways of pyrimidines and purines with its relationship with protein biosynthesis.

1.8 Aims of study

In the past few decades, it has been shown that various studies were employed for protein analysis in bovine milk as well as in bovine mastitis. Although clinical mastitis cases have been widely studied, it is important to take into account the subclinical cases of mastitis for the discovery of important protein biomarkers of mastitis. With the development of new and advanced non-gel approaches (peptidomics and metabolomics), a number of lower molecular weight molecules such as peptides and metabolites can be discovered which are significant in detecting the presence of bovine mastitis. Thus, the overall objective of this study is to apply various proteomic and other non-gel methodologies (peptidomics and metabolomics) to discover significant disease biomarkers in bovine mastitis.

Chapter II: Acute Phase Proteins in Bovine Mastitis

2.1 Introduction

2.1.1 Bovine Acute Phase Proteins and Acute Phase Reactions

As described in Chapter 1, Section 1.4, acute phase proteins (APP) are a group of blood proteins that are synthesized and change in concentration following various stimuli such as infection, inflammation, surgical trauma, stress, neoplastic growth or immunological disorders (Gordon & Koy 1985; Gruys *et al.* 1999, Murata *et al.* 2004) which initiate the acute phase reaction (APR) which is also part of non-specific innate immune responses that involves restoration of homeostasis and inhibits the growth of pathogens (Murata *et al.* 2004; Eckersall & Conner 1988).

Following internal or external challenges, the body reacts by the release of pro-inflammatory cytokines as well as the activation of vascular system and inflammatory cells at the site of microorganism invasion (Gruys *et al.* 2005). The systemic reaction activated by cytokines caused an induction of hepatic metabolism which leads to the released of APP (Gruys *et al.*1994). Of the pro-inflammatory cytokines, interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β) are known to mediate the synthesis of APP (Nakagawa-Tosa *et al.* 1995; Alsemgeest *et al.* 1996; Yoshioka *et al.* 2002). Some of the APPs are increased during the APR while others, such as albumin, transthyretin (TTR, formerly called prealbumin), retinol binding protein (RBP), cortisol binding globulin, transferrin and albumin, which represent the negative APPs, are decreased (Eckersall & Conner 1988; Gruys *et al.* 2005). The positive APPs, which are mainly glycoproteins synthesized by hepatocytes, include haptoglobin, C-reactive protein (CRP), SAA, ceruloplasmin, fibrinogen and α 1-acid glycoprotein (α1-AGP) (Murata *et al.* 2004; Gruys *et al.* 2005).

Overall, the released of APP during inflammatory condition helps to bind and remove harmful substances following tissue damage, to protect host from further damage and lastly to eliminate pathogenic organisms (Baumann & Gauldie 1994).

2.1.2 Bovine Haptoglobin

Haptoglobin (Hp) is a major APP in bovine which strongly binds to haemoglobin, has anti-inflammatory properties and binds to CD11b/CD18 receptor of effector cells (El Ghmati *et al.* 1996). It also reduces the oxidative damage associated with haemolysis (Yang *et al.* 2003). Upon binding with haemoglobin (Hb), this stable complex of Hp-Hb is scavenged by phagocytes after recognition via CD 163, the specific surface receptor on macrophages (Schaer *et al.* 2002).

Bovine plasma Hp was recognized by Liang (1957) and Neuhaus and Sogoian (1961). However, negligible levels of Hp are seen in healthy cows with physiological levels in the plasma being less than 0.1mg/ml (Ritcher 1974). Upon immune stimulation, its circulating level can increases up to 100-fold (Conner *et al.* 1988; Conner *et al.* 1989).

Other than binding to haemoglobin, Hp also has an inhibitory effect on granulocyte chemotaxis, phagocytosis and bactericidal activity (Rossbacher *et al.* 1999). According to Xie *et al.* (2000), Hp is able to prevent spontaneous maturation of epidermal Langerhans cells or suppress T-cell proliferation (Baseler and Burrell 1983).

Hp is often associated with inflammatory conditions in cows. Increased Hp levels were seen in cattle with mastitis (Spooner & Miller 1971; Conner *et al.* 1986), liver abscesses and other inflammatory diseases including pyometra, reticulitis, traumatic pericarditis (Panndorf *et al.* 1976; Makimura & Suzuki, 1982). Moreover, detection of Hp as a highly polymeric complex with Hb was found in cattle with acute inflammation (Morimatsu *et al.* 1991). APP was not stimulated by viral diseases for example in inflammation by rinderpest virus (Spooner & Miller 1971) or in bovine leukosis (Panndorf *et al.* 1976). However, Eckersall *et al.* (1988) found smaller Hp response in some viral infections compared with bacterial infections. Just like any other APP, Hp concentration can be used to determine the severity status of the disease and the extent of tissue damage in affected cows (Murata et *al.* 2004). Thus, Hp has the potential on being a significantly useful parameter to measure the occurrence and severity of inflammatory responses.

Apart from infection, the level of Hp is raised in cows with fatty liver syndrome (Yamamoto *et al.* 2000; Katoh 2002), by starvation, from dexamethasone treatment (Yoshino *et al.* 1993), at parturition (Uchida *et al.* 1993), and in stressed calves associated

with travelling (Murata & Miyamoto 1993). However, Skinner *et al.* (1991) found that in cows with normal births, there was no significant increase in Hp indicating that Hp elevation at parturition is due to infection rather than trauma.

2.1.3 Haptoglobin in bovine mastitis

Bovine Hp has been demonstrated to be a selective indicator for the acute phase responses in animals. Skinner *et al.* (1991) demonstrated that Hp can be used to diagnose early bacterial infection, particularly mastitis. The Hp test is also known to be accurate, can be performed quickly and has similar sensitivity as other major bovine APP; the SAA (Boosman et al 1989). Skinner *et al.* (1991) concluded that the concentration of Hp between 0.2g/litre-0.4g/litre and above was an indication of infection.

Many studies have demonstrated that bovine Hp is one of the important APPs that could be used to diagnose bovine mastitis. It also has been proven that serum Hp levels raised with both experimental and naturally occurring diseases (Conners *et al.* 1986; Salonen *et al.* 1996; Hirvonen *et al.* 1996; Skinners *et al.* 1991; Hirvonen and Pyorala 1998; Eckersall *et al.* 2001; Ohtsuka *et al.* 2001).

Previously, serum bovine Hp was used to investigate its potential in diagnosing bovine mastitis. This is due to the fact that Hp is liver-derived protein (Koj 1974), synthesized by the hepatocytes in the event of acute phase reaction. As described previously, during the activation of acute phase reactions, the concentration of Hp can increase up to 100-fold from its normal level. Furthermore, its level in the normal serum is negligible and thus, the measurement of bovine Hp in serum has been used to analyse the effect of acute phase reaction during inflammatory processes. The increase of bovine serum Hp concentrations during mastitis is supported by various studies (Conner *et al.* 1986; Salonen *et al.* 1996; Horadagoda *et al.* 1999; Ohtsuka *et al.* 2001).

However it is now known that Hp can be detected in milk during mastitis. The first study that determined the presence of Hp in milk was performed by Eckersall *et al.* (2001). Measuring Hp level in milk to investigate the presence of intramammary inflammation was suggested to provide a more readily and accessible indication of infection. According to Nielsen *et al.* (2004), the use of APP in milk to assess the APR following inflammatory processes provides a more specific and sensitive diagnostic method than bacteriology and it is less influenced by the physiological stage of the cow than SCC or EC (Sheldrake *et al.* 1983; Biggadike *et al.* 2002). According to Grönlund *et al.* (2003), although increase in

serum Hp occurred during mastitis, it was not specific as increases in serum APP can be due to other acute inflammatory conditions, but measuring Hp in milk provides specificity.

It is first thought that an increased of Hp level in milk during bovine mastitis was due to transfer of these serum proteins synthesized largely by the hepatocytes into the mammary gland due to increased permeability of blood-milk barrier (Eckersall *et al.* 2001). This is based on the presence of other types of serum proteins such as serum albumin and protease inhibitor α-antitrypsin in the infected quarters as reported by Honkanen-Buzalski *et al.* (1981) and Honkanen-Buzalski & Sandholm (1981). Grönlund *et al.* (2003) also stated it was not clear whether APP found in the infected milk are locally produced or by an influx of these proteins from blood due to the change in the permeability of the blood-milk barrier.

However, Nielsen *et al.* (2004) suggested that higher Hp concentrations in the milk from infected quarters could be due to the production of these proteins by the mammary tissues. They also suggested that increased serum Hp concentration in the cows with mastitis was due to the access of Hp synthesized by the mammary tissues to the blood serum. Hiss *et al.* (2004) confirmed that bovine mammary gland can be considered as another extrahepatical source of Hp based on the observed Hp mRNA in mammary gland tissues (the teat, the cisternal region, the glandular parenchyma). The authors also suggested that the migrated leukocytes might contribute or solely account for the detection of Hp mRNA. Thielen *et al.* (2005) further demonstrated that Hp mRNA was present in bovine peripheral blood leukocytes and milk somatic cells and confirmed that leukocytes are another cellular source of mammalian Hp mRNA expression.

When comparing the increased level of Hp in milk to that of serum, it was found that the former occurred at 15h after experimentally induced mastitis by *S. aureus* while the earliest detection of increased serum Hp was at 24h postinfusion. Blood was therefore unlikely to be the source of immediate Hp elevation in milk (Eckersall *et al.* 2006). The probability of local production of Hp in the mammary gland was further supported by quantitative RT-PCR.

A study by Lai *et al.* (2009) proposed that Hp levels correlated well with SCC in bovine milk due to the fact that SCC >500 000 cells/ml indicated 10-fold increase in milk Hp concentrations. Thus, neutrophils which were the predominant cell type in a group with

>500 000 cells/ml, are the major source of Hp synthesis. As the conclusion, Lai *et al.* (2009) suggested four combined pathways for the presence of Hp in milk during mastitis which were: activation and migration of neutrophils by inflammatory cytokines into the infected mammary gland where they secrete cellular Hp; direct entrance of plasma Hp into mammary epithelial cells via a receptor-mediated process and secreted into alveolus; passive diffusion of plasma Hp into udder; endogenous synthesis and expression of Hp by mammary epithelial cells.

Åkerstedt *et al.* (2007) found the presence of Hp was significantly more common at higher SCC in quarter and composite milk samples but not in bulk tank milk samples due to dilution that took place from quarter to bulk tank level.

2.1.4 Quantification of bovine Hp

The determination of bovine serum Hp was first demonstrated by Bremner (1964) but the concentrations were too low to be detected in healthy cattle but local inflammation induced by turpentine injection was able to increase the level of Hp in serum. Various types of assays have been described to determine Hp levels directly or indirectly. There were haemoglobin binding assay (Owen *et al.* 1960) modified by Jones & Mould (1984) using a microtitre plate format while Skinner *et al.* (1991) used an automated method based on an RA500 analyser. Immuno assay has also been described, for instance single radial immunodiffusion (Morimatsu *et al.* 1992). It is known that Hp binds with free Hb with high affinity. Measurement of the inherent Hb peroxidase activity which is protected from acid inactivation by formation of the Hb-Hp complex was used to determine Hp levels in plasma (McNair *et al.* 1995). Amongst many methods Hb binding assay is the most common method used to determine Hp level in cattle.

Makimura & Suzuki (1982) determined the level of Hp by performing the modified method of Tarukoski 1966 based on the peroxidase activity of haptoglobin-haemoglobin (HpHb) complex. This modified method provided more reliable values even for haemolysed serum samples. Polyacrylamide gel disc electrophoresis (PAGE) employed in this study only provided a qualitative but simple and reliable method to determine Hp.

However, due to bovine Hp being highly polymerised polymorphic protein (Eckersall & Conner 1990; Morimatsu *et al.* 1991), it can disturb both Hb-binding and immunochemical assays (Salonen *et al.* 1996) due to its large and heterogeneous molecular sizes.

Depolymerisation of bovine haptoglobin with 2-mercaptoethanol produced homogenous haptoglobin with a high cyanmethaemoglobin (HbCN)-binding capacity.

Hp can also be analysed photometrically based on the principal that cyanomethaemoglobin (HbCN) when bound to Hp was protected from denaturation in an acidic medium (formate buffer, pH 3-7) as described by Elson (1974). Studies by Salonen *et al.* (1996), Hirvonen *et al.* (1996) and Hirvonen *et al.* (1997) used this photometric assay to analyse serum Hp and the absorption of the acidified Hp-HbCN complex was measured at 405nm (Harvey 1976) and 380nm for the sample blank. The haemoglobin binding capacity was calculated in order to determine Hp concentrations.

High performance liquid chromatography (HPLC) can also be used to analyse serum Hp by binding of depolymerised Hp by excess HbCN and separating Hp-HbCN complex and free HbCN by gel filtration on HPLC (Salonen *et al.* 1996). This method proved not to be influenced by haemolysis in the sample, haemoglobin is converted to HbCN by Drabkin's solution and the excess did not interfere with the assay thus making it more sensitive and reproducible than that of photometric assay in detecting bovine serum Hp.

Due to the development of monoclonal antibodies specific for bovine Hp (Sheffield *et al.* 1994; McNair *et al.* 1995; Godson *et al.* 1996), antibody-based methods promised the advantages of direct and specific measurement of Hp (McNair *et al.* 1997). Indeed, Sheffield *et al.* (1994) used monoclonal antibody against bovine Hp in ELISA to measure bovine Hp level in sera.

Morimatsu *et al.* (1992) developed a single radial immunodiffusion method (SRID) to overcome problems of measuring bovine serum Hp using Hb-binding assay. Purified bovine Hp from acute phase bovine sera and specific antisera from rabbits were used in this method. Hp was treated with 20mM cysteine or glutathione (GSH) to partially reduce the Hp to smaller molecules or homogenous form.

McNair *et al.* (1995) introduced a highly sensitive and specific competitive time resolved fluorimetric assay to determine bovine serum Hp. It was shown to enhance the level of detection using a solid phase competitive immunoassays in combination with lanthanide chelate labelling. This method used mouse monoclonal antibody (1D9) specific for bovine Hp which was labelled with lanthanide chelate. It has been shown that this method has a

superior sensitivity when compared with Hp binding assay since there was a direct measure of Hp by specific antibody while the Hp binding assay peroxidase activity may be affected by the presence of haemoglobin, active and inactive forms of Hp (Cerda & Oh 1990) and other serum peroxidase inhibitors such as cysteine and glutathione.

Young *et al.* (1995) further developed the immunoassay described by Sheffield (1994) using either three formats; (a) a direct binding format; (b) a direct Hb binding format and (c) a competitive inhibition Hp immunoassay. These three formats provide simple, rapid results but with different reproducibility. Furthermore, the reagents used in these immunoassays were inexpensive, non-hazardous and readily available. It was concluded that Format b gave a very good correlation with Hp levels determined by the standard peroxidase activity method with good precision and reproducibility as compared with other two formats.

Immunoassay detected elevated Hp concentrations (62.5%) compared with binding assay (19.5%) although a close relationship (r=0.94) was found between the two methods (McNair *et al.* 1997).

However the majority of methods available relate to its measurement in serum and cannot be used to measure Hp in milk due to lack of sensitivity of the test and interfering substances present in the milk which can overestimated its concentration in milk (Eckersall *et al.* 2001). Milk Hp may be quantified using agarose immunodiffusion plates as performed by Eckersall *et al.* (2001).

Subsequently, several studies have employed the use of ELISA to measure milk Hp (Grönlund *et al.* 2003; Nielsen *et al.* 2004; Eckersall *et al.* 2006; Safi *et al.* 2009; Milica *et al.* 2010; Larsen *et al.* 2010; Haghkhah *et al.* 2010) but Hiss *et al.* (2004) claimed that the immunodiffusion assay and the sandwich ELISA were not sensitive enough to detect Hp level in milk from healthy cows. They eventually established an ELISA for bovine Hp that is highly sensitive to detect Hp levels in milk from healthy cows throughout lactation and physiologically increased Hp levels (e.g; colostrum) (Hiss *et al.* 2004).

In contrast, ELISA measurement of bovine milk Hp was said to be unsuitable for routine large-scale analysis (Åkerstedt *et al.* 2006). They developed a biosensor method which

appeared to be rapid and sensitive based on the interaction between Hp and Hb using surface plasmon resonance (SPR) biosensor technology.

2.1.5 Bovine Serum Amyloid A (SAA)

The other major bovine APP is SAA which is an acute phase protein complexed to high density lipoprotein (HDL) as an apolipoprotein in serum (Benditt & Eriksen 1977; Skogen *et al.* 1979; Husebekk *et al.* 1988). In order to form a complex with HDL, it has to compete for the binding sites with other apoproteins (Husebekk *et al.* 1987). However, a study by Husebekk *et al.* (1988) found that no formation occurred of complexed amyloid A (AA) protein-HDL which suggested removal of SAA from the HDL complexes before degradation to amyloid A in amyloid deposits took place. Serum amyloid A (SAA) is thought to be a precursor protein for AA, a major constituent in bovine amyloid fibrils (Husebekk *et al.* 1985). However, one of the most important features of SAA is that it can increase more than one hundred-fold in concentration during inflammatory reactions (Horadagoda *et al.* 1999).

The molecular weight of SAA in its native form is estimated at 18 kDa (Pepys & Baltz 1983). SAA is involved in the transport of cholesterol from dying cells to hepatocytes (Liang & Sipe 1995), has an inhibitory effect on fever (Shainkin-Kestenbaum *et al.* 1991), on the oxidative burst of neutrophilic granulocytes (Linke *et al.* 1991) and on in vitro immune response (Aldo-Benson & Benson 1982; Benson & Aldo-Benson 1979). It has a chemotaxic effect on monocyte, polymorphonuclear leukocytes and T cells (Badolato *et al.* 1994; Xu *et al.* 1995), induction of calcium mobilization by monocytes (Badolato *et al.* 1995) and inhibition of platelet activation (Zimlichman *et al.* 1990)

SAA is synthesized predominantly in the liver with an induction of various pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Jensen & Whitehead 1998; Sipe 2000). It is also known to be produced extra-hepatically; by macrophages, endothelial cells and smooth muscle cells (Dibartola & Benson1989), intestinal epithelium (Vreugdenhill *et al.* 1999) as well as mammary epithelial cells (McDonald *et al.* 2001).

According to Nazifi *et al.* (2008), several isotypes of SAA are found in bovine; type 1 and 2 which represent positive acute phase proteins in bovine, while Jacobsen *et al.* (2004) also successfully determined different types of SAA isoforms in plasma/serum of cows with mastitis. The acute phase response also caused hepatic expression of SAA1 and SAA2 which are collectively known as the A-SAA proteins in a study by Jensen & Whitehead

(1998). Meanwhile, in a range of human tissues which include breast, pancreas and intestine, the mRNA encoding SAA1, SAA2 and SAA 4 proteins has been detected by in-situ hybridization (Urieli-Shoval *et al.* 1998).

Amongst many isoforms, SAA3 seems to be the predominant SAA isoform in most mammals which expressed the protein extra-hepatically (Ramadori *et al.* 1985; Meek and Benditt 1986; Benditt and Meek 1989). McDonald *et al.* (2001) discovered the mammary-associated SAA3 (M-SAA3) isoform with a pI value of 9.6 was present in high levels in colostrum of equine, bovine and ovine. Although it is found in high concentrations in colostrum, M-SAA3 expression in the mammary gland is part of natural physiological process that is not only limited in disease-associated responses. Its presence provides beneficial effects for the newborn as well as for the maintenance of mammary gland.

2.1.6 Serum Amyloid A in Bovine Mastitis

SAA as discussed previously can be used as a marker of tissue injury in cows (Horadagoda *et al.* 1999) and thus is considered to be a potential diagnostic marker due to its low level in normal animals but with a significantly increased concentration during an acute phase response and a later decrease in concentration with the resolution of the disease (Gruys *et al.* 1994).

SAA response during inflammation/infection is known to be longer in duration when there were co-infections with virus and bacteria rather than in pure viral infections (Ganheim *et al.* 2003). Its magnitude and duration also depended on the severity of the clinical signs (Ganheim *et al.* 2003; Heegaard *et al.* 2000).

The reliability of SAA in comparing between acute and chronic inflammation has been studied by Horadagoda *et al.* (1999) where it was observed that SAA levels were significantly higher in cows with acute inflammation than in cows with chronic inflammation. It was proved that SAA was the most reliable indicator in differentiating cattle with acute and chronic inflammation and confirmed that the use of APP measurement was better than a haematological test, such as neutrophil count for the presence of infection.

Many studies have been done in order to investigate the potential of SAA as one of the important disease markers in bovine mastitis. Eckersall *et al.* (2001) first measured the SAA in mastitic milk as well as serum of cows with mastitis. It has been shown that the

measurement of SAA in milk is able to discriminate between mild and moderate mastitic cows compared with SAA in serum. The ability of the SAA in providing sensitive results is due to the local synthesis of SAA in the mammary gland or milk which induces a greater response in moderate mastitis. The presence of different isoform of SAA in mammary gland was confirmed by McDonald *et al.* (2001) in which M-SAA3was identified in healthy bovine colostrum. There is debate on the naming of the isoform in milk. While it should be named M-SAA3 if it can be definitely identified as this isoform, most assays measure all isoforms and here it will be referred to as M-SAA3 depending on the publication being cited.

As shown by Grönlund *et al.* (2003), SAA levels in serum elevated dramatically during acute mastitis but these results can be due to other acute inflammatory conditions (Conner *et al.* 1988; Heegaard *et al.* 2000). Compared with the results from Horadagoda *et al.* (1999), Grönlund *et al.* (2003) found much lower levels in serum APPs during chronic phase of mastitis than serum APP levels during acute mastitis. Milk serum amyloid A3(M-SAA3) however provided a good marker in chronic subclinical mastitis as it was significantly higher in infected quarters than in pre-infected and healthy udder quarters (Grönlund *et al.* 2003).

To be useful as a mastitis indicator, SAA needs to accumulate in milk during episodes of intramammary infection (Nielsen *et al.* 2004). Its high level in milk was found to coincide with the onset of clinical signs of acute mastitis as well as increases in SCC in milk (Grönlund *et al.* 2003). However, in some cases, APPs in milk started to be elevated in concentration in milk from clinical mastitic cows even before the SCC started to rise (Hogarth *et al.* 2002). This is in agreement with Pedersen *et al.* (2003) where the concentration of M-SAA3 increased prior to increase in SCC in cows induced with *S. uberis.*

High levels of SAA in infected quarters can be attributed to its local production in mammary gland as well as from leakage of serum proteins across the blood-milk barrier due to increased permeability during mastitis (Nielsen *et al.* 2004). According to Petersen *et al.* (2003), it can be assumed that SAA is produced locally in the mammary gland due to the lack correlation between SAA in milk and serum (Eckersall *et al.* 2001), the expression of serum amyloid protein homologue in the mammary gland (Molenaar *et al.* 2002) and the discovery of milk specific form of bovine SAA by McDonald *et al.* (2001).

SAA levels were also found to be increased during endotoxin-induced mastitis in both serum and milk of the cows (Lehtolainen *et al.* 2004). However, serum SAA was not related to M-SAA3 as SAA seemed to peak earlier in milk than in serum. It was also thought in this study that some SAA that was present in serum may originate from milk due to changes in permeability of blood-milk barrier. However, Jacobsen *et al.* (2004) disagreed on this by saying that udder-specific SAA was unable to cross the blood-milk barrier and thus be present in the plasma. Lehtolainen *et al.* (2004) also concluded that M-SAA3 was less able to assess the severity of mastitis because of the slow rise of M-SAA3 concentration during intramammary infection.

In the study by Jacobsen *et al.* (2004) using *E. coli*-induced mastitis, they found several SAA isoforms in both plasma and milk of the cows. However, the presence of alkaline pI, tissue-specific SAA3 isoforms in the milk did not appear in any of plasma or serum samples examined confirming the synthesis of SAA in the mammary gland. Furthermore, M-SAA3 levels in milk started to rise earlier at 12-hour post inoculation than SAA levels in plasma (between 12-hour to 24-hour post inoculation). Thus, M-SAA3 became a suitable marker for early mastitis detection as well as an indicator of tissue damage severity.

Larson *et al.* (2005) showed that bacterial LPS that caused a severe mammary gland infection were able to induce differential expression of M-SAA3 by bovine mammary epithelial cells. It was shown that inflammatory cytokines induced by LPS played an important role in the upregulation of M-SAA3 expression in the mammary gland and initiate a cascade of events during the acute phase response. The A-SAA proteins expressed hepatically also present in the mastitic milk as part of leakage of serum proteins into the mammary gland from increased permeability of the blood-milk barrier.

SAA levels also known to be increased both in serum and milk with repeated challenges of *E.coli* inoculation in udder quarters. However, serum SAA levels (24-hour post challenge) increased later than the levels of M-SAA3 in milk (12-hour post inoculation) but peaked at the same time in two challenges. They further confirmed the use of APP as mastitis indicator and to assess the severity of inflammation (Soujala *et al.* 2008).

S. aureus infection often leads to subclinical and chronic mammary gland inflammation

and further promotes the spread of disease to other cows (Weber *et al.* 2005). Lipotechoic acid (LTA) from gram-positive bacterial cell wall components mimicked a mastitic episode as induced by Gram-positive bacteria (*S. aureus*) without having the confounding factors of bacterial growth as well as epithelial cell invasion. LTA was shown to be able to stimulate the differential production of SAA by mammary ductal cells with M-SAA3 (pI 9.6) being the predominant SAA isoform produced (Weber *et al.* 2005).

Another experimental study of *S. aureus* induced mastitis by Eckersall *et al.* (2006) supported the finding that increases in M-SAA3 level in milk occurred earlier than increases of SAA in serum. The level of M-SAA3 was also greater in quarters that received 2 infusions of bacteria compared with quarters that only received single infusion of bacteria. Immunocytochemistry results revelead that M-SAA3 is present in the secretory epithelium, gland cistern and at significantly lower levels in the teat canal and it was concluded that the origin of M-SAA3 in milk was within the secretory epithelial cells and cells lining the gland cistern. Less M-SAA3 detected in the teat canal was probably due to infusion of bacteria directly into the gland cistern by-passing the teat canal.

Milica *et al.* (2010) further demonstrated that the magnitude of both local and systemic APR in *S. aureus* mastitis was higher if more than 1 udder quarter were affected. Higher magnitude of local tissue response was observed due to different strain of *S. aureus* (Dingwell *et al.* 2006), number of *S. aureus* (Eckersall *et al.* 2006) and additional exudation of very high plasma SAA that leads to increased in concentration of MAA in milk.

When comparing the reliability of APP measurement in diagnosing bovine mastitis with other mastitis diagnostic methods, APP showed significant correlations with ATP as in a study by Grönlund *et al.* (2005), about 52% of quarters of negative and positive ATP agreed with the results on APP level in udder quarters (ATP-Hp-SAA- group and ATP+Hp+SAA+). However, discrepancy in results was observed between APP level and SCC in affected udder quarters possibly due to different mechanisms of entry into the udder. Leukocytes migrate actively into the mammary gland by means of adhesion molecules while APP is presumed to leak passively from blood into milk from altered membrane permeability or is produced locally by the mammary gland.

O'Mahony *et al.* (2006) revealed that there was a good correlation between the

concentration of SAA in bovine milk and established cellular indices of mastitis particularly the CMT and SCC. CMT in full and SCC have a strong correlation as both represent the cell concentrations in milk. However, there were some indications that negative correlation happened between the level of milk SAA and SCC in a group of cows when the level of SAA was increased. Nevertheless, this study suggested that milk SAA as useful in detecting quarters with high SCC as serum SAA did not significantly influence the M-SAA3.

Milk APP also showed high correlation with SCC as rise in milk APP concentrations was seen with increasing in SCC thresholds (Kovac *et al.* 2007). Thus, measurement of milk APP can be used in monitoring udder health as SCC was not suitable to be used alone as a marker to identify infected quarters (Zecconi & Piccini 2002).

Åkerstedt *et al.* (2007) further confirmed the significant relationships between SAA and SCC at quarter, cow composite and bulk tank level of healthy milk samples. SAA was also detected more frequently than Hp regardless of the types of milk samples (quarter, cow composite and bulk tank) at higher SCC.

The detectable level of M-SAA3 in milk can also be used to predict the protein quality of cow composite milk samples in which samples with detectable SAA level had lower casein number, lower lactose level and higher SCC. Casein number provided a good and specific milk quality parameter especially in cheese-making industry as total protein may include less valuable serum proteins that are known to increase in concentrations during mastitis. Reduced casein number could be contributed by proteolysis while reduced lactose content might be due to the regulation of lactose secretion by specific peptides released during casein hydrolysis by plasmin (Silanikove *et al.* 2000).

Gerardi *et al.* (2009) revealed that by using milk a SAA ELISA kit to measure the level of SAA in milk, a significant correlation was found between SCC and M-SAA3 in lower SCC levels from quarter milk samples compared with the level of M-SAA3 in milk determined by serum SAA ELISA kit. Thus, M-SAA3 has been suggested as a physiological marker of subclinical mastitis as it provided statistically better response in distinguishing subclinical from clinical mastitis.

2.1.7 Quantification of bovine SAA

In early studies, a variety of SAA assays have been employed but faced with certain

difficulties because most of them require some form of plasma or serum denaturation by heat, acid or alkali so that complete dissociation of SAA from the HDL 3 fraction of lipoprotein occurred (Marhaug *et al.* 1983; Sipe *et al.* 1989). This resulted in considerable variation in the reproducibility of the assays.

In one example, Horadagoda *et al.* (1993) purified amyloid A from acute phase bovine serum and quantified the SAA concentration in serum by an indirect ELISA using rabbit anti-human SAA and horseradish peroxidase conjugated donkey anti-rabbit IgG. However, this type of assay was considered to be laborious, time consuming and depended on the commercial availability of antisera to human amyloid A with cross-reactivity to the bovine protein (Horadagoda *et al.* 1999). They suggested that by using immunoturbidimetric or latex agglutination immunoassay it was quicker and based on antiserum to bovine SAA. Latex agglutination nephelometric immunoassay has been used to measure human SAA on an automated analyser in less than 6 minutes (Yamada *et al.* 1993).

McDonald *et al.* (2001) then developed a monoclonal antibody sandwich immunoassay for SAA that did not require sample denaturation and can be performed in 3 hours. It also has other advantages such as the hybridomas producing the monoclonal antibodies are self-renewing, inexpensive to maintain and yield a highly consistent, easily purified product, the microtitre plates can be coated and stored so that the assay is available within 3 hours, it provides an accurate and reproducible results and lastly requires equipments and skills that normally available in a research or a clinical laboratory.

Later, a commercialized SAA ELISA (Tridelta Development plc, Ireland) was produced based on the assay first developed by McDonald *et al.* (1991). With that, studies of SAA measurement whether in serum or milk have been done using commercialized SAA ELISA.

In the subsequent chapters in this thesis, measurement of Hp and M-SAA3 in milk has been used to confirm the origin of milk samples from udder quarters with mastitis. The immunoassay for bovine Hp used in previous studies (Eckersall *et al.* 2006) was no longer available and a novel ELISA was developed for this purpose. While attempts were made to establish an ELISA for M-SAA3, this was not successful and a commercial assay for SAA was used in these investigations.

2.1.8 Aims of study

The aims of this chapter were to develop sandwich bovine Hp assay before validating and optimizing this assay to test its reliability to measure Hp. Commercialized SAA assay using Tridelta™ kit was used to measure bovine milk SAA. This type of SAA assay was used due to several problems encountered in developing an in-house bovine milk SAA assay especially in getting a good and acceptable standard SAA curve. After validating and optimizing these assays, they were used to measure APP in milk for the next three chapters to confirm the disease status of the animals.

2.2 Materials and Methods

2.2.1 Reagents

General chemicals and other materials were obtained from Sigma Chem. Co, Poole, UK and Bio-Rad Laboratories Inc. for PAGE unless otherwise stated. Double distilled water was used throughout.

2.2.2 Milk samples

Milk samples were obtained from Cochno Farm, West of Scotland and Vale Veterinary Laboratory, Dorset, UK. Samples from subclinical mastitic cases (n=10) were chosen based on their previous somatic cell count records, no obvious clinical signs as the time of sampling but produced positive California Mastitis Test (CMT) results. Samples from clinical mastitic cases (n=40) were chosen based on their clinical signs and further confirmed by bacteriological culture results which mainly identified major mastitis pathogens such as *E. coli*, *S. aureus* and *Strep uberis.* Samples from healthy animals (n=25) (SCC < 100000 cells/ml) were used in comparison with animals with mastitis cases. Samples were stored at -20°C until required.

2.2.3 California Mastitis Test

The CMT procedure was conducted as discussed in the previous chapter on Section 1.2.2. The CMT reagent dissolved or disrupted the outer cell wall and the nuclear cell wall of any leukocyte and DNA was released from the nuclei. A stringy mass was developed and the amount of gel formation increased with an increase number of leukocytes (Mellenberger 2001). The result was interpreted as presented in Table 1.1 in Chapter 1.

2.2.4 Development of Bovine Haptoglobin ELISA

2.2.4.1 Biotinylation of antibody

The biotinylated rabbit anti-bovine Hp was prepared using a biotinylation kit according to the manufacturer's instructions as described below. Bovine anti-haptoglobin was purchased from Life Diagnostic Inc., USA. This antibody was stored at the concentration of 3.43mg/ml. Different biotinylation of antibody was made up at least for 4 times and the reason will be discussed in the discussion part (Section 2.4).

2.2.4.1.1 Preparation of Elution buffer and Antibody Binding Solution

Elution buffer was prepared by adding 50µl of 4M Imidazole stock solution to 950µl PBS. The Antibody Binding Solution (ABS) was prepared by adding 29µl of anti-bovine Hp to 210µl of PBS.

2.2.4.1.2 Antibody Binding

The disc provided was put in the bottom of a 1.5ml microcentrifuge tube. The prepared ABS was added to the tube containing the disc. To hydrate the resin, the tube was incubated at room temperature for 30 seconds. The tube was inverted several times to suspend the resin and further 10 minutes incubation at room temperature with gentle rocking motion on a rotating platform was performed (no vortex). It must be noted that the resin must remain suspended during binding and the tube was manually inverted every 2-3 minutes to keep the resin in suspension. The tube was centrifuged at 500 *x g* for 30 seconds to pellet the resin. The liquid was carefully removed with a pipette to discard. 1mL of PBS was added to the tube and the tube was inverted several times to wash the resin. The tube was centrifuged at 500 *x g* for 30 seconds to pellet the resin and the liquid was carefully removed with a pipette to discard. To complete washing, 1mL of PBS was added to the tube as before and step was repeated. 300µl of PBS was then added to the tube containing antibody and resin and pipetting the liquid gently up and down to resuspend the resin. A Handee Spin Column was placed in a new 1.5ml microcentrifuge tube. The entire volume of resin slurry was pipetted into the spin column. The column was centrifuged at 500 *x g* for 30 seconds and the flow-through was discarded before placing the column back into the tube.

2.2.4.1.3 Antibody Biotinylation

A bottom plug was applied to the separation column. A No-Weigh NHS-PEO4 Biotin Microtube was punctured with a pipette tip and the tube contents were dissolved by adding 200µl of PBS with gentle pipetting. 188µl of PBS was added to 12µl of biotin reagent

directly to the separation column. PBS was added to the column first before adding the biotinylation reagent. The column was capped with a screw cap and gently mixed by flicking. The column was incubated for 1 hour at room temperature with the column being flicked occasionally during incubation to keep the resin from settling. The bottom cap of the column was removed and it was centrifuged at 500 *x g* for 30 seconds. The flow-through was discarded and the column was placed back into the same tube. 400µl of PBS was added to the column and further centrifuged at 500 *x g* for 30 seconds. Again, the flow-through was discarded and the column was placed back into the same tube. The PBS was added as before and the step was repeated three times to wash the column.

2.2.4.1.4 Antibody Elution

The column was capped at the bottom and placed in a new 1.5ml microcentrifuge tube. 200µl of Elution buffer was added to the column. The tube was incubated at room temperature for 10 minutes before centrifugation at 500 *g* for 30 seconds to elute antibody. After elution, some antibody will remain bound to the column. To increase yield of biotinylated antibody, 200µl of Elution buffer was added and incubated for 10 minutes at room temperature and elution repeated. The resin was discarded after used. The biotinylated antibody was stored at 4°C for up to one month. Biotinylated antibodies are generally stable when stored in Elution buffer (0.2M Imidazole in PBS) at 4°C. If biotinylated antibodies were not used within one month, they were stored in single-use aliquots at -20°C.

2.2.4.2 Newly prepared biotinylated antibody

To test for the availability of newly prepared biotinylated antibody, serial dilutions of standard bovine Hp (3.43mg/ml, Life Diagnostics Inc., USA) were prepared in 1:100, 1:200, 1:400, 1:800 and 1:1600. Standard bovine Hp at a different dilution was blotted onto a nitrocellulose membrane. 10% of Marvel milk in TBS Tween buffer was used to block the membrane for 60 minutes. The membrane was rinsed with wash buffer and the newly prepared biotinylated antibody at 1:1000 was blotted onto the membrane and further incubated at 60 minutes. 1:1000 of streptavidin peroxidise (Tridelta Development Ltd., Ireland) was blotted after rinsing and incubated for another 60 minutes. The membrane was rinsed and substrate (Opti-4CN^{TM} Substrate Kit, USA) was added to develop bands.

2.2.4.3 Assay Procedure

The assay conditions were optimised as described in section 2.2.4 so that the best performance from the assay is achieved. The following protocol described the finalised conditions.

2.2.4.3.1 Coating

The Life Diagnostic rabbit anti-bovine haptoglobin as described earlier was diluted to a final concentration of 1 µg/ml in 0.05M $NaHCO_3$, pH 9.6. 100 µl was dispensed into each well of a Nunc-Maxisorp 96 MicroWell™ plate (Nunc International, Rochester, NY) and incubated at 4°C overnight.

2.2.4.3.2 Washing

Wells were washed using 0.02M Tris-HCl pH 7.4 with 0.05% Tween-20 by adding 250µl per well for 4 times.

2.2.4.3.3 Blocking

Unoccupied binding sites were blocked by adding 250µl of 10% (w/v) Marvel milk protein in wash buffer and incubated at 37ºC for 60 minutes.

2.2.4.3.4 Washing

Wells were washed again as above.

2.2.4.3.5 Dilution and addition of Standard Bovine Haptoglobin and milk samples

Standard bovine haptoglobin (0.845mg//ml, Life Diagnostics Inc., USA) was prepared at 0.25µg/ml in washing buffer and double diluted until concentration reached 1.95ng/ml. Milk samples were also diluted at 1:10000 or 1:1000 in washing buffer. 100µl of each standard bovine haptoglobin and milk samples were added into duplicate wells and incubated at 37 °C for 60 minutes.

2.2.4.3.6 Addition of biotinylated antibody

After washing, diluted biotinylated rabbit anti-bovine haptoglobin at 1:1000 in washing buffer was added at 100µl per well and incubated at room temperature for 60 minutes.

2.2.4.3.7 Addition of secondary antibody

After washing, diluted Streptavidin-peroxidase at 1:1000 in washing buffer was added at 100µl per well and further incubated at room temperature for 60 minutes.

2.2.4.3.8 Addition of substrate

After washing, Tetra methyl benzidine (TMB) peroxidise substrate was prepared according to manufacturer instruction (KPL, Inc., USA). The substrate was added at 100µl per well and developed for 5-10 minutes. The peroxidase-enzyme reaction was stopped by adding 100µl per well of 1M hydrochloric acid.

2.2.4.3.9 Absorbance reading

The absorbance was read at 450 nm using Fluostar OPTIMA platereader (BMG Labtech Ltd.) and the results analysed and calculated using the associated FLUOstar OPTIMA Software V1.32 R2 using a 4 parameter-fit standard curve plotted on a linear scale.

2.2.5 Haptoglobin Assay Optimisation

During assay development a number of parameters were altered to come to the optimised assay. A number of these variations are given below.

2.2.5.1 Antibody concentrations

The concentration of primary antibody (anti-rabbit bovine Hp) was varied to achieve the highest optical density (OD) value for the maximum standard while producing a steep standard curve, with low OD for blanks. Other variables were kept constant with varying antibody concentrations of 1µg/ml, 5µg/ml and 25µg/ml. Though the curve for 1µg/ml of antibody gave the lower curve (Fig 2-2) it was chosen for the optimised assay due to its lower background reading.

2.2.5.2 Varying batch of biotinylated antibodies

With each batch of antibody prepared, different dilutions of antibody were tested so that a straight standard curve with low OD values for blanks were achieved.

2.2.5.3 Biotinylated antibody concentrations

The concentration of biotinylated antibody (anti-rabbit bovine Hp) was varied to achieve aims as previously described. Other variables were kept constant with varying antibody concentrations of 1:250 and 1:1000. Figure 2-4 shows the Hp standard curve for the first batch of biotinylated anti-bHp at 1:250 and 1:1000, the latter was chosen for the optimised assay due to a lower background. Figure 2-5 shows the comparison between batch 1 and batch 2 of the biotinylated antisera at 1:1000. With batch 3 and batch 4 of the biotinylated antibody a dilution of 1:500 was optimal.

2.2.5.4 Optimisation of Standard and Sample Dilution

The standard bovine Hp was diluted at different concentrations and serially diluted to achieve standard curves over different ranges, so that a steep standard curve over was produced. Figure 2-6 shows a typical standard curve. Milk samples were diluted at different concentrations until their reading were on the linear section (0-1000ng/ml) of the standard curve. It shows that samples of milk with elevated Hp gave parallel dilution curves with the standard curve giving an indication that the assay is measuring haptoglobin.

2.2.6 Haptoglobin Assay Validation

2.2.6.1 Precision

For intra-assay precision, the coefficient of variance was determined as the mean of coefficient of variance (CV) for of duplicate results. Inter-assay precision was determined where CVs were calculated from the mean and standard variation of the same milk samples (control samples) over 10 times during the use of the assay.

2.2.6.2 Accuracy

Linearity of dilution of 3 milk samples with varying Hp concentrations was assessed for accuracy of the assay.

2.2.6.3 Limit of detection

The limit of detection was determined as the lowest Hp concentration which could be distinguished from a zero value, and was taken as the mean +3 standard deviations of 4 replicates of blank samples.

2.2.6.4 Specificity

The specificity of the assay was based on the commercial anti-bovine Hp anti-serum assessed by Ouchterlony immunodiffusion in agarose gel.

2.2.7 Serum Amyloid A Assay

The concentration of SAA in milk samples were quantified using a commercial Multispecies SAA ELISA kit (PhaseTM Range, Tridelta Development Limited) according to the manufacturer's instructions.

2.2.7.1 Reagents

General chemicals and other materials were obtained from Sigma Chem. Co., (Poole UK) while Tridelta Development Ltd supplied the Phase™ Range SAA ELISA kit.

2.2.7.2 Reagent Preparation

All buffers should be prepared beforehand according to the instruction manual. These include 1X diluents buffer, 1X wash buffer, 1X biotinylated anti-SAA conjugate and 1X Streptavidin-peroxidase.

The SAA calibrator provided in the kit was reconstituted by adding distilled water to the vial and vortexed vigorously for 3-4 times for 3-5 seconds to dissolve the pellet completely. Top calibrator was prepared first before serially diluted.

Quality controls from both with highest and lowest SAA values were diluted at 1:500 using 1X diluent buffer.

All milk samples were diluted in 1X diluents buffer before analysis.

2.2.7.3 SAA Assay

50 µl of diluted milk samples, diluted SAA calibrators and quality controls were added to duplicate wells. 50µl of 1X biotinylated antibody conjugate were also added to each well and incubated at 37ºC for 60 minutes. After incubation, the plate was washed 4 times using 1X wash buffer before tapping the plate dry on absorbent paper. 100µl of 1X Streptavidin-peroxidase was added to each of the wells and further incubated at room temperature in the dark for 30 minutes. The plate was washed as above. 100 µl of TMB Substrate were added before incubated at room temperature in the dark for 30 minutes. Finally, 50µl of Stop solution was added and the absorbance was read at 450nm.

2.2.8 SAA Assay Validation

The specificity and accuracy of the SAA ELISA was based on the data supplied with the commercial ELISA (Tridelta Development Ltd., Ireland).

2.2.8.1 SAA Assay Precision

For intra assay precision, the CV was calculated from the mean CV of duplicate samples (n=42). Inter-assay precision was determined where CVs were calculated from the mean and standard variation of the same milk samples (control samples) over 10 times during the use of the assay

2.2.8.2 Limit of Dectection

The limit of detection was determined as the lowest Hp concentration which could be distinguished from a zero value, and was taken as the mean +3 standard deviations of 15 replicates of blank samples.

2.2.9 Clinical Validation

Milk samples were collected from two different locations; the Cochno Farm, University of Glasgow, Clydebank, UK and Vale Veterinary Group Devon, UK. Milk samples acquired were grouped into healthy which comprised of 25 milk samples (Cochno Farm), subclinical mastitis group which comprised of 10 milk samples (Cochno farm) and clinical mastitis group which were comprised of 40 milk samples (Vale Veterinary Group). Both healthy and subclinical mastitic milk samples were chosen based on their monthly SCC recording data whilst cows with clinical mastitis were chosen based on the general clinical signs of mastitis and further confirmed by bacteriological culture.

2.2.10 Data Analysis

Statistical analysis was carried out using Predictive Analytics Software (PASW) Statistics (SPSS) version 18 (IBM Corporation, NY, US).

2.3 Results

2.3.1 California Mastitis Test

All subclinical mastitic milk samples (n=10) were subjected to CMT and all results revealed positive CMT reaction (2+ or 3+).

2.3.2 Immuno dot blot of newly prepared biotinylated antibody

The newly prepared biotinylated antibody was tested for cross reaction as shown in Figure 2-1. The immuno dots developed with the greatest intensity at lowest dilution (1:100) of standard bovine Hp and decreased as the dilution of Hp increased.

2.3.3 Haptoglobin Assay Optimisation

2.3.3.1 Coating Antibody concentrations

During optimisation, varying concentrations of coating antibodies were assessed as shown in Figure 2-2. A final concentration of 1µg/ml of coating antibody was chosen due to its reasonably steep curve and covered a wide range of OD values whilst giving lower blank readings than other different dilutions.

2.3.3.2 Biotinylated antibody concentrations

The biotinylated antibody was optimised as the previous step with different dilutions of 1:250 and 1:1000 using two different types of antibodies as shown in Figure 2-3. The final concentration of 1:1000 of biotinylated antibody was chosen as it resulted in a steep curve, provided a good range of OD values with lower blank readings than other different dilutions.

2.3.3.3 Varying batch of biotinylated antibodies

For the Batch 1 of prepared biotinylated antibodies, several dilutions were tested (1:1000 and 1:250). The chosen concentration was 1:1000 of as previously mentioned. An example of standard curve is shown in Figure 2-4.

For the Batch 2 of prepared biotinylated antibody using anti-rabbit bovine Hp, the 1:1000 dilution still give the best standard curve as shown in Figure 2-4.

But for the Batch 3 and 4 of prepared biotinylated antibody using anti-rabbit bovine Hp, the 1:1000 dilution did not produce a very reasonable standard curve thus a range of different dilutions were tested (1:250, 1:500 and 1:1000). However, a good standard curve was not achieved.

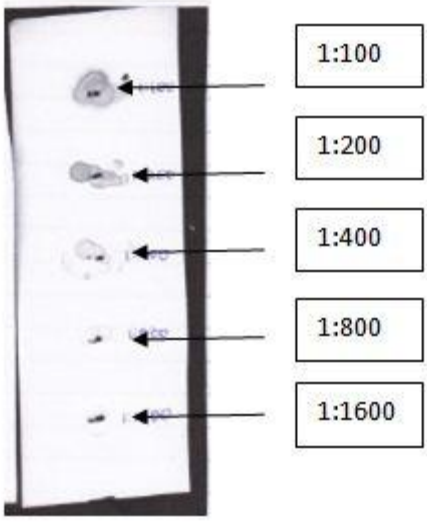


Figure 2-1: Immuno dot blot of biotinylated anti-bovine Hp reacting with bovine Hp at dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1600.

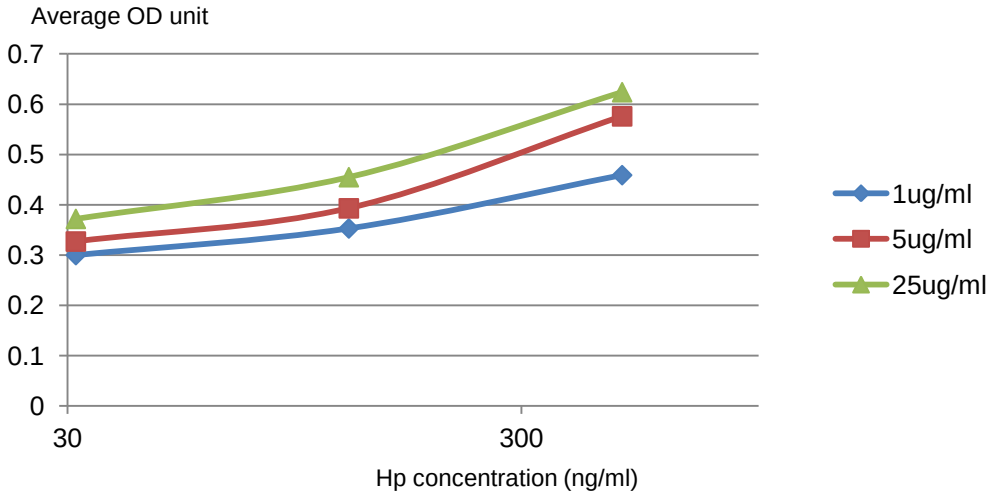


Figure 2-2: Optimisation of coating antibodies concentrations using different concentrations for the developed ELISA. The effect of anti-rabbit bovine Hp concentrations at 1µg/ml, 5µg/ml and 25µg/ml on bovine Hp ELISA. The 1µg/ml concentration gave lower blanks and a steeper curve than others.

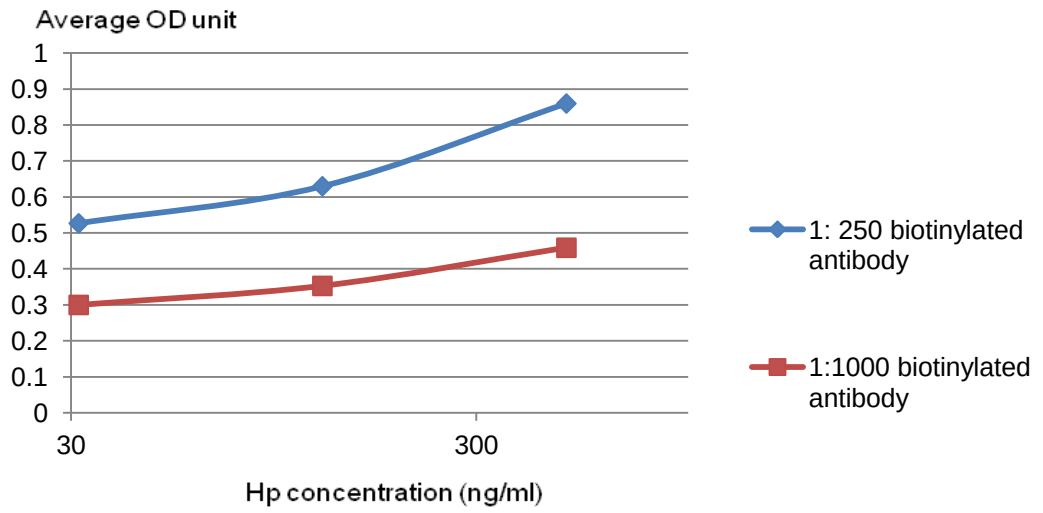


Figure 2-3: Optimisation of biotinylated antibody concentrations for the developed ELISA. The effect of the biotinylated antibodies at 1250 and 1:1000 on bovine Hp ELISA. The biotinylated antibody at 1:1000 provided lower blanks and a steeper curve than others.

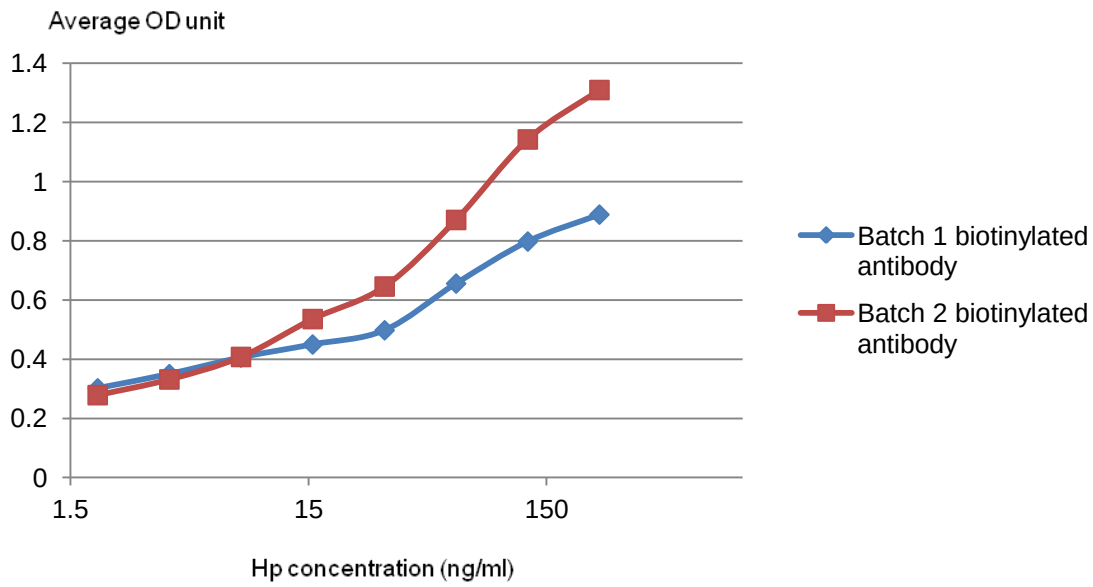


Figure 2-4: Standard curve obtained from the optimised first and second batch of biotinylated antibodies (3.43mg/ml) diluted at 1:1000.

2.3.3.4 Optimisation of standard and sample dilution

A working standard curve is needed as part of assay optimisation. This was achieved by diluting the standard bovine Hp (0.845mg/ml) in assay buffer to 0.25µg/ml and serially diluting the standards to achieve concentrations between 1.95-250ng/ml. An example of working standard curve is given on Figure 2-5. Milk samples were diluted accordingly based on their Hp content until the readings were on the linear portion of the standard curve.

2.3.4 Haptoglobin Assay Validation

2.3.4.1 Precision

The intra assay CV for the bovine Hp assay was 2.5% which was calculated as the mean CV of duplicate samples (n=36). Table 2-1 showed the values, means and the calculated CVs for the Hp ELISA assays which were 29.9% and 47.1% for mean milk Hp of 21 and 3.3µg/ml respectively.

2.3.4.2 Accuracy

Serial dilution of three milk samples as shown in Figure 2-6 was used to assess the assay accuracy. The three samples show linearity then plateau out at low dilution and are parallel to the standard curve. Diluted sample concentrations were taken from the linear portion of the curve as it is the most precise and accurate area of the curve.

2.3.4.3 Limit of detection

The minimum detection limit significantly different from the blank samples was taken as the Hp concentration at 3 standard deviations (SD) from the mean of blank sample (n=4). This resulted in a limit of detection of Hp in milk of 0.37ng/ml.

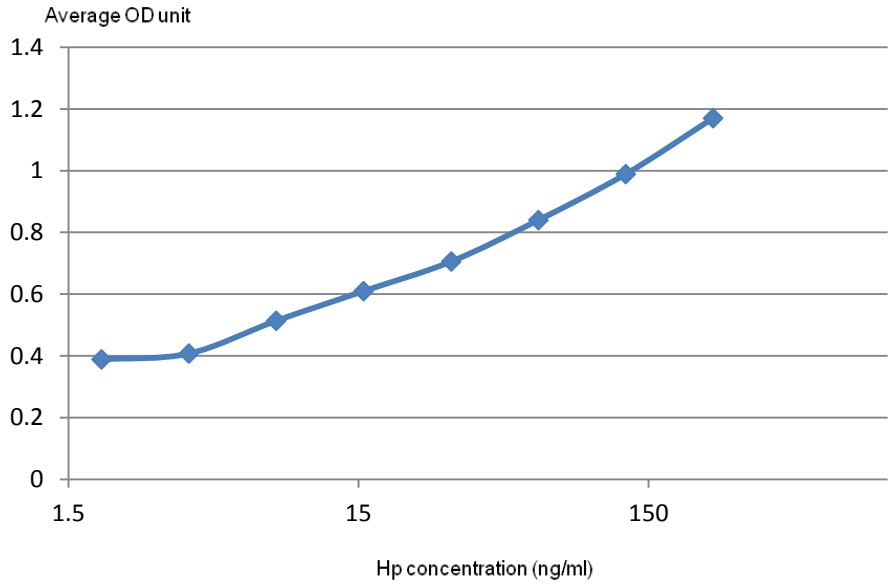


Figure 2-5: Standard curve obtained from optimised standard bovine Hp dilution. Standard bovineHp was diluted from 0.845mg/ml to 0.25µg/ml, then by 1:2 dilution.

Sample ID	Hp Conc. (µg/ml)	Plate
92	15.4	1
92	25.2	2
92	15.2	3
92	19.4	4
92	11.8	5
92	21.3	6
92	32	7
92	29.3	8
92	20.1	9
Mean	21.16	
SD	6.33	
% CV	29.9	

Sample ID	Hp Conc. (µg/ml)	Plate
41	6.5	1
41	5.5	2
41	3.3	3
41	3.9	4
41	2.5	5
41	2	6
41	2	7
41	2.9	8
41	2.3	9
		10
Mean	3.31	
SD	1.56	
% CV	47.1	

Table 2-1: Inter-assay precision results obtained for ELISA with milk samples. The CV for Hp concentration of 21.2µg/ml was 29.9% and the CV for Hp concentration of 3.31µg/ml was 47.1%.

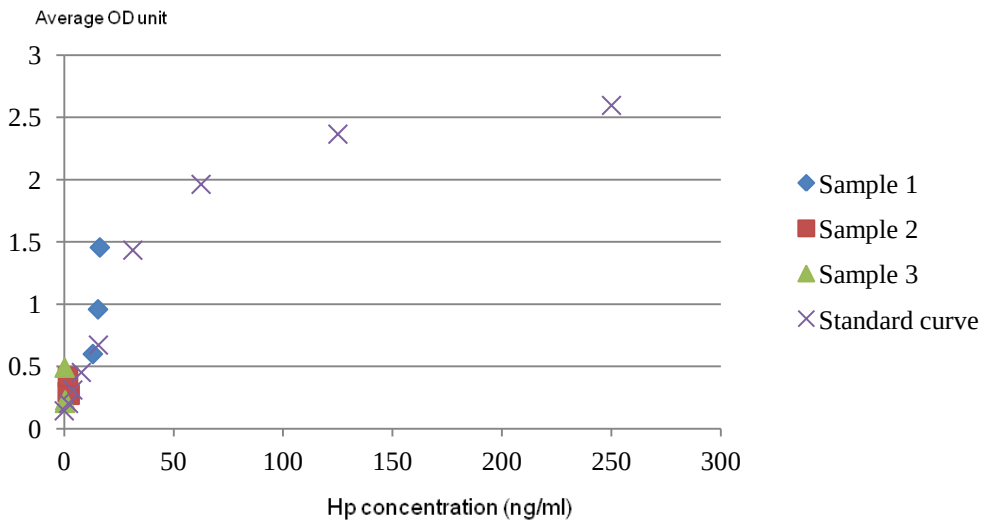


Figure 2-6: Linearity of 3 bovine milk samples after serial dilution. Example of standard curve included for comparison.

2.3.4.4 Specificity

The specificity of the assay was based on the commercial anti-bovine Hp anti-serum and was assessed by Ouchterlony immunodiffusion in agarose gel (Figure 2-7).

2.3.5 SAA Assay Validation

The specificity and accuracy of the SAA ELISA was based on the data supplied with the commercial ELISA (Tridelta Development Ltd., Ireland). In use the precision and limit of detection of the SAA ELISA for assessment of the protein in milk were as follows.

2.3.5.1 Precision

The intra assay CV for the SAA assay was 6.8% calculated from the mean CV of duplicate samples (n=42). Results were from too few assays to calculate the inter assay just for this investigation but when included in a series of ELISAs run in the laboratory (n=35) the CVs were 46% at a concentration of 7.2 + 3.3 ng/ml and 30% at a concentration of 40.6 + 12.1 ng/ml. These values are for post dilution of samples and are equivalent to 1.44µg/ml and 8.12µg/ml in the undiluted milk samples respectively. While the CVs were high, they were deemed to be satisfactory in view of the large increased being measured between healthy and diseased samples.

2.3.5.2 Limit of detection

The limit of detection of the SAA was 4ng/ml being the value determined as 3 SD from the mean of replicate blank samples (n=15).

2.3.6 Clinical Validation

The descriptive statistics of all variables in each group are presented in Table 2-2 while the distribution of SAA, Hp concentrations and SCC measurement in all samples are presented in Figure 2-8, 2-9 and 2-10. For healthy group, the range of SAA concentration was between 0.03-2.96µg/ml with a median of 0.36µg/ml, mean of 0.54µg/ml and standard deviation of 0.61µg/ml. The range of Hp concentration fell

between 0.07-31.95µg/ml with the average of 9.17µg/ml with a median of 3.52µg/ml and standard deviation of 9.88µg/ml. The range of SCC measurement was between 12 x 10^3 to 81 x 10^3 cells./ml with a median of 43 x 10^3 cells/ml, mean of 43 x 10^3 cells/ml and standard deviation of 19 x 10^3 cells/ml on at least one SCC recording one month before the sampling took place.

For subclinical mastitic group, the range of SAA concentration was between 0.18-14.05µg/ml with a median of 1.44µg/ml, mean of 3.19µg/ml and standard deviation of 4.27µg/ml. The range of Hp concentration fell between 0.31-14.91µg/ml with a median of 7.41µg/ml, average of 7.49µg/ml and standard deviation of 5.13µg/ml. The range of SCC measurement was between 67 x 10^3 to 1224 x 10^3 cells/ml with a median of 268 c 10^3 cells/ml with the average of 486 x 10^3 cells/ml and standard deviation of 447 x 10^3 cells/ml on the SCC recording one month before the sampling took place.

For clinical mastitic group, the range of SAA concentration was between 1.78-1780.5µg/ml with a median of 63.55µg/ml, average of 422.14µg/ml and standard deviation of 649.08µg/ml. The range of Hp concentration fell between 1.95-413.85µg/ml with a median of 86.52µg/ml, average of 99.36µg/ml and standard deviation of 90.16µg/ml. The cultured bacterial pathogens were amongst the common causal of bovine mastitis such as *E. coli*, *S. aureus*, *S. uberis* and *S. dysgalactiae* (Table 2-3)

As the data was not normally distributed, the Spearman's correlation was performed to analyze the correlation between these variables (Table 2-4). The Spearman's correlation between SAA and Hp concentration in all groups of milk samples was 0.653. The Spearman's correlation between SAA and SCC in all groups of milk samples was 0.438 whilst the Spearman's correlation between Hp and SCC in all groups of milk samples was 0.08.

Mann-Whitney's test was used to analyze for significant differences between diseased groups on SAA, Hp and SCC measurements (Table 2-5). Healthy and clinical mastitic groups showed significantly difference in the SAA concentration ($p<0.05$) with the z value of -6.737and healthy and subclinical mastitic groups also showed significant difference in SAA concentration ($p<0.05$) with the z value of -2.41. Clinical mastitic and subclinical mastitic groups had significant differences in SAA concentration ($p<0.05$) with the z value of -4.573.

Healthy and clinical mastitic groups showed significantly difference in the Hp concentration ($p<0.05$) with the z value of -5.437 whilst the Hp concentration between healthy and subclinical mastitic groups was not significantly different ($p>0.05$) with the z value of -0.087. Clinical mastitic and subclinical mastitic groups however had significant difference in Hp concentration ($p<0.05$) with the z value of -4.131.

Finally, healthy and clinical mastitic groups showed significantly difference in the SCC measurements ($p<0.05$) with the z value of -4.439.

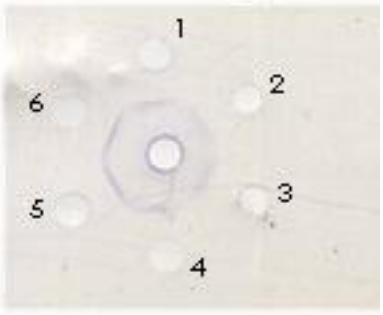


Figure 2-7: Ouchterlony immunodiffusion gel of anti-bovine Hp. The centred well was anti-bovine Hp, well 1 was purified bovine Hp, well 2 was low Hp bovine serum, well 3 was low Hp bovine serum, well 4 was high Hp bovine serum, well 5 was high Hp bovine serum and well 6 was high Hp bovine serum. A single line of immunoprecipitate was observed showing that the antiserum was specific for bovine Hp and that there were no non-specific reactions with other proteins in the serum samples.

Group		N	Minimum	Maximum	Mean	Median	Std. Deviation	Variance
Healthy	**SAA**	25	.03	2.96	.5435	.3600	.60944	.371
	Hp	22	.07	31.95	9.1665	3.5150	9.88022	97.619
	SCC	25	12000	80500	42800.00	43000.00	19812.664	3.925E8
	Valid N	22						
Subclinical	**SAA**	10	.18	14.05	3.1870	1.4350	4.27359	18.264
	Hp	9	.31	14.91	7.4848	7.4120	5.13332	26.351
	SCC	10	67000	1224000	485900.00	286000.00	446707.573	1.995E11
	Valid N	9						
Clinical	**SAA**	40	1.78	1780.50	422.1374	63.5480	649.08256	421308.170
	Hp	40	1.95	413.85	99.3570	86.5200	90.16136	8129.070
	SCC	0						
	Valid N	0						

Table 2-2: The descriptive statistics for the Hp concentration (µg/ml), SAA concentration (µg/ml) and SCC of milk samples taken from healthy, subclinical mastitic and clinical mastitic cows. The values for mean, maximum and minimum values, median, standard deviation and variance of each Hp, SAA and SCC for each group of milk samples were presented.

Sample IDs	Bacteriological culture
475	*E. coli*
76	*E. coli*
1111	*E. coli*
Star	*Strep. uberis*
1510	*Strep. dysgalactiae*
814	*E. coli*
438	*S. aureus*
940	*Coryne. bovis*
74	NA
669	*Strep. uberis*
544	*Strep. uberis*
708	*Strep. uberis*
200	*Strep. uberis*
185	*Strep. uberis*
14	*S. aureus*
692	*E. coli*
1061	NA
697	*E. coli*
519	*E. coli*
31	*Strep. uberis*
980	*S. aureus*
453	*E. coli*
496	*E. coli*
1222	*E. coli*
828	NA
49	*Strep. dysgalactiae*
4493	*Strep .uberis*
669	*Strep. uberis*
708	*S. aureus*
474	*E. coli*
346	*S .aureus*
7	*Strep. uberis*
230	*Strep. uberis*
1512	*Strep. dysgalactiae*
715	*S. aureus*
452	*E. coli*
932	*E. coli*
770	*Strep. uberis*
656	*Strep. uberis*
339	*Strep. uberis*

Table 2-3: The bacteria causing mastitis cultured from the clinical mastitic milk samples. NA stands for not available.

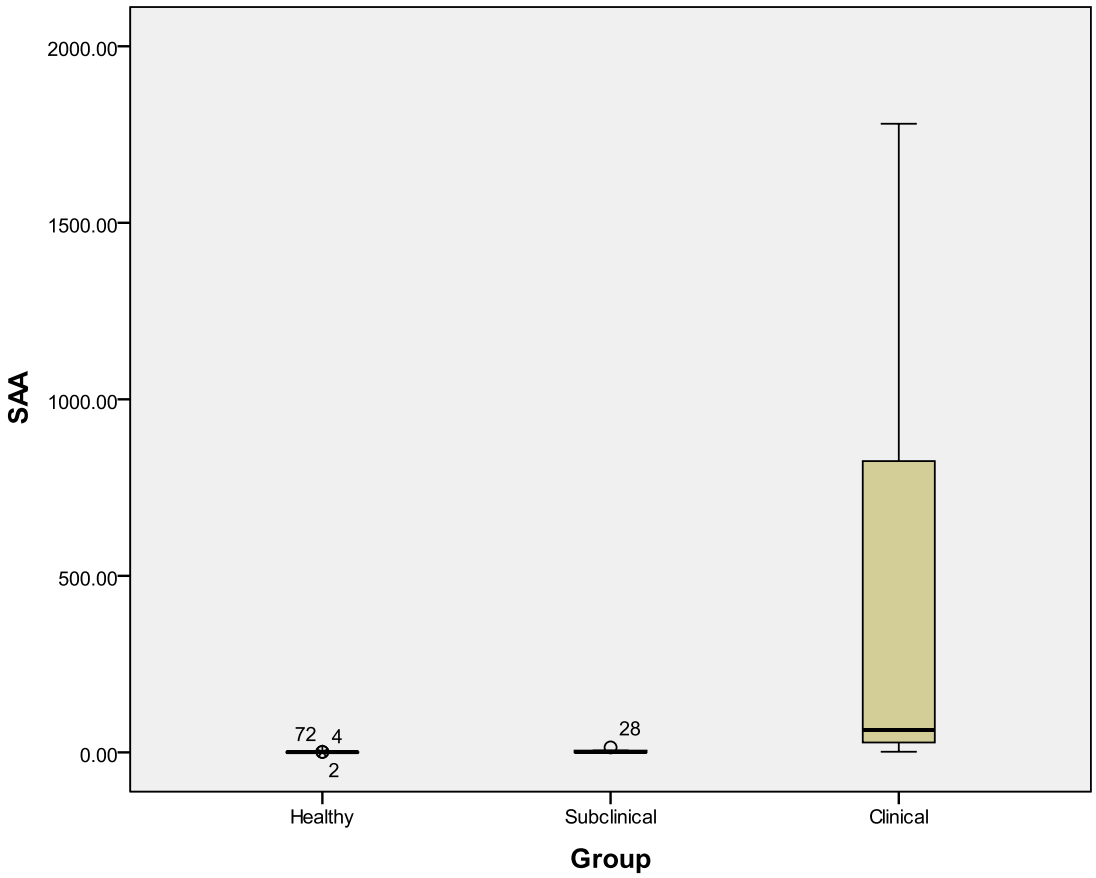


Figure 2-8: The box-plot showed the distribution of SAA levels in bovine milk samples between healthy, subclinical mastitis and clinical mastitis groups. The median of SAA concentration in healthy group was 0.36µg/ml whilst the maximum and minimum values were 2.96 and 0.03µg/ml respectively. The median of SAA concentration in subclinical mastitic group was 1.435µg/ml whilst the maximum and minimum values were 14.05 and 0.18µg/ml respectively. The median of SAA concentration in clinical mastitic group was 63.55µgml whilst the maximum and minimum values were 1781 and 1.78µg/ml respectively.

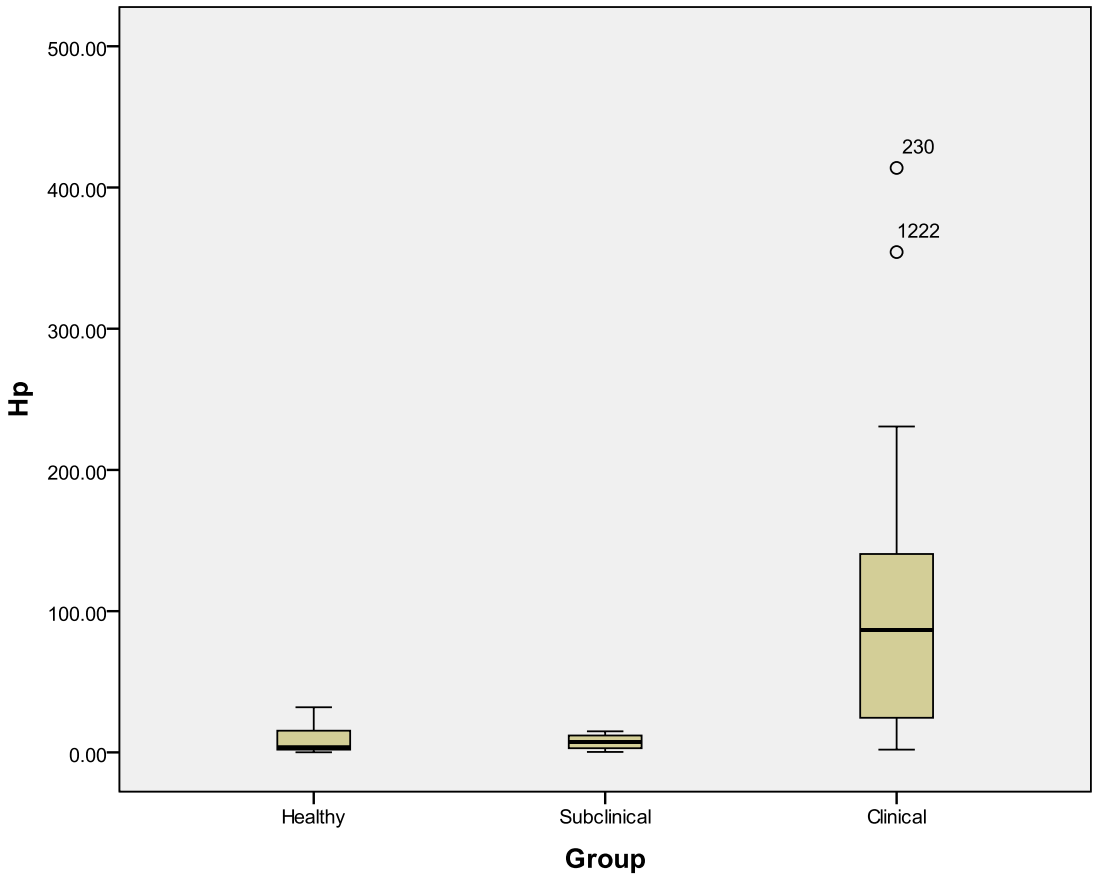


Figure 2-9: The box-plot showed the distribution of Hp levels in bovine milk samples between healthy, subclinical mastitis and clinical mastitis groups. The median of Hp concentration in healthy group was 3.52μg/ml whilst the maximum and minimum values were 31.95 and 0.07μg/ml respectively. The median of Hp concentration in subclinical mastitic group was 7.41μg/ml whilst the maximum and minimum values were 14.91 and 0.31μg/ml respectively. The median of Hp concentration in clinical mastitic group was 86.52μg/ml whilst the maximum and minimum values were 413.85 and 1.95μg/ml respectively.

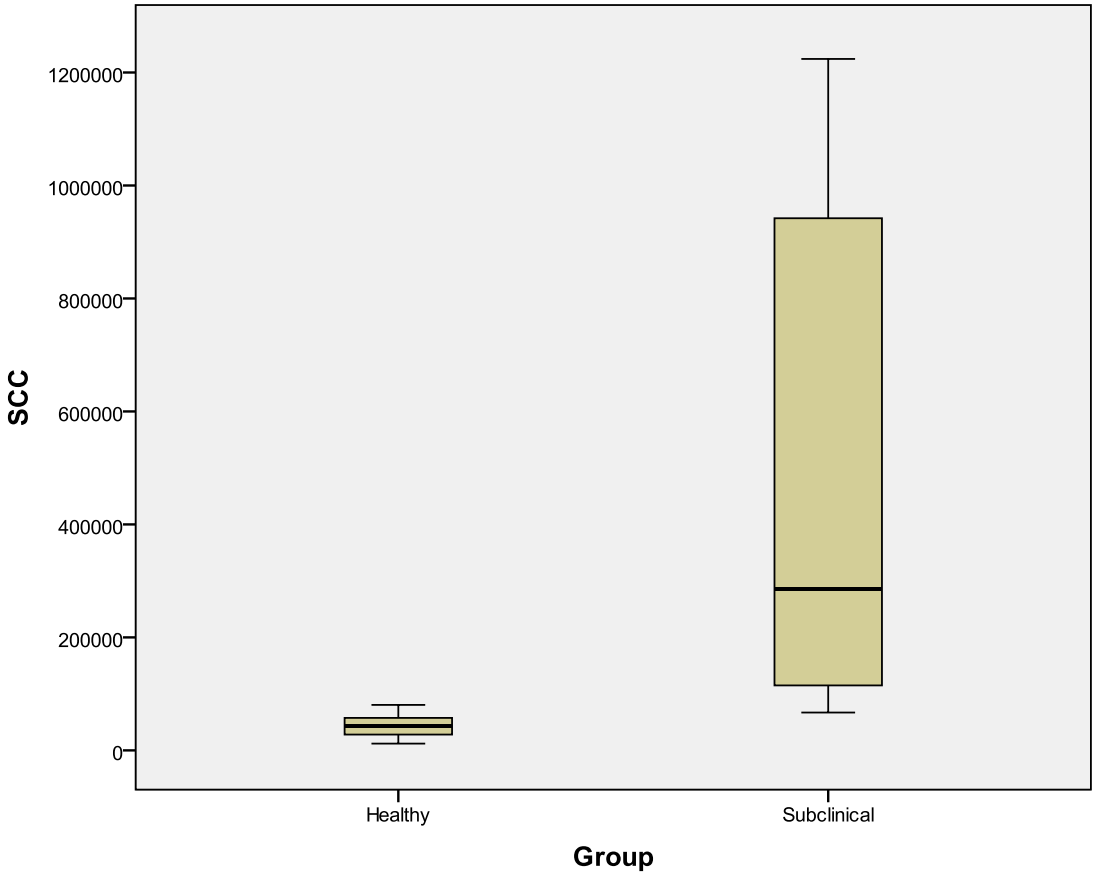


Figure 2-10: The box-plot showed the distribution of SCC values in bovine milk samples between healthy and subclinical mastitis groups. The median of SCC measurement in healthy group was 43 x 10^3cells/ml whilst the maximum and minimum values were 80.5 x 10^3 and 12 x 10^3 cells/ml respectively. The SCC measurement in subclinical mastitic group was 286 x 10^3 cells/ml whilst the maximum and minimum values were 1224 x 10^3 and 67 x 10^3 cells/ml respectively

Correlations

			SAA	Hp
Spearman's rho	SAA	Correlation Coefficient	1.000	.653**
		Sig. (2-tailed)	.	.000
		N	75	71
	Hp	Correlation Coefficient	.653**	1.000
		Sig. (2-tailed)	.000	.
		N	71	71

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

Correlations

			SAA	SCC
Spearman's rho	SAA	Correlation Coefficient	1.000	.438**
		Sig. (2-tailed)	.	.008
		N	75	35
	SCC	Correlation Coefficient	.438**	1.000
		Sig. (2-tailed)	.008	.
		N	35	35

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

Correlations

			SCC	Hp
Spearman's rho	SCC	Correlation Coefficient	1.000	.080
		Sig. (2-tailed)	.	.670
		N	35	31
	Hp	Correlation Coefficient	.080	1.000
		Sig. (2-tailed)	.670	.
		N	31	71

Table 2-4: Spearman's correlation between SAA concentration, Hp concentration and SCC measurements in milk samples. The Spearman's correlation between SAA and Hp was 0.653 whilst the Spearman's correlation between SAA and SCC was 0.438. The Spearman's correlation between SCC and Hp was 0.08.

	Healthy vs. Clinical mastitis	**Healthy vs. Subclinical mastitis**	**Clinical mastitis vs. Subclinical mastitis**
SAA Conc. (µg/ml)	*z*= -6.737 *p*=0.00	*z*= -2.41 *p*= 0.00	*z*= -4.573 *p*= 0.00
Hp Conc. (µg/ml)	*z*= -5.437 *p*= 0.00	*z*= -0.087 *p*= 0.931	*z*= -4.131 *p*= 0.00
SCC (x 10^{-3}/cells)	NA	*z*= -4.439 *p*= 0.00	NA

Table 2-5: Mann-Whitney's Test was performed to test for differences in SAA concentration and Hp concentration as well as SCC measurements between healthy, subclinical mastitic and clinical mastitic group.

2.4 Discussion

Hp and SAA have been studied in this chapter to evaluate their effectiveness in diagnosing bovine mastitis prior to use their use to confirm the status of samples used in the following chapters. After optimising and validating the developed assays for Hp and SAA, their concentrations in different groups of milk samples were then compared statistically. Correlation between SCC values and APP concentrations were also analysed statistically.

For the development of bovine Hp ELISA, several stages were optimised to make sure the assays were reproducible. A final concentration of 1µg/ml of 3.43mg/ml rabbit anti-bovine Hp was chosen whilst 1:1000 or 1:500 (depending on batch) of biotinylated antibody was used to achieve an optimised assay. Both gave a reasonably steep standard curve and covered a wide range of OD values with acceptable low background. Each time a new biotinylated antibody was prepared, a series of optimisation on the varying batch of biotinylated antibodies were done. Different batch of biotinylated antibodies at different dilutions (1:1000 or 1:500) were used due to the large number of milk samples assayed throughout this project. The amount of biotinylated antibody was often less than 0.5ml after its preparation.

The Hp assay was validated by determining the intra-assay CVs of 36 duplicates milk samples and inter-assay CVs of the internal controls. Inter-assay CVs were within the range of 29.9-47.1% with the highest value occurring for a control with lowest level of Hp in milk. In general, CV values for biochemical tests should be lower than 10%, but in the case of immunoassays, the value of 20-30% are generally acceptable, especially if the concentrations of the analyte are low (FDA, 2001). In this study, even though the CV value was above 20%, the large changes in Hp concentration meant that the assay could be used to identify samples with high concentrations of this APP in milk samples.

The PhaseTM Range SAA Assay developed by Tridelta Development Limited, was used to detect the M-SAA3 in the milk samples. It had acceptable precision with an intra assay CV of 6.8% in 42 duplicate samples and a limit of detection adequate to measure M-SAA3 in the healthy milk samples.

2.4.1 Clinical Validation: Assessment of APP level in healthy, subclinical and clinical mastitic milk samples

The final stage of validation of the Hp assay and demonstration of the SAA assay was by clinical validation on assays of the APPs in different groups of milk samples. Some of these samples were used in the following chapters. Hp and SAA assays as described in previous section were performed to assess any differences and correlations between these APP levels in three different groups of milk samples.

2.4.2 Hp level in three different groups of milk samples

Based on the quantification of Hp levels in this study, Hp was detected in milk samples regardless of its disease status. Statistically, it was found that the Hp concentration in healthy milk samples (n=22) was significantly different ($p<0.05$) from clinical mastitic milk samples. This was in agreement with many publications (Eckersall *et al.* 2001; Gronlund *et al.* 2003) on the ability of Hp as a disease marker in bovine mastitis. Hp level also showed significantly higher in concentrations ($p<0.05$) in clinical mastitic milk samples in comparison with subclinical mastitic milk samples. Thus, Hp may be able to discriminate between clinical and subclinical mastitic animals as reported by others (Gronlund *et al.* 2003; Ohtsuka *et al.* 2001). However, Hp levels in the milk examined here produced no significant difference between healthy and subclinical mastitic milk samples ($p>0.05$). This is in an agreement with Gronlund *et al.* (2003) in which they only observed significantly higher level of SAA in infected quarters than pre-infection and healthy quarters but not Hp. The reason might be due to different types of cytokines produced which lead to variation in the secretion and synthesis of APP (Horadagoda et al 1999) in milk samples with different disease status. Furthermore, during subclinical mastitis, Hp production might not be as great as that produced during clinical mastitis. The other possible reason is might be due to the immunoassay which is not sensitive enough to detect a slight elevation of Hp concentrations in sublinical mastitic group. Other factors such as pathogen virulence and host response of the cows can play a role in the production of APP in infected quarters (Gronlund et al 2004). Nevertheless the Hp ELISA developed in the project was able to identify milk samples from cows with clinical mastitis and its use will be demonstrated in subsequent chapters.

2.4.3 SAA level in three different groups of milk samples

With the commercial ELISA to measure SAA levels in this study, this APP was detected in all milk samples regardless of its disease status. This ELISA was sensitive enough to detect a low amount of SAA in healthy animals.

Statistically, SAA levels were found to be significantly different ($p<0.05$) between healthy and clinical mastitic milk samples. The high concentration of SAA found in all clinically infected milk samples further confirmed the role of SAA as a biomarker in bovine mastitis. SAA levels also showed significantly higher concentrations in clinical mastitic milk samples compared with subclinically infected milk samples ($p<0.05$). Furthermore, SAA can also be employed to discriminate between clinical and subclinical mastitis and milk SAA concentrations can also be used to differentiate between healthy and subclinical mastitis as it showed statistically significant difference in milk SAA levels between those two groups ($p<0.05$). These results are in agreement with several studies which have shown that SAA (or M-SAA3) are elevated in milk during mastitis (Eckersall et al 2001; Grönlund et al 2003; Nielsen et al 2004) and in particular those that show that milk-SAA3 is able to differentiate healthy from subclinical mastitis samples (Grönlund et al 2003; Grönlund et al 2005). Thus use of the SAA ELISA was validated to monitor the concentration of this APP in milk for the investigations in the following chapters.

2.4.4 Correlation between SAA and Hp levels in milk samples

As the APP and SCC had been measured in multiple samples it was of interest to determine the correlations between the analytes. In general, SAA level showed a strong and positive correlation ($r= 0.653$) with the level of Hp in clinical mastitic group. This finding confirmed that SAA and Hp can be used as good mastitis indicators in which they both present in a very low amount in healthy milk samples but can increase significantly after induction of acute phase response. This finding also supported the fact that the measurement of APP during intramammary infection can be done by using milk samples rather than serum/plasma due to the ease of sampling and sample preparation. It also showed that the ELISA used to measure these major bovine APP is reliable and sensitive enough to detect its concentration in milk samples.

2.4.5 Correlation between SCC and APP levels in milk samples

Milk samples of healthy and subclinically infected cows were chosen on the basis of SCC measurement. Thus, it is also important to assess the relationship observed between SCC values and APP measurements. SCC has been a common method for mastitis diagnosis and is still being widely used in most of commercial dairy farms as part of disease monitoring system.

According to Hillerton (1999), the limit of SCC for a healthy quarter should be around 100 000 cells/ml and the SCC for composite milk should not exceed 100 000 cells/ml (Laevens et al 1997). In this current study, the average of SCC in the healthy group is 43 x 10^3 cells/ml. Statistiscally, the SCC measured between healthy and subclinical mastitic milk samples were significantly different ($p<0.05$). It proved that SCC is still reliably trusted to diagnose subclinical mastitis as changes of milk composition microscopically are the only signs of subclinical mastitis (Radostits et al 1994). The SCC was not measured in the clinical group as measurement can be misleading in clinical mastitis due to the interference of milk protein clots distorting the cell counting results.

The correlation between SCC and APP in was done and showed a positive but not very strong relationship. The correlation between SCC and SAA ($r=0.438$) gave a medium relationship whilst the correlation between SCC and Hp ($r=0.08$) gave a very small relationship. The interpretation was based on Cohen (1988).

Previous studies showed a strong and positive relationship between SCC and Hp (Thielen et al 2005; Lai et al 2009). They claimed that leukocytes of milk somatic cell act as one of the cellular source of mammary Hp mRNA expression and also that these circulating bovine leukocytes might be able to synthesize the Hp and contribute towards the measurement of Hp in milk (Thielen et al 2005). It was also supported by Lai et al (2009) that somatic cells were responsible for the increase of milk Hp as Hp levels measured using ELISA were positively correlated with the SCC and also Hp mRNA levels in cells of high SCC groups were significantly elevated than in cells from normal group.

Although the relationship between Hp and SCC was very low, it should be noted that this correlation was calculated based on all milk samples regardless of the type of milk samples (control or infected). Furthermore, various cut-off values for SCC have been

suggested depending on the conventions used, countries, breed and type of milk samples (Mattila et al 1986; Holdaway et al 1996).

As a summary, the ELISA developed for quantification of Hp in milk samples was a reliable and sensitive enough assay to investigate the Hp levels in milk samples of different groups being able to differentiate clinical mastitic samples from subclinical and healthy samples. Commercial SAA ELISA also proved to be able to analyse SAA concentration in all milk samples studied. The measurement of APP levels in diagnosing bovine mastitis appears to be a useful tool in monitoring udder health for its ability to discriminate the healthy milk samples from infected milk samples. However, at present SCC should also be used in detecting the presence of subclinical mastitis in a herd of cows, although various factors should be evaluated (e.g; stages of lactation, milk production level, stress, breed) before any decision-making, especially on therapeutic measures. Furthermore, SCC and APP measurements should be used together as part of udder health inspection in a herd of dairy cows. For the investigations in subsequent chapters, the ELISAs for M-SAA3 and milk Hp along with SCC and bacteriology were used to provide definitive characterisation of milk samples as being from healthy dairy cows or from udder quarters with subclinical or clinical mastitis

Chapter III: Proteomics in Bovine Mastitis

3.1 Introduction

Proteomics has come forward as a rapidly maturing essential tool in the *omics* age following the huge success of genomic studies. As mentioned previously, proteomics is a study that involved in separation and identification of a set of proteins existing in a cell, organ or organism (Wang *et al.* 2007). Studying proteins has been a subject of interest for many researchers as any cell responses to stimuli or stress are manifested by the alteration of protein expression levels (Lippolis & Reinhardt 2010). Protein information is also valuable as protein acts as structural building blocks, conveys information, controlling many chemical reactions as well as being involved in antimicrobial defence mechanisms.

Proteomics can also be defined as a large-scale study of protein expression, protein-protein interactions or post-translational modifications (Cravatt *et al.* 2007; Lippolis & Reinhardt 2010). Unlike other methodologies, proteomics is able to analyze thousands of proteins in a single study and thus one can demonstrate how cells dynamically change upon stimulation or changes in the environment. In particular, proteomics stands out in its ability for a high resolution and simultaneous analysis of the composition of milk. (O'Donnell *et al*. 2004).

Previous studies on the milk proteome have utilized many different approaches of proteomic analyses. Details on each study have been discussed earlier in chapter 1. By using various proteomic methods such as 2D gel electrophoresis and MALDI analyses (Anderson *et al.* 1982; Galvani *et al.* 2000,2001; Baeker *et al.* 2002), 2D and microsequencing (Murakami *et al.* 1998; Yamada *et al.* 2002) as well as LC-MS/MS (Palmer *et al.* 2006), a complete milk proteome which also included low abundance milk proteins has been achieved. In this section, the technical aspects of proteomic analyses will be discussed further.

3.1.1 Sample Collection and Preparation

The protein level in any biological fluid is influenced by many factors such as normal metabolism, circadian rhythms of protein production, degradation and environmental conditions (Hale *et al.* 2003). Upon sampling, protein modifications may also occur which include proteolysis, protein aggregation and chemical modifications. Thus, to minimize variation, sample collection and storage must be standardized.

However, most biological fluids are dominated by relatively few proteins for example in blood, albumin and the immunoglobulins (Igs) predominate (Anderson & Anderson 2002). Thus, removal of these proteins is advantageous to retrieve other lower abundance proteins and to reduce sample complexity. For example, the use of columns containing Cibacron blue (Hale *et al.* 2003) and anti-albumin antibodies (Steel *et al.* 2003) or albumin-specific peptides (Pingali *et al.* 1996) can be applied to remove albumin.

Despite the sensitivity of the mass spectrometers used in proteomics study, several factors concerning the complexity of the sample must be analyzed. These include the number of protein in a proteome, the diversity of protein expression, the degree of protein solubility and the copy number of protein classes to assess the changes (Reinhardt & Lippolis 2008; Graham *et al.* 2005).

Thus, various fractionation protocols were introduced to reduce the complexity of the mixtures for a more complete identification of proteins. This can be achieved via subcellular fractionation, enrichment strategies, chromatography or gel electrophoresis (Stasyk & Huber 2004). The subcellular fractionation provides important information on the nuclear membrane proteome; for example the isolation of MFGM from bovine milk. With enrichment methods, antibodies or metal ions can be used to isolate the protein of interest from the cell or pathogens. Size exclusion filtration may be applied to separate peptides subsequently sequenced by mass spectrometry (Hunt *et al.* 1992; Lippolis *et al.* 2002). Chromatography and gel electrophoresis will be discussed next in this section.

3.1.2 Protein separation

Protein separation is the first main technology in proteomic study and two most popular methods are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrell 1975; Görg *et al.* 1998; Rabilloud 2002) and the gel-free procedure; liquid phase separation (Isaaq 2001; Isaaq *et al.* 2002).

2D-PAGE has been utilized for many years and was first established in the mid 70's (O'Farrell 1975; Klose 1975). It is able to resolve thousands of proteins and the procedure consists of two steps; separation of proteins based on their isoelectric points (pI) and finally separation based on their molecular weight (Mr). Basically, this method combines isoelectric focusing (IEF) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (O'Donnell *et al.* 2004; Hiendleder *et al.* 2005). The IEF gels are available

in broad or narrow pH ranges to achieve different levels of resolution (Lippolis & Reinhardt 2008).

Following gel electrophoresis, a spot pattern on a gel is generated and each spot represents a different protein or its modification form (Heindleder *et al.* 2005). These protein spots can be cut out and further analyzed by mass spectrometry (Lippolis & Reinhardt 2008).

According to O'Donnell *et al.* (2004) the advantage of using 2D-PAGE includes higher resolution and ability to separate proteins of similar relative molecular mass (Mr) and pI. However, major drawbacks of this method limit its usage such as an inability to separate single polypeptide chains of masses greater than 150 kDa or less than 8 kDa, limited dynamic range and loading capacity of the immobilized pH gradient (IPG) strip while it is unable to resolve proteins with an extremely basic (pI >12) or acidic (pI < 3) in nature.

To overcome the irreproducibility problem of 2DE, difference gel electrophoresis (DiGE) was developed to minimize the effects of gel-to-gel variation by labelling two samples with two different fluorescent dyes before running them on the same gel (Unlu *et al.* 1997). DiGE is extremely sensitive as it is able to detect over a >10 000-fold protein abundance range (Viswanathan *et al.* 2006; Lilley *et al.* 2002).

In DiGE, equal amount of protein samples are differentially labelled using synthetic N-hydroxysuccinimidyl (NHS) ester derivatives of the cyanine dyes Cy3 and Cy5 before being subjected to 2DE separation and finally scanned to produce two super-imposable images (Timms & Cramer 2008). In order to increase the accuracy of the results, standard can be included and labelled with a third fluorophore through internal standardization (Hiendleder *et al.* 2005).

Other protein separation methods are based on liquid phase separation such as capillary electrophoresis (CE) and liquid chromatography (LC). They are known to have greater sensitivity, provide a wide dynamic range, are easily automated and can be faster than 2D-PAGE (O'Donnell *et al.* 2004). A number of separation mechanisms can be applied such as size exclusion, reversed-phase (RP) and ion exchange so that strongly acidic/basic proteins can be analysed. Peptides are separated into fractions based on their hydrophobicity (RP-HPLC) or based on their charge state (strong cation exchange chromatography) (Lippolis & Reinhardt 2008).

A rather new approach to provide better separation is multidimensional protein identification (Mud-PIT) where two or more LC techniques (e.g. ion exchange chromatography and RP HPLC) are used with on-line MS (Washburn *et al.* 2001).

3.1.3 Protein characterization

Protein characterization is mainly accomplished by mass spectrometers. Basically, all current mass spectrometers can detect and identify peptides in the femtomole (10^{-15}) to attomole (10^{-18}) range (Moyer *et al.* 2003). After initial protein digestion with trypsin, peptides must be ionized upon entering the mass spectrometer. The two most frequently used ionization methods are electrospray ionization (ESI) (Fenn *et al.* 1989) and MALDI (Tanaka *et al.* 1988; Karas & Hillenkamp 1988).

ESI allows direct connection between LC and MS due to the volatility of the HPLC solutions. MALDI requires mixing of the digested peptides with a UV-absorbing molecule to form a crystalline structure. A laser will strike this structure for sublimation of the matrix and ionization before releasing the associated peptides (Lippolis & Reinhardt 2008).

After ionization, the peptide mass is determined by mass analyser. Peptide mass is usually expressed as a ratio of mass divided by the charge of the peptide (m/z). Mass spectrometers are completed by detectors which can range from simpler quadrupole (Q), time-of-flight (TOF) to more complex Fourier transform ion-cyclotron resonance (FTICR) analysers (O'Donnell *et al.* 2004).

Information gained from mass spectrometers includes mass and sequence of the proteins. MALDI-TOF is used primarily for detecting only protein masses while quadruopole-time of flight tandem mass spectrometry (Q-TOF MS/MS) can be used to provide sequence information (Roepstorff & Fohlman 1984). Post-source decay (PSD) is another technique used to sequence proteins (Spengler *et al.* 1992).

The next step is protein identification by matching the masses of digested tryptic petides with known masses from a non-redundant protein database (Beavis & Fenyo 2000). This technique is simply achieved by peptide mass fingerprinting (PMF). Peptide fingerprinting is often associated with 2D-PAGE and protein identification is based on the measurement of multiple peptides from one particular protein (Lippolis & Reinhardt 2008). The second method to identify proteins is by using tandem mass spectrometers when PMF data are inconclusive (O'Donnell *et al.* 2004; Lippolis & Reinhardt 2008).

Generally, proteomics experiments can be either direct identification or characterization of a certain known proteins of interest or indirect/shotgun approach. The ultimate goal of

shotgun approach is to identify all proteins present in a sample. It can be divided into survey or expression proteomics (Lippolis & Reinhardt 2010). In survey proteomics, it gives an overview of types of proteins and grouped them based on their biological process, cellular compartment or molecular function (Lippolis & Reinhardt 2010) while expression proteomics involved with relative quantification where two or more samples are compared with one served as a reference (Bantscheff *et al.* 2007; Lippolis & Reinhardt 2008; Ong & Mann 2005).

3.1.4 Quantitative proteomics

The next stage of proteomics is quantification for estimates of differential protein expression. DiGE is one of the techniques which represent a real advance in protein quantification by 2DE electrophoresis as described above. Fluorescence characteristics of the CyDyes are used to determine the relative abundance of proteins from two different samples (Wu *et al.* 2009).

The other two commonly used quantitative techniques use molecular tags to label peptides via an interaction with a free thiol or amine group. The first molecular tags is called isotope-coded affinity tags (ICAT) that uses isotopic labelling of cysteine residues by light or heavy tag containing biotinylated reagents (Lippolis & Reinhardt 2008; Gygi *et al.* 1999). One sample is labelled with the light tag and the other is labelled with the heavy tag. They are combined and enzymatically cleaved before further analyzed by LC-MS/MS. A newer tag approach called iTRAQ has been commercially available since 2004 and uses a set of 4 tags that possess the same molecular weight but different fragmentation points. It permits relative quantification of many samples (up to 8 samples) at one time (Choe *et al.* 2007).

Other less commonly used methods to obtain metabolic information on bacterial growth are stable isotope from amino acids in cell culture (SILAC) (Ong *et al.* 2002; Couté *et al.* 2007) and surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) to identify enzymes that change expression in response to the presence of substrates such as lactose, glucose and galactose (Gagnaire *et al.* 2009).

3.1.5 Aims of study

The aims of this study were to determine the changes of milk proteome during the presence of intramammary inflammation (subclinical and clinical bovine mastitis). For comparison, the milk proteome of normal milk samples as controls were also studied using 1DE, 2DE,

DiGE and MALDI-MS analyses. The discovery of new biomarkers especially in subclinical mastitis was also an important objective of this study.

3.2 Materials and Methods

3.2.1 Reagents

General chemicals and other materials were obtained from Bio-Rad Laboratories Inc. unless otherwise stated. Double distilled water was used throughout.

3.2.2 Milk samples

Milk samples used in this study were of subclinical mastitic cases from Cochno Farm of School of Veterinary Medicine, University of Glasgow, UK (n=5) and clinical mastitis cases from Vale Veterinary Laboratory, Devon, UK (n=12). Bacteriological culture was performed for clinical mastitic milk samples in the Bacteriology Laboratory of Vale Veterinary Laboratory to determine the bacterial cause of bovine mastitis. Meanwhile, control milk samples (n=12) came from healthy cows from the Cochno Farm of School of Veterinary Medicine, University of Glasgow. The healthy milk samples were confirmed by somatic cell counts (<100 000 cells/ml) (Kromker *et al.* 2001) obtained from the Cochno Farm of School of Veterinary Medicine, University of Glasgow, UK. Prior to proteomic analyses, SAA and Hp assays (Chapter 2) were performed to determine the concentration of both acute phase proteins in each milk sample as confirmation of the health status. Samples were stored at -20°C prior to analysis. California Mastitis Test (CMT) was carried out on the subclinical mastitic milk samples at the Cochno Farm.

3.2.3 California Mastitis Test

The California Mastitis Test (CMT) was performed as described in the previous chapter on Section 2.2.3.

3.2.4 Removal of high abundance milk proteins

3.2.4.1 Rennet Coagulation

Casein, the most abundant milk protein can be coagulated with the enzyme rennin found in rennet (an extract from the stomach of calves) to split a specific bond in the amino acid chain of the κ-casein macromolecule and form para-κ-casein and a glycol-macropeptide (Leonil & Molle 1991). Thus, the removal of casein from milk can be performed by rennet coagulation to allow the investigation of low abundance proteins. A total of 10µl of 15% (w/v) of calcium chloride was added into 1ml of milk sample in order to improve coagulation before adding the rennet. The sample was incubated at room temperature for 1

hour before pre-heated at 30ºC using a water bath for 20 minutes. An aliquot of 5 µl of 7.5mg/ml rennet (Sigma-Aldrich, Dorset, UK) was added into 1ml milk sample and incubated at 30ºC for 30 minutes. Finally, the sample was centrifuged at 21 000 x *g* for 10 minutes and supernatant/whey was kept frozen until further analysis.

3.2.4.2 Acid Precipitation

Acid precipitation was used to remove major milk proteins such as caseins as another approach to investigate the properties of noncasein milk proteins (Murakami *et al.* 1998; Baeker *et al.* 2002, Yamada *et al.* 2004). A 0.5ml aliquot of milk sample was used and its pH was altered to pH4 using 1M acid hydrochloric (HCl). After pH adjustment, milk sample was centrifuged at 21 000 *g* for 10 minutes to remove precipitates. Supernatant/whey was kept frozen until further analysis.

3.2.4.3 Ammonium sulphate (NH_4SO_4) Fractionation

Ammonium sulphate fractionation was employed as another method to remove major milk proteins such as caseins. It was thought that this method was able to reduce any potential for acid induced protein hydrolysis (Hogarth *et al.* 2004). Saturated NH_4SO_4 was prepared by dissolving NH_4SO_4 in ice cold distilled water till no more dissolved. NH_4SO_4 (100%) was added into milk sample to make up 35% of NH_4SO_4. The sample was incubated for 1 hour in an ice bath before being centrifuged at 21 000 g for 10 minutes. Supernatant/whey was kept frozen until further analysis.

3.2.5 One-dimensional gel electrophoresis

Milk samples used for 1D GE were 5 from individual subclinical mastitic and another 6 from individual healthy cases obtained from Cochno Farm, Glasgow, UK.

3.2.5.1 Reagent Preparation and Stock Solutions

All stock solutions and buffers were prepared according to Mini-PROTEAN® 3 Cell Instruction Manual. 12% PAGE gels were also prepared according to the manual (see method in Appendix 1, Chapter 3).

3.2.5.2 Sample Preparation

Prior to 1DE analysis, the protein content was measured using a BioRad Bradford Assay (see Appendix 2, Chapter 3). Milk samples were diluted to give a sample at 2-3mg/ml and mixed with SDS Reducing Buffer (10% (w/v) SDS containing 0.5M Tris-HCl, pH 6.8, 2.5ml glycerol and 0.05% (v/v) bromophenol blue; Bio-Rad Laboratories, Inc. USA) at a ratio of 1:1 and heated at 95°C for 4 minutes.

3.2.5.3 Running the gel electrophoresis

After the assembly of the electrophoresis module, the sample was loaded into wells with a pipette using gel loading tips. Power was applied to the Mini-Protean® 3 cell and electrophoresis was run at 200 volts for 40 minutes.

3.2.5.4 Gel staining and Image Acquisition

After electrophoresis, the gels were stained using colloidal Coomassie blue G dye according to manufacturers' instructions (Sigma-Aldrich, Dorset, UK) and de-stained in 10% (w/v) acetic acid in 25% (v/v) methanol overnight. Gel images were acquired using Umax Power Look III.

3.2.5.5 In-gel trypsin digestion of Coomassie-stained proteins

Chosen gel bands from 1D gel electrophoresis were excised and placed in 1.5ml Eppendorf tubes. These bands were then cut into several pieces using pipette tips. Gel pieces were washed for 45 minutes in 500µl of 100mM ammonium bicarbonate and the wash was discarded. Gel pieces were further washed in 50% acetonitrile/100mM ammonium bicarbonate for another 45 minutes. The wash was again discarded. For reduction, 150µl of 100mM ammonium bicarbonate and 10µl of 45mM DTT were added and incubated at 60ºC for 30 minutes. For alkylation, 10µl of 100mM iodoacetamide was added and incubated in the dark for 30 minutes. Solvent was discarded and gel pieces were washed in 500µl 0f 50% (v/v) acetonitrile/100mM ammonium bicarbonate with shaking for 1 hour. The wash was discarded and 50µl of 100% (v/v) acetonitrile was added to shrink the gel pieces. After 10 minutes, solvent was removed and gel pieces were dried completely in a vacuum centrifuge for 30 minutes. The dried gel pieces were left at 4ºC for 15 minutes before adding 0.2µg/µl sequencing grade modified Porcine Trypsin (Promega #V111) in 25mM ammonium bicarbonate to rehydrate each gel piece. Additional trypsin solution was added if trypsin was completely absorbed so that the gel band became fully rehydrated. Finally about 20µl of 25mM ammonium bicarbonate was added over the gel pieces which were incubated overnight to allow protein digestion at 37ºC. 30µl of 100% acetonitrile was then added to the gel pieces and incubated for 20 minutes. Each solution in each tube was transferred to separate wells in a 96-well plate and dried in the Speed Vac. About 20 µl of 1% (v/v) formic acid was added into each Eppendorf tubes containing gel pieces and incubate for another 20 minutes. Each solution was transferred to the corresponding wells. These two steps were repeated for better recovery. About 40µl of acetonitrile was added and further incubated for 10 minutes before the solution was transferred to the corresponding wells. This step was also repeated for one time. The plate was checked at least after 90 minutes to make sure the sample had been dried up.

3.2.5.6 Sample Plating

The MALDI plate should be cleaned and rinsed before use. A saturated solution of cyano-4-hydroxy cinnamic acid (CHCA) in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) was prepared in 1.5ml Eppendorf tube and covered with aluminium foil. The tube was vortexed for 5 minutes before being centrifuged for 5 minutes. The saturated solution of CHCA was diluted at 1:10 in 50% acetonitrile and 0.1% TFA. 10µl of 50% acetonitrile and 0.1% TFA was loaded into the plate and 1µl of dried peptide was dissolved into the same spot. 1µl of 90% (v/v) CHCA solution (matrix solution) was added to the peptide on the plate and was mixed well by pipetting up and down for 5-7 times. After sample loading, the plate was left to dry before being analysed by the MS which was supervised by Kamburapola Jayawardena of the Sir Henry Wellcome, Functional Genomic Facilities, University of Glasgow, UK.

3.2.6 Two-dimensional gel electrophoresis

Milk samples used for 2D GE were pooled together from six of subclinical mastitic milk samples to make up pooled subclinical mastitic sample and another six from healthy cases were pooled together to make up pooled healthy samples.

3.2.6.1 Sample preparation

Each supernatant/whey sample from rennet coagulation was precipitated with acetone overnight for 500µg protein. To a sample volume containing 500 µg, a 10 times volume of ice cold acetone was added and incubated at -20°C overnight. The protein solution was then centrifuged at 14 000 x *g* for 10 minutes. Supernatant was removed after centrifugation. 1ml of 80% acetone was used for washing before suspending the pellet in 80% acetone. The dissolved pellet was centrifuged at 14 000 x *g* for another 10 minutes. Supernatant was discarded before dissolving the pellet in 1ml of lysis buffer (see Appendix 3, Chapter 3). To make sure the pellet did not stick to the centrifuge tube's wall, another brief centrifugation was performed. For the pellet to fully dissolve, the tube was put on the vibrating platform.

3.2.6.2 Isoelectric Focusing

A protein concentration assay was run on each sample so that an equal amount (100µg) was present before adding 3-10 IPG buffer (0.5% v/v), DTT 0.001 g/µl (0.02% w/v) and rehydration buffer (6M urea, 2M thiourea, 4% CHAPS and 0.02% (w/v) bromophenol blue) for 100µg. Sample was centrifuged at maximum speed for 5 minutes. About 450 µl of prepared sample was applied on pH3-10, 24cm IPG strip. Isoelectric focusing was run on the IPGphor system using this protocol:

50μA per strip at 20°C

S1: Step-and-hold 30V 12 hours

S2: Step-and-hold 300V 1 hour

S3: Gradient 1000V 2 hours

S4: Gradient 8000V 2 hours

S5: Step-and-hold 8000V 8 hours

S6: Step-and-hold 1000V 24 hours

S7: Step-and-hold 1000V 24 hours

After the focusing finished, the strip was put in a plastic tube with the gel part facing upward and stored at -20°C before running the SDS-PAGE gel electrophoresis.

3.2.6.3 SDS-PAGE Gel Electrophoresis

Both IEF Equilibration Buffers (EB) I and II (see Appendix 4 and 5, Chapter 3) were prepared prior to gel electrophoresis. 10ml of EB I was added onto the strip and mixed for 15 minutes. The strip was then washed with running buffer and then 10ml of EB II was added onto the strip and mixed for another 15 minutes. The strip was washed once again before 1ml of running buffer was added on top of the gel (large format SDS PAGE gel). The strip was placed horizontally on top of the precast Ettan DALT® gels (26 x 20cm) and added 1ml of 0.5% agarose (heated at 105°C at least 5 hours prior to gel electrophoresis) was placed on top of the gel. The prepared gel was placed in the tank and the remaining running buffer was added in the tank. The electrophoresis was run overnight at higher and constant power.

3.2.6.4 Gel staining and Image Acquisition

After electrophoresis, the gel was fixed for 1-2 hours using 10% methanol and 7.5% acetic acid. Gel was then stained with freshly prepared Colloidal dye solution and put on the rocker overnight. Gel images were acquired using a Typhoon™75 multicolour fluorescence and phosphor image scanner.

3.2.7 Difference Gel Electrophoresis

Milk samples used for DiGE comprised 12 clinical mastitic and 12 healthy milk samples which were then pooled together to make up a pooled clinical mastitic milk samples and

pooled healthy milk samples. Concentration of Hp and M-SAA3 were determined as in Section 2.2.3 and 2.2.6.

3.2.7.1 Sample Preparation

Cold acetone (-20ºC) at four times sample volumes was added to protein (i.e: 250µl of each sample into 1000µl of cold acetone). This mixture was incubated overnight and it was then centrifuged for 10 minutes at 13 000 *x g*. The supernatant was decanted and the pellet was washed with 4 times volume of 80% acetone. Centrifugation was performed again and supernatant was decanted. Brief centrifugation was done and residual supernatant was removed with pipette tip. The dry pellet was air dried for approximately 5 minutes at room temperature but was not overdried the pellet. The pellet was resuspended in 10µl of DiGE lysis buffer (see Appendix 6, Chapter 3) with protease inhibitor cocktail with brief spinning. The milk samples were solubilised by probe sonification for 3 times brief cycles (1-2 second each cycle). Between sonification, the sample was cooled on ice for 1 minute. Foaming and heating during sonifcation was avoided. After sonification, the samples were incubated for 10 minutes at room temperature with gentle mixing. Lastly, to remove insoluble material, the samples were centrifuged for 10 minutes at 13 000 *x g*. The supernatant was transferred to a fresh microcentrifuge tube.

3.2.7.2 CyDye Labelling

1µl of CyDye was added into 10µl of sample and incubated in the dark for 30 minutes. To stop the reaction, 1µl of 10mM lysine was added and further incubated in the dark for 10 minutes. The pooled milk samples from healthy cows were labelled with Cy3 and the pooled milk samples from cows with clinical mastitis were labelled with Cy5.

3.2.7.3 Isoelectric focussing

The samples (1 labelled Cy3 and 1 labelled Cy5; ~24µl of each) were mixed together and 500µl of rehydration buffer was added into the sample mixture. Before centrifugation, the sample mixture was left at room temperature for 10 minutes. 450µl of sample mixed in rehydration buffer was pipetted along the length of the strip holder, avoiding any formation of bubbles. The IPG strip was put onto the strip holder before layering mineral oil over the top of the strip, just enough to cover the strip. The lid was placed on the strips which were placed on the IPGphor system (GE Healthcare UK) and isoelectric focussing was run for between 70-80000V hours on the IPGphor as described in Section 3.2.6.2

3.2.7.4 SDS-PAGE

After isoelectric focussing, the strips were taken out of the holders and put into the plastic tubes. 10ml of SDS Equilibration Buffer I (see Appendix 4, Chapter 3) were placed onto

the strips and the plastic tubes was put on a large flat rocker desk for 15 minutes. The buffer was poured off and SDS Equilibration Buffer II (see Appendix 5, Chapter 3) was added and was rocked gently for another 15 minutes. The buffer was poured off and the strip was placed on the gel.1ml of 0.5% agarose was added on top of the gel (precast Ettan DALT® gels; 26 x 20cm) before putting the gel in the tank. Electrophoresis was run overnight at 1-2V/gel in the running buffer.

3.2.7.5 Gel Scanning

DiGE gels were fixed before scanning on a 3 Laser Typhoon 9400 scanner. A preliminary scan was performed with one 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 (see DiGE manual GE Healthcare). Gels can be scanned in pairs. Each high resolution (100 microns) scan takes > 1 hr to complete but it was found that DiGE gels can be stored wrapped in the fridge overnight without noticeable loss of resolution. Once satisfactory images were obtained, gels were discarded and plates cleaned. After staining and scanning, the gel was wrapped and stored at 4°C until spot picking.

3.2.7.6 Image manipulation

DiGE images were cropped using Imagequant software on the Typhoon scanner. The cropping removed all edges of the gel image (gel spacers, IPG strip and dye front). Images were then loaded into DeCyder for processing.

3.3 Results

3.3.1 SCC measurement and acute phase proteins concentration

In order to confirm the disease status of milk samples used in this study, SCC measurement and acute phase proteins (SAA and Hp) concentrations were determined. Table 3-1 showed the SCC measurement and CMT scores in all control (n=6) and subclinical mastitic (n=5) milk samples used in this study for 1D and 2D gel electrophoresis. In control milk samples, the range of SCC measurement in control milk samples (n=6) was between 6 and 27 x 10^3 cells/ml with an average of 18 x 10^3 cells/ml and median of 19 x 10^3cells/ml. The range of SCC measurement for subclinical mastitic milk samples (n=5) was between 211 and 4472 x 10^3 cells/ml with an average of 1355 x 10^3 cells/ml and median of 389 x 10^3 cells/ml. The CMT scores for all subclinical mastitic

milk samples were 2+ and all control milk samples gave negative results on CMT score. Table 3-2 showed the SCC measurement concentrations in all control milk samples (n=12) and APP concentrations as well as bacteriological culture results in clinically infected milk samples used in this study for DiGE. In control milk samples, the range of SCC measurement in control milk samples (n=6) was between 6 and 105 x 10^3 cells/ml with an average of 40 x 10^3 cells/ml and median of 27 x 10^3 cells/ml. The range of Hp concentration was between 560 and 16803.62 μg/ml with an average of 5532μg/ml and median of 40234μg/ml. The range of SAA concentrations in clinically infected milk samples was between 16.57 and 10635μg/ml with an average of 1041μg/ml and median of 107.7μg/ml. From the results of bacteriological culture of clinical mastitic milk samples, bacteria that had been successfully identified include *S. uberis* and *E. coli* although in some milk samples, no growth of bacteria was identified.

3.3.2 Comparison between rennet coagulation, acid precipitation and salt precipitation (ammonium sulphate) to remove casein

After each of casein precipitation methods, whey samples from normal milk were run on 1D gel electrophoresis for protein separation. For comparison, whole milk without any casein precipitation was run on the 1D gel electrophoresis as a control. Based on Figure 3-1, Lane 2, protein bands of casein were found between 25-37 kDa by comparing to Mr standard (Lane 1) showing the presence of major milk protein in the whole bovine milk. As for Lane 3, 4 and 5 where various methods of casein precipitation were applied, protein bands of casein were completely absent from the 1D gel. Furthermore, both rennet coagulation (Lane 3) and acid precipitation (Lane 4) showed increased intensity of protein bands between 10-15 kDa of molecular weight. These protein bands are likely to be β-lactoglobulin (15 kDa) and α-lactalbumin (10 kDa). However, more low intensity of higher molecular weight protein bands were seen between 50-250 kDa on both rennet precipitation (Lane 3) and acid precipitation (Lane 4).The intensity of those high Mr protein bands was higher with rennet precipitation as compared with acid precipitation. The rennet coagulation do had a very big Mr protein band at Mr 250kDa. Ammonium sulphate precipitation gave a low yield of whey protein (Lane 5).

Healthy milk samples

ID	SCC (x 10^3 cells/ml)	CMT Score
31	24	Negative
5	6	Negative
4	27	Negative
16	13	Negative
73	27	Negative
110	9	Negative
Min	6	
Max	27	
Mean	17.67	
Median	18.5	

Subclinical mastitic milk samples

ID	SCC	CMT Score
78	4472	2+
75	211	2+
80	482	2+
27	255	2+
97	NA	2+
Min	211	
Max	4472	
Mean	1355	
Median	368.5	

Table 3-1: The SCC measurement in all control (n=6) and subclinical mastitic milk samples (n=5) and CMT scores in subclinical mastitic milk samples used in this study for 1D and 2D gel electrophoresis. The range of SCC measurement for healthy milk samples was between 6 and 27 x 10^3 cells/ml with an average of 18 x 10^3 cells/ml and median of 19 x 10^3 cells/ml. The range of SCC measurement for subclinical mastitic milk

samples was between 211 and 4472 x 10^3 cells/ml with an average of 1355 x 10^3 cells/ml and median of 369 x 10^3 cells/ml. The CMT scores for all subclinical mastitic milk samples were 2+ and all control milk samples gave negative results on CMT score. NA means not available.

Control milk samples

ID	**SCC (x 10^3 cells/ml)**
31	24
5	6
4	27
16	13
73	27
110	9
43	19
60	80
49	105
110	46
71	43
95	75
Min	6
Max	105
Mean	39.5
Median	27

Clinical mastitic milk samples

ID	Hp (µg/ml)	SAA (µg/ml)	Bacteriological culture
24	16803.62	20.52	*Strep. uberis*
9	11277.6	19.92	*Strep. uberis*
17	559.5	10635.28	No growth
18	6296.3	99.5	No growth
3	2905.1	559.5	*E. coli*
7	7980.4	115.81	*Strep. uberis*
78	4419.15	16.57	No growth
47	2815.77	131.34	No growth
A94	1517.24	17.11	No growth
2	4772.38	502.07	*E. coli*
4	3411.28	42.47	*Strep. uberis*
6	3628.2	336.46	No growth
Min	559.5	16.57	
Max	16803.62	10635.28	
Mean	5532.211667	1041.37917	
Median	4023.675	107.655	

Table 3-2: The SCC measurement in all control (n=12) milk samples and APP concentration in clinically infected milk samples (n=12) used in this study for DiGE.

The range of SCC measurement for healthy milk samples was between 6 and 105 x 10^3 cells/ml with an average of 40 x 10^3 cells/ml and median of 27 x 10^3 cells/ml. The range of Hp concentration was between 559.5 and 16803.62 µg/ml with an average of 5532.21µg/ml and median of 4023.68µg/ml. The range of SAA concentrations in clinically infected milk samples was between 16.57 and 10635.28µg/ml with an average of 1041.38µg/ml and median of 107.66µg/ml. From the results of bacteriological culture of

clinical mastitic milk samples, bacteria that had been successfully identified include *S. uberis* and *E. coli* although in some milk samples, no growth of bacteria was identified.

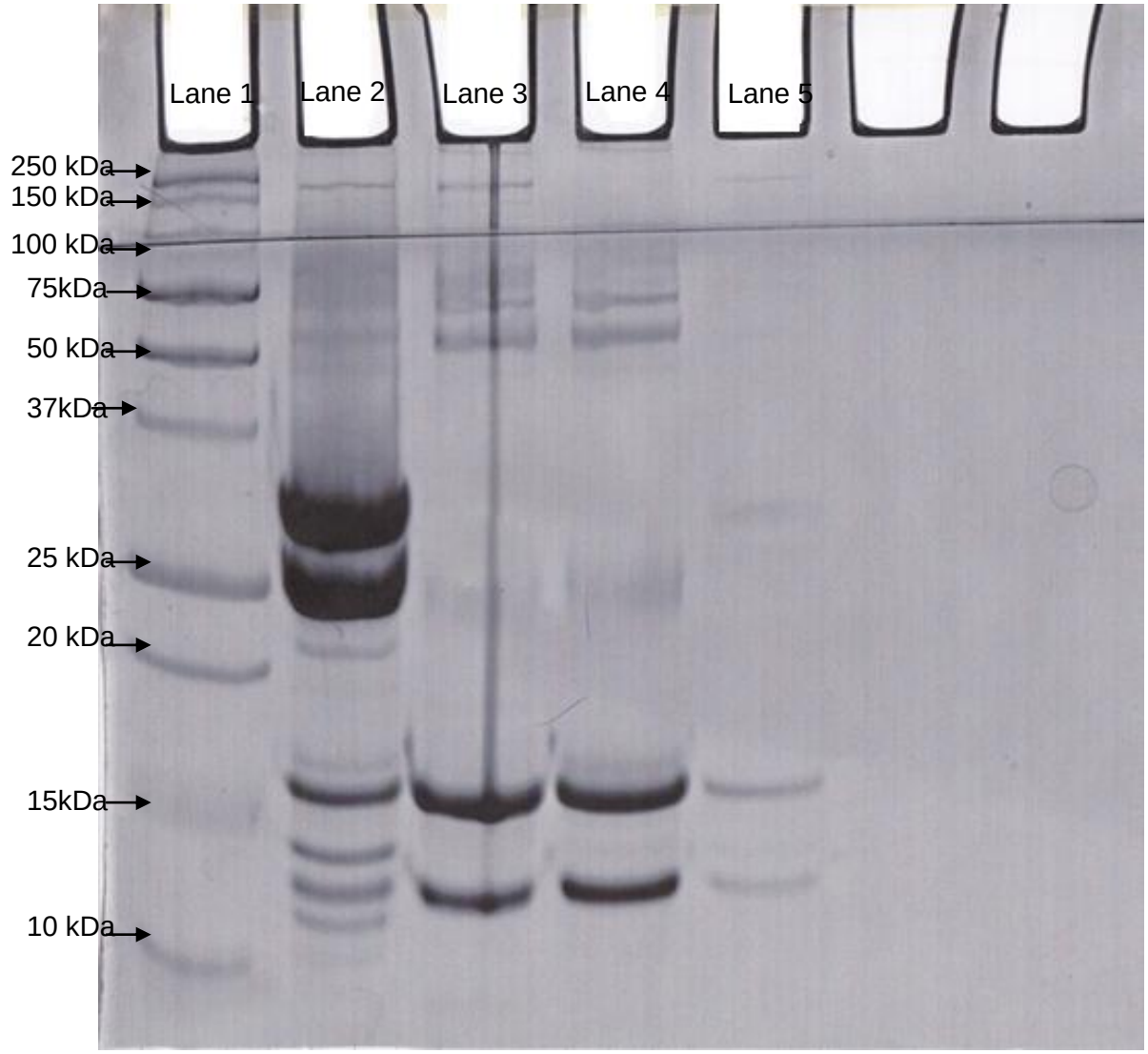


Figure 3-1: 1D gel electrophoresis whey samples after various methods of casein precipitation in co3mparison with whole milk sample without any treatment. Mr standard on Lane 1, whole milk on Lane 2, whey sample after rennet coagulation method on Lane 3, whey sample after acid precipitation method on Lane 4 and whey sample after ammonium sulphate fractionation method on Lane 5.

3.3.3 1D gel electrophoresis on individual subclinical mastitic and healthy milk samples

Rennet precipitation was subsequently used to remove major casein protein to allow analysis of lower abundant protein in whey. At the concentration used, rennet did not give a protein band stainable with Coomassie blue (not shown). The separation of proteins from individual subclinical mastitic and healthy milk samples on 1D gel electrophoresis is shown in Figure 3-2a, 3-2b, 3-2c. In comparison of mastitic to healthy milk, no clear difference in terms of presence or absence of protein bands observed (Fig 3-2a). However, despite having no significant difference of protein bands, visualized protein bands in one individual sample of each group (subclinical mastitis in Lane 3; healthy in Lane 10) was digested with trypsin and identified by MALDI-MS. Upon, protein identification searched against Mascot database, only major milk proteins and some serum-derived proteins were successfully identified such as α-lactalbumin, β-lactoglobulin, albumin, lactoferrin and immunoglobulins (Fig 3-2b and Fig 3-2c). These results were similar between subclinical mastitic and healthy milk samples. No protein was successfully identified from the database for high molecular protein bands such as band 1, 2, 11 and 12 or for low Mr protein bands such as 10, 21, 22 (Fig 3-2b). The whole list of successful protein identification by Mascot database is presented on Table 3-3.

3.3.4 2D gel electrophoresis on pooled subclinical mastitic and healthy milk samples

The second dimension protein separation on both pooled subclinical mastitic and healthy milk samples after casein removal by rennet precipitation is shown in Figure 3-3a and 3-3b. The IPG strip used was between pH 3 and pH 10. Upon colloidal Coomassie staining of the gel, there was no clear difference in terms of presence, absence, increase or decrease intensity of protein spots observed between these two pooled milk samples. There was only increased in the intensity of albumin protein spot (Spot 1) seen on 2D gel of pooled subclinical mastitic milk sample in comparison with pooled healthy milk sample. Based on previous study by Hogarth *et al.* (2004), it was concluded that protein spot 2 and 3 were the major whey proteins; β-lactoglobulin and α-lactalbumin respectively. Thus, no further mass spectrometry analysis was done on this 2D gel.

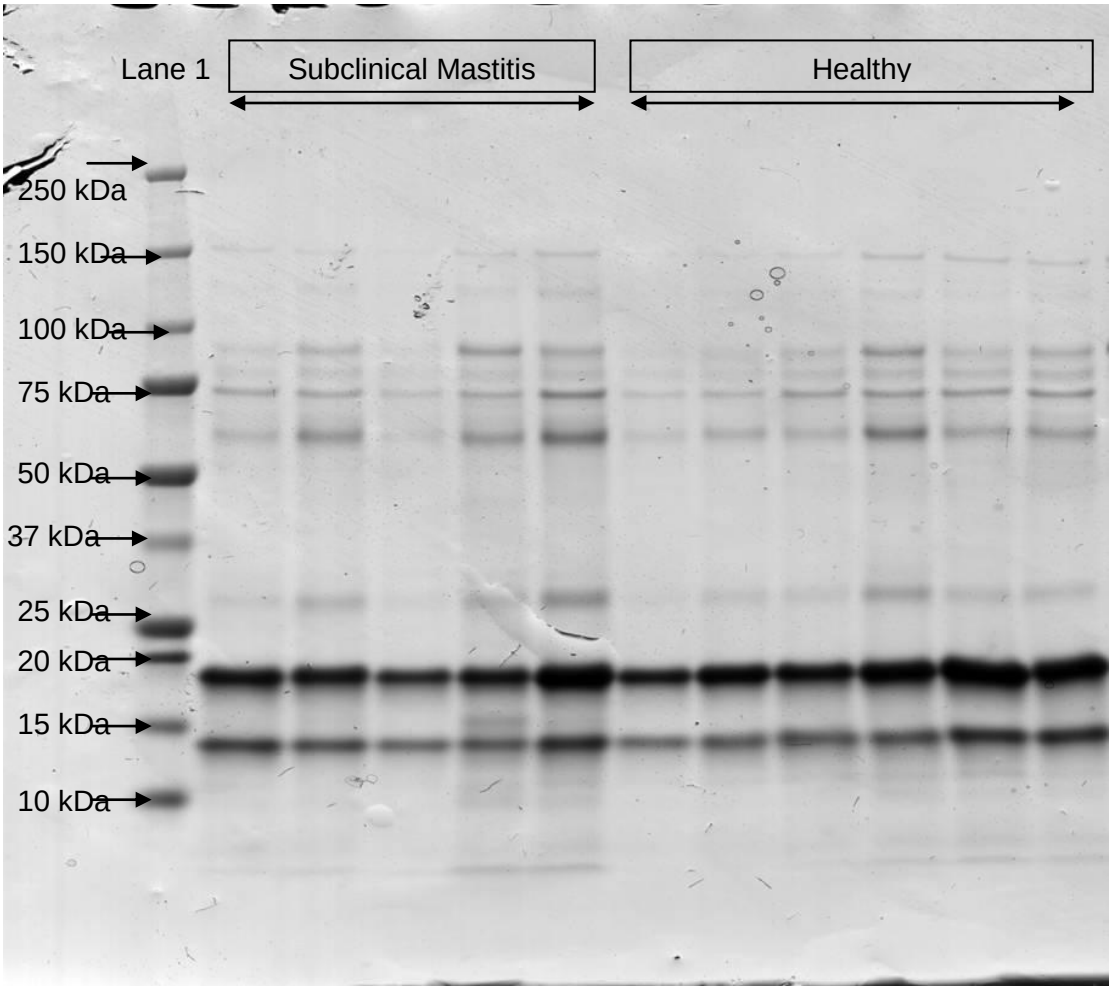


Figure 3-2a: 1D gel electrophoresis of 5 individual subclinical mastitic and 6 individual healthy milk samples. Showing protein bands for each of milk samples and the Mr standard was on Lane 1.

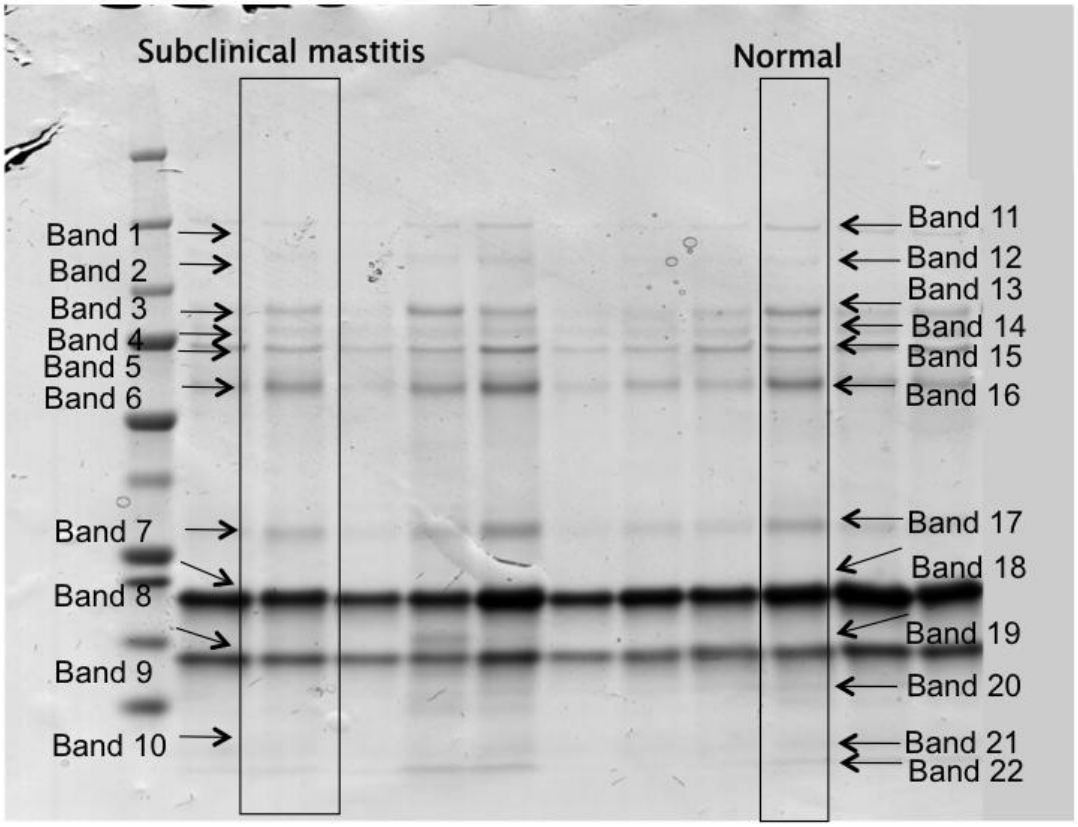


Figure 3-2b: 1D gel electrophoresis of a subclinical mastitic and a healthy milk sample. Showing protein bands used for MS analysis**.**

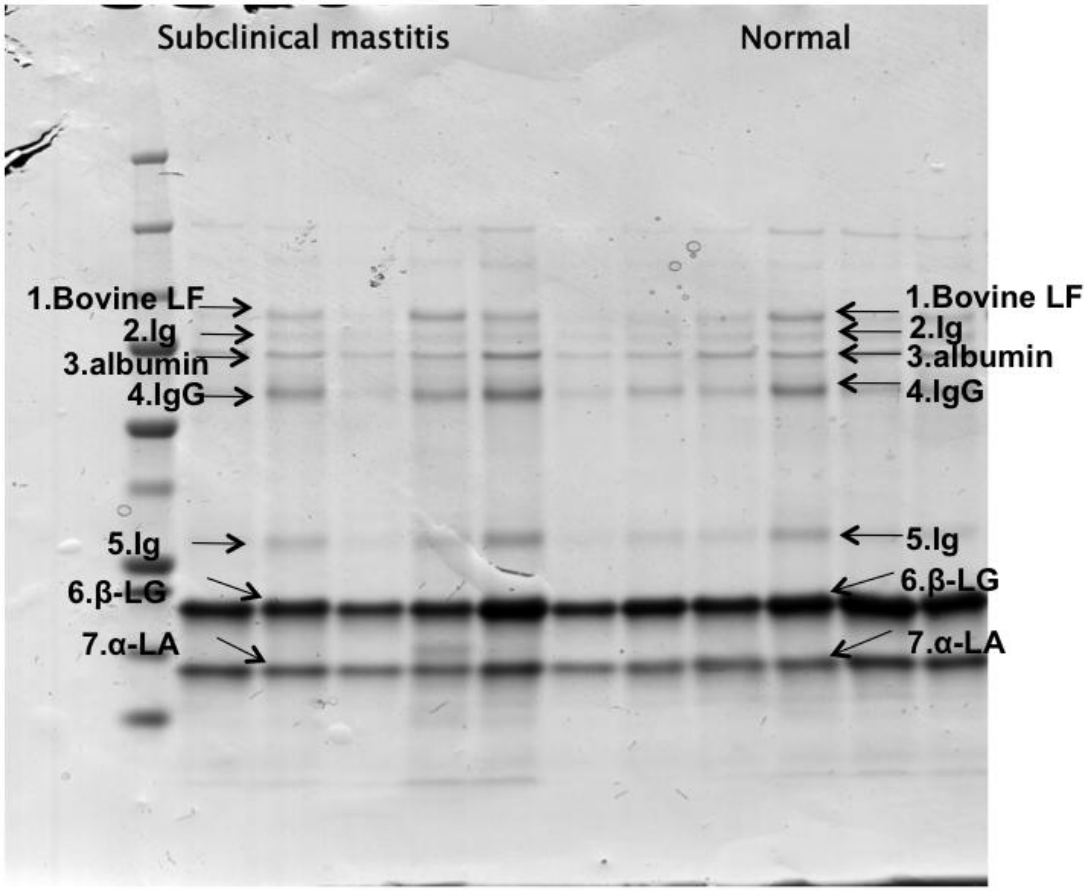


Figure 3-2c: 1D gel electrophoresis of a subclinical mastitic and a healthy milk sample. Showing all the proteins identified by the MS based on the Mascot database.

	Protein Name	Protein MW	Protein pI	Peptide count	Protein score
Band 1	ND	ND	ND	ND	ND
Band 2	ND	ND	ND	ND	ND
Band 3	Bovine lactoferrin	77129.2	8.52	17	108
Band 4	polymeric Ig receptor	83694.6	7.07	18	118
Band 5	albumin (*Bos taurus*)	71244.2	5.82	13	78
Band 6	IGHGI protein (*Bos taurus*)	52154.6	6.1	9	193
Band 7	anti-testosterone Ab (*Bos taurus*)	24786.1	7.53	7	92
Band 8	Bovine beta-lactoglobin	18554.5	4.83	13	458
Band 9	alpha-lactalbumin	11541.4	4.61	8	156
Band 10	ND	ND	ND	ND	ND
Band 11	ND	ND	ND	ND	ND
Band 12	ND	ND	ND	ND	ND
Band 13	Bovine lactoferrin	78033.8	8.67	22	183
Band 14	polymeric Ig receptor	83694.6	7.07	15	128
Band 15	albumin (*Bos taurus*)	71244.2	5.82	15	126
Band 16	IgG1 heavy chain constant region	36509.7	6.09	8	101
Band 17	IGL@ protein (*Bos taurus*)	24910.1	5.84	7	125
Band 18	Bovine beta-lactoglobin	18640.6	4.76	13	321
Band 19	alpha-lactalbumin	11541.4	4.61	7	75
Band 20	ND	ND	ND	ND	ND
Band 21	ND	ND	ND	ND	ND
Band 22	ND	ND	ND	ND	ND

Table 3-3: Proteins identified after in-gel trypsin digestion of protein bands on 1D gel electrophoresis. Mascot peptide database was used to identify the proteins. Mr and pI data were from Mascot database result.

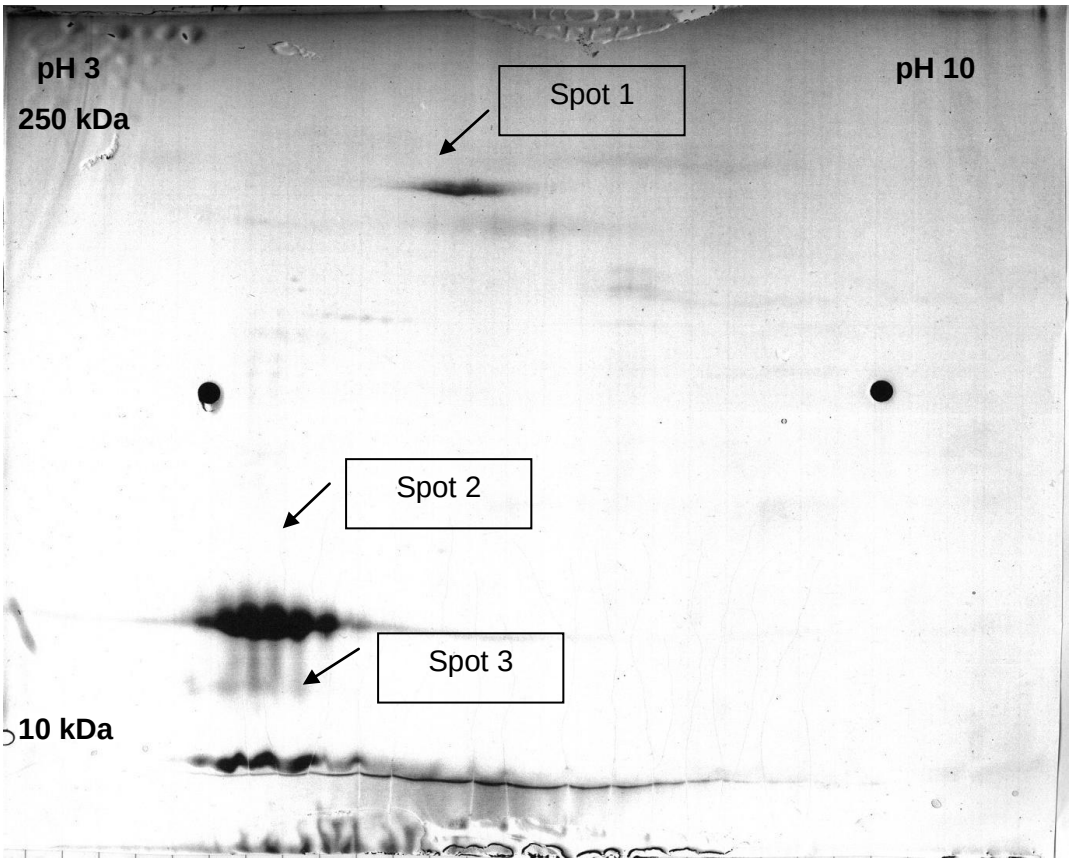


Figure 3-3a: 2D gel electrophoresis of pooled subclinical mastitic whey sample. Showing major milk proteins such as albumin on Spot 1, β-lactoglobulin on Spot 2 and α-lactalbumin on Spot 3 (Hogarth *et al.* 2004).

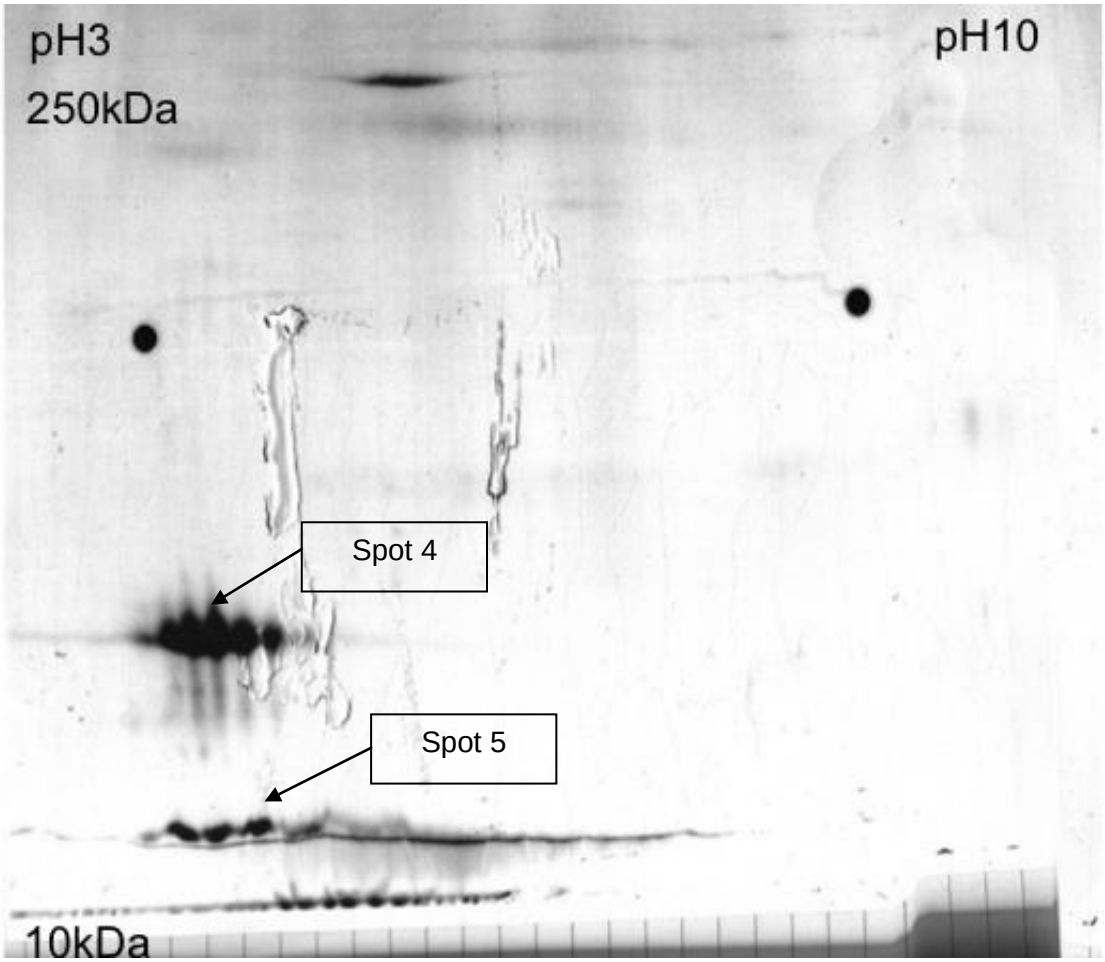


Figure 3-3b: 2D gel electrophoresis of pooled healthy whey sample. Showing major milk proteins such as β-lactoglobulin on Spot 4 and α-lactalbumin on Spot 5 (Hogarth et al 2004).

3.3.5 Difference gel electrophoresis (DiGE) on pooled clinical mastitic and healthy milk samples

Difference gel electrophoresis was performed on clinical mastitic samples in comparison with healthy samples to increase the likelihood of identifiable difference and after casein removal by rennet precipitation to increase the sensitivity of lower-abundance proteins detection. The scanned gel of the overlay image where both samples labeled with Cy3 and Cy5 fluorescent dyes can be analyzed on the same image as shown in Fig 3-4a. The gel stained with Cy5 dye for the mastitic milk is shown in Fig 3-4b and with Cy3 dye for normal milk is shown in Fig 3-4c. No clear difference was observed in terms of presence, absence, increase or decrease intensity of protein spots between the pooled two milk samples except for increase in the intensity of protein spot of albumin and reduced intensity of the protein spot of whey proteins in mastitic milk sample as compared with healthy milk sample. Otherwise, no other significant protein spot was detected on these gels.

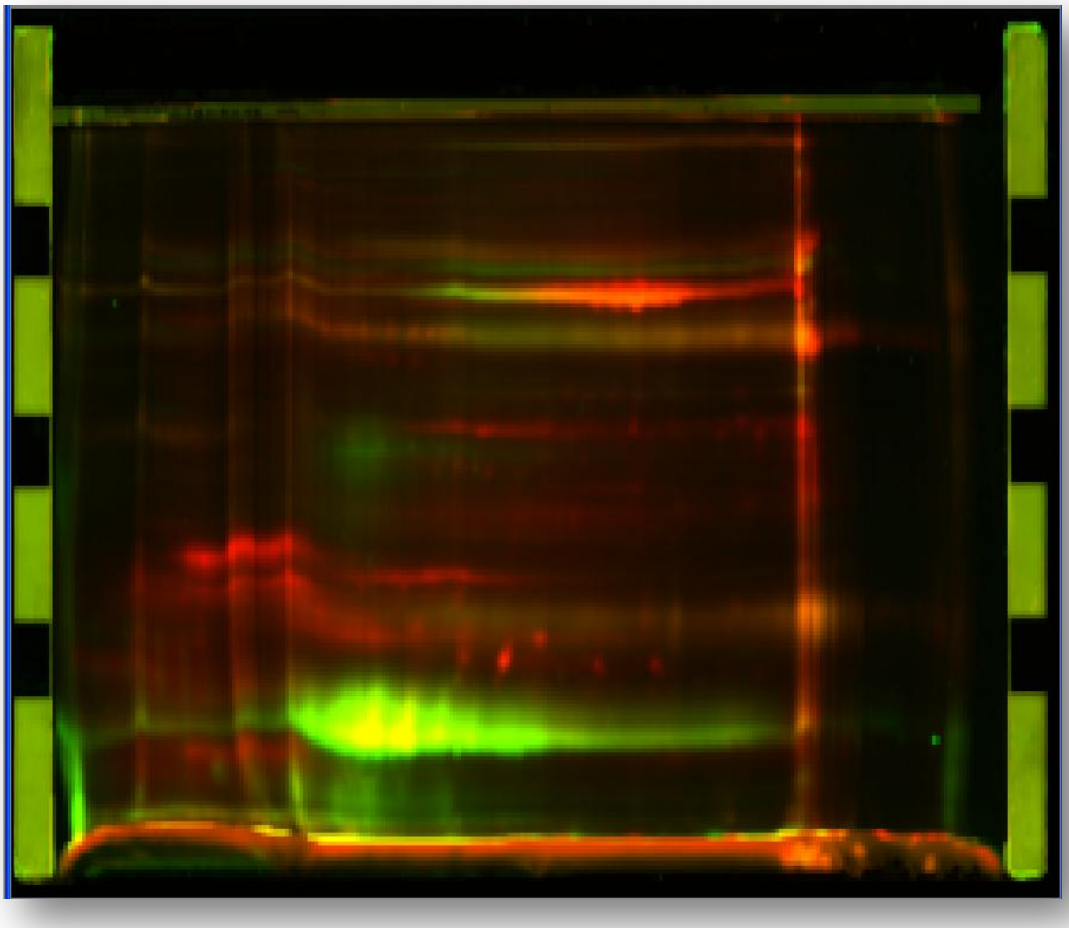


Figure 3-4a: Difference gel electrophoresis on clinical mastitic and healthy milk samples. Showing clinical mastitic milk sample was labeled with Cy5/red fluorescent dye and healthy milk sample was labeled with Cy3/green fluorescent dye.

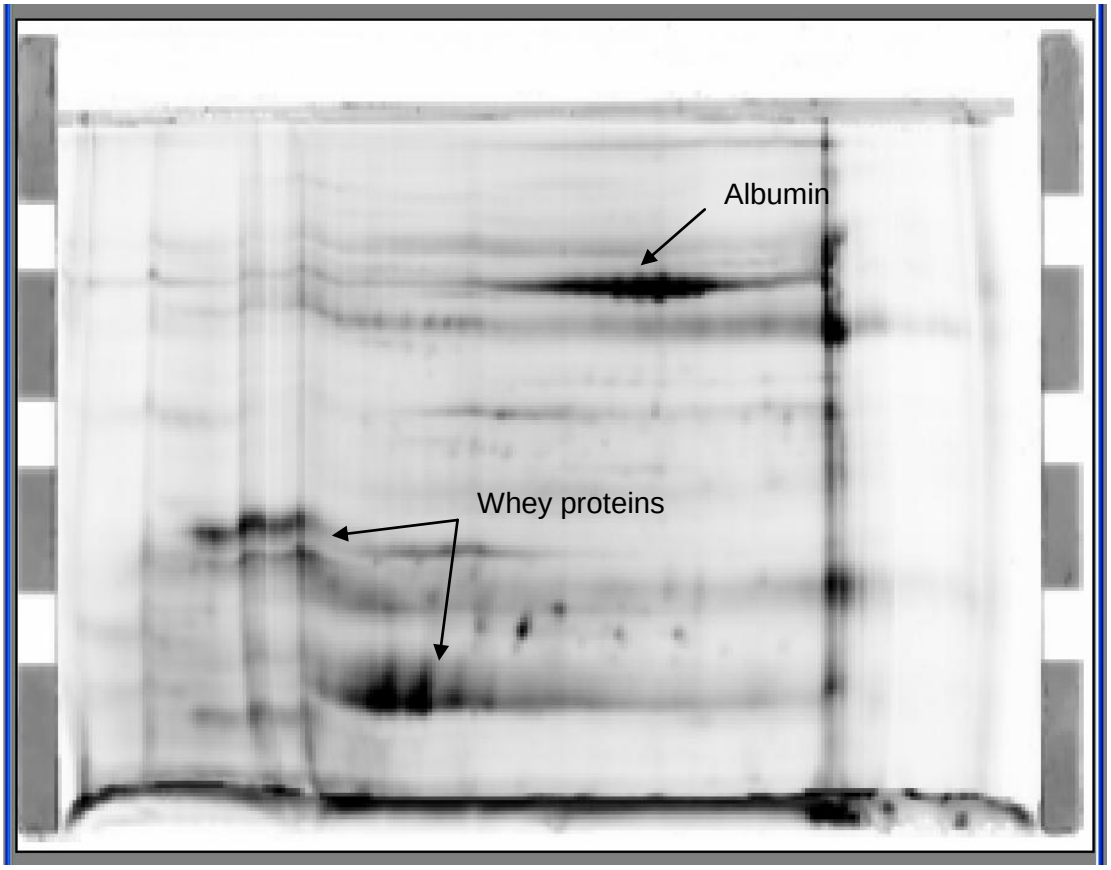


Figure 3-4b: Difference gel electrophoresis on pooled clinical mastitic milk sample on Cye5 fluorescent dye. Increased in albumin and decreased in whey proteins spot intensities are shown by the arrows as compared with pooled healthy milk sample in Figure 3-4c.

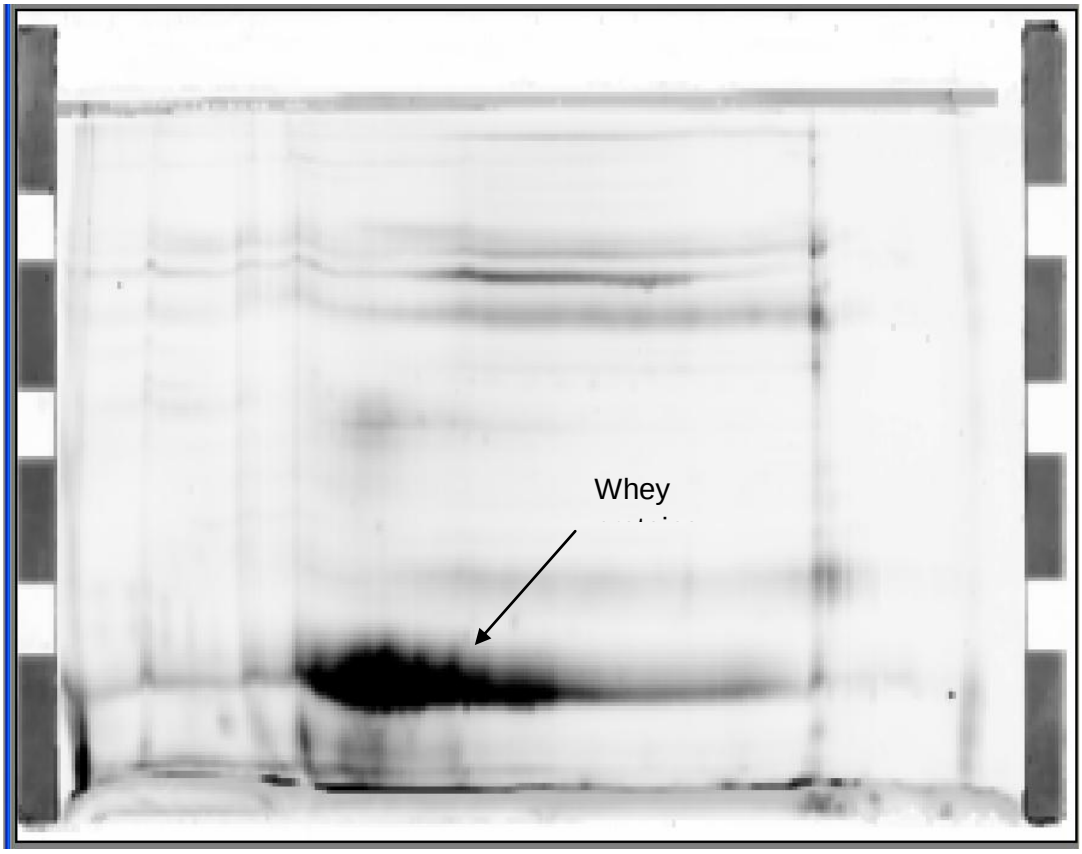


Figure 3-4c: Difference gel electrophoresis on pooled healthy milk samples on Cye3 fluorescent dye. Increased in whey proteins spot intensity is shown by the arrow as compared with pooled healthy milk sample in Figure 3-4b.

3.4 Discussion

The clinical mastitic milk samples chosen in this study had high concentrations of acute phase proteins for both SAA and Hp. The mean values for APP concentrations in clinically infected milk samples were 5532μg/ml and 1041μg/ml for Hp and SAA respectively. The range in values for APP concentrations in clinically infected milk samples were 560 to 16804μg/ml and 16.6 to 10635μg/ml for Hp and SAA respectively. As the distribution of these data was not normally distributed, the median values for each parameter were obtained. The median values for APP concentrations in clinically infected milk samples were 4024μg/ml and 107.7μg/ml for Hp and SAA respectively. Because no APP concentration was obtained for either the healthy or the subclinical mastitic milk samples as these analyses were carried out retrospectively after development of the Hp ELISA assay and the specific samples were no longer available, no significance test was performed. However, the Spearman's correlation test was performed which revealed a negative and low relationship between SAA and Hp concentrations ($r = -0.364$). It was surprising and interesting and may reflect different origins of Hp and SAA in milk, notable that recent evidence suggests that Hp in milk may be derived from neutrophils (ie somatic cells) as well as epithelial tissue (Cooray *et al.* 2007). The SCC measurement for all healthy milk samples was less than 100 000 cells/ml (average was 40 000 cells/ml) which confirmed the healthy status of the cows. The Mann Whitney's test revealed significant difference ($p<0.05$) in the SCC measurement of control and subclinical mastitic milk samples with a significance level (p) of $p=0.001$.

The objective of this study was to characterize the milk proteome associated with bovine mastitis on gel-based proteomic techniques and to characterize and identify low abundance protein in the milk proteome using mass spectrometry. Previous studies as described in chapter one clearly demonstrated that proteomic analyses have successfully characterized and identified several important milk proteins which might be served as protein biomarker of bovine mastitis (Yamada *et al.* 2002; Smolenski *et al.* 2007). Furthermore proteomics has also helped to study the physiological functions of those identified proteins in the maintenance of mammary gland in terms of providing nutrients immunity and development to the newborns.

To examine changes in low abundance proteins, rennet coagulation was chosen over acid precipitation or ammonium sulphate fractionation. Based on 1D gel electrophoresis, rennet coagulation successfully removed the majority of milk casein. Furthermore, the intensity of

lower abundance proteins at 50kDa to 250kDa of molecular weight was increased when rennet coagulation was used. However, other major milk proteins such as α-lactalbumin and β-lactoglobulin were still present in the milk samples. Potential interference in protein pattern for the protein of the rennet enzyme was discounted. A rennet precipitation of a similar concentration to that used for casein precipitation was run on 1D SDS PAGE and gave no Coomassie blue stained band.

For a preliminary analysis, 1D gel electrophoresis of individual milk samples was performed on subclinical mastitis in comparison with healthy cases. However, there was no distinctive difference in terms of protein bands between subclinical mastitic and healthy milk samples (Figure 3.2a, b, and c). Furthermore, no lower abundance protein bands were seen in milk samples after casein removal in the healthy or mastitic sample. Unfortunately, the high molecular weight protein bands but with lower intensity were not successfully identified by the MALDI-MS.

Colloidal Coomassie blue staining might not be sensitive enough to detect all lower abundance proteins which may still include an important protein biomarker in bovine mastitis. However, there was no significant difference in terms of protein levels that could differentiate subclinical mastitic from healthy milk sample in this study. Another possible reason was due to the limited usage of 1D gel electrophoresis as it only separates milk proteins based on their molecular weight so that 2DE may be more effective. Low intensity but high molecular weight protein bands (Band 1, 2 ,11 and 12) were not successfully identified due to very low level of proteins exist in those protein bands. Limited number of *B. taurus* database entries in the database used (Mascot) might contribute to no possible protein identification of these protein bands.

However, for further characterization of milk proteins on 1DE, separated protein bands in a milk sample from each group were cut from the gel and sent for MS analysis. MALDI-TOF successfully identified more of these protein bands though it appeared that the identified proteins were the normal milk proteins that can be found in bovine milk. These include albumin, α-lactalbumin and β-lactoglobulin. This result was in accord with previous proteomic analyses (Hogarth *et al.* 2004; Smolenski *et al.* 2007; Boehmer *et al.* 2008; Boehmer *et al.* 2010). No casein was identified on the 1DE gel showing that rennet precipitation had successfully removed casein.

Beta-lactoglobulin which comprises about 10% of the total milk protein was identified by MALDI-TOF with Mr of 18554 and pI value of 4.83 for subclinical mastitic milk sample

and slightly higher Mr of 18640 with the pI value of 4.76 for healthy milk sample. Beta-lactoglobulin binds to retinol and thus implicates its function in the transport of vitamin A to newborns (Wong *et al*. 1996). Alpha-lactalbumin which accounts for about 13% of total whey protein was identified by MALDI-TOF in this study with a Mr of 11541.4 kDa with pI values of 4.61. It acts as a specifier protein which catalyzes the biosynthesis of lactose in addition of galactosyltransferase (Wong *et al.* 1996). During intramammary infection, there is a possible reduction in the levels of α-lactalbumin and β-lactoglobulin in milk has been reported due to depletion in the synthesis and secretion of these proteins following damage to the mammary epithelial cells by bacterial toxins or by the presence of infection in the mammary gland (McFadden *et al.* 1988).

It is known that serum-derived proteins such as albumin, immunoglobulins, complement proteins (Ward *et al.* 2002) and lactoferrin are present in low levels in bovine milk (Lacy-Hulbert *et al.* 1999; Smolenski *et al.* 2007). These may have antimicrobial properties (Ward *et al*. 2002). Albumin is also known to increase in concentration during mastitis as a result of leakage from blood circulation into the mammary gland (Hogarth *et al.* 2004). It has also been demonstrated that albumin can be synthesized and secreted in the bovine mammary gland as local expression of albumin in the mammary gland increased upon exposure to LPS as well as in mastitic condition (Shamay *et al.* 2005). However, because no quantification of specific protein levels in milk samples was made, it is not known whether the presence of albumin in subclinical mastitic milk was significantly different from its presence in healthy milk in this study. Other than albumin, immunoglobulins and lactoferrin were also present in both subclinically infected and healthy milk samples. Epithelial cells of the mammary gland are known to produce lactoferrin as well as cytokines (IL-8 and TNF-α) in response to LPS stimulation (McClenahan *et al.* 2005; Pareek *et al.* 2005).Study by Chaneton *et al.* (2008) suggested that different bacteria species may stimulate different mammary gland responses mediated by Lf secretion. On the other hand, immunoglobulins are one of the major proteins in colostrum which confer passive immunity to the newborn (Smolenski *et al.* 2007) which also serve as defence mechanism against microbial invasion of mammary gland.

As no differences were observed on the gel pattern of 1D gel electrophoresis between subclinical mastitic and healthy milk samples, 2D gel electrophoresis was applied to similar samples. It was hope that lower abundance proteins would be identified because 2D electrophoresis is generally more effective in separating as in bovine milk. Further pools of

milk samples from subclinical and healthy groups were used prior to 2D gel electrophoresis.

Unfortunately, there was again no observable difference in low abundance proteins between the pooled subclinically infected milk and pooled healthy milk samples on the 2D gel. Mass spectrometry analysis was not undertaken as the protein spots in both gels represent the same major milk proteins; albumin, α-lactalbumin and β-lactglobulin and have previously been identified (Hogarth *et al.* 2004). However, it was interesting to find that albumin was not observed on the 2D gel of healthy samples. This result further supported the findings that albumin was increased in concentration during bovine mastitis as a result of either an increase in leakage from serum into the mammary gland or in albumin expression in the mammary gland. No reduction in the intensity of protein spots of other whey proteins in subclinical sample was shown in this 2D gel.

In this study, no low abundance protein was seen on the 2D gel of subclinical mastitic sample and the possible explanation may derived from the evidence or the potential binding of other proteins to serum albumin. Serum albumin is known to bind as well transport long-chain fatty acids and other small molecules such as bradykinin, interferon and even acute phase proteins (SAA and α-1-acid glycoprotein) (Peters 1977; Gundry *et al.* 2007). Furthermore, 2D gel electrophoresis was thought to have difficulty to separate low abundance proteins, acidic and basic proteins, proteins with very small or very large in size as well as hydrophobic proteins (Beranova-Giorgianni 2003). This can be explained of why no low abundant protein particularly APP seen on 2D gel of subclinical mastitic sample.

In contrast to studies which have removed casein, it has been suggested that in order to increase the likelihood of the detection of lower abundance proteins, no sample fractionation or depletion of high-abundance proteins should be performed (Boehmer *et al.* 2008). Although several other studies suggest the use of fractionation or immunoprecipitation (Yamada *et al.* 2002; Hogarth *et al.* 2004) to selectively remove casein, β-lactoglobulin, α-lactalbumin or other major milk proteins, the possibility of other lower-abundance proteins also being removed might occur. To investigate subclinical mastitis, more milk samples should also be examined to increase the potential of discovering protein biomarkers important in bovine mastitis. Another way to improve and increase the separation and detection of lower abundance proteins is by using more sensitive staining methods than colloidal Coomassie including silver or sypro-ruby staining (Hogarth *et al.* 2004). As investigation of milk samples from sub clinical mastitis cases did

not give any significant difference in the levels of milk proteome, the examination of milk samples from clinical mastitis should be considered to find identity possible biomarker after which change in the subclinical mastitis could be determined.

Therefore, milk samples from clinical mastitis cases were collected for comparison with healthy milk samples and analyzed using difference gel electrophoresis (DiGE). DiGE enables the visualization of multiple protein samples in one 2D gel. After labelling the milk samples with the differing fluorescent dyes and running on the same gel, spot patterns were visualized by alternately illuminating the gel with the excitation wavelengths of each dye. Overlay image was then analyzed using standard proteomics software (Van den Bergh *et al.* 2003).

Unfortunately, although clear distinction were found between clinical mastitic and healthy samples on DiGE gels with lower abundance proteins and increased albumin in the mastitic sample, all of the changes had been observed before (Hogarth *et al.* 2004). Thus, no further mass spectrometry analysis was done and based on the previous study (Hogarth *et al.* 2004), the protein spots seen on the Cy dye stained gels were actually the major milk proteins (albumin, β-lactoglobulin and α-lactalbumin).

The protein concentration of the non-casein but still high abundance proteins (albumin or whey proteins) of these milk samples might also contribute to failed detection in other lower abundance proteins. Thus, in order to increase the potential of using difference gel electrophoresis, several important factors must be taken into consideration. The use of more clinical mastitic milk samples may increase the sensitivity to detect lower abundance proteins. Milk samples should be handled more cautiously in terms of transporting the samples from farm to laboratory as well as minimizing the thawing process of milk samples. This would ensure no depletion of important milk proteins should occur.

In conclusion, among milk proteins successfully identified by MALDI-MS on 1D gel electrophoresis were albumin, α-lactalbumin, β-lactoglobulin, lactoferrin and immunoglobulins. However, no clear distinction was observed between the 2D gel electrophoresis of subclinical mastitic and healthy milk samples. Additionally, from DiGE analysis, albumin was found increased in clinical mastitic sample whilst β-lactoglobulin was reduced in mastitic sample when compared with healthy sample. From this study, it was evident that gel-based proteomic techniques as used here were not able to identify novel protein biomarker in milk in bovine mastitis or other lower-abundance proteins involved in inflammation or host defence mechanism. The technical requirement and

resource implication needed for consistent analytical result in a gel-based approach to proteomic analysis of milk are further aspects to consider. Accordingly, non-gel approaches were undertaken to continue the investigation such as CE-MS and LC-MS/MS in the next chapters.

Chapter IV: Peptidomic Analysis on Bovine Mastitis

4.1 Introduction

Peptidomic analysis involves extraction, separation, detection and identification of peptides in samples. In earlier studies, less manipulation of the samples was employed to allow peptides to be analysed by mass spectrometry (Baggerman *et al.* 2004). Tissues were directly placed on the MALDI target plate together with the matrix solution (Clynen *et al.* 2001; Verhaert *et al.* 2001; Uttenweiler-Joseph *et al.* 1998; Sweedler *et al.* 2000) although in the case of ESI, a simple extraction was performed in the spraying solvent (Kollisch *et al.* 2000). However, due to the presence of multiple biological peptides at different concentrations in cells, tissues or body fluids, it is desirable that peptide extraction and separation are used prior to mass spectrometric analysis. Indeed, the aim of several studies is to enrich the low-abundance peptide composition of samples (Chertov *et al.* 2005; Baggerman *et al.* 2002; Skold *et al.* 2002; Schulz-Knappe *et al.* 2001).

As the main difference between protein and peptide is based on their molecular weights, the isolation of smaller peptides from higher concentration of larger proteins is essential (Hu *et al.* 2009). Several extraction steps for analysis of endogenous peptides have been employed to meet this aim. Some methods which have been applied include ultrafiltration, organic solvent precipitation, solid phase extraction, restricted-access material (RAM) - trap column-based extraction and others (Hu *et al.* 2009).

In ultrafiltration, a membrane with an accurate molecular weight cut-off (MWCO) is used to extract peptides of interest while removing other high molecular weight (HMW >20kDa) peptides and proteins. However, in this method, the ultrafiltration times will increase dramatically if huge amount of sample is applied and also, other LMW (<20kDa) contaminants (e.g. salt) may also be concentrated which may affect the efficiency of the method (Hu *et al.* 2009).

By using organic solvent precipitation, the electrostatic interactions between molecules are increased and the smaller peptides are dissolved in the high concentration of solvents (Polson *et al.* 2003). An example of an organic solvent that has been used is acetonitrile (Chertov *et al.* 2004). The use of solid-phase extraction (SPE) has also been employed as

part of sample clean-up preparation while functionalized magnetic particles were used as adsorbents to enrich specific peptides in various studies (Villanueva *et al.* 2004; Fiedler *et al.* 2007; Baumann *et al.* 2005). In addition, conventional chromatographic packing materials such as reversed-phase polymers and nanomaterials with different properties have been employed as part of this methodology (Aristoteli *et al.* 2007; Li *et al.* 2007; Tian *et al.* 2009).

All these methods are examples of off-line mode sample preparation which may be laborious, time-consuming and have poor reproducibility (Hu *et al.* 2009). The use of a special type of chromatographic packing materials called restricted access materials (RAMs) with two functions during chromatographic separation has been proposed. It was suitable for online extraction of smaller peptides and removal of other high abundance peptides (Boos & Grimm 1999). Other techniques including selective electrophoresis and continuous elution electrophoresis have similar characteristics in their ability to concentrate LMW peptides (Camerini *et al.* 2007; Ly *et al.* 2008).

The next step of peptidomics involves peptide separation. Baggerman *et al.* (2004) reviewed several types of peptide separation methodologies that can be employed. One of them is the use of coupling an HPLC to electrospray sources. In this method, the peptides are separated by the use of reversed-phase chromatography (Schoofs *et al.* 1993) and by applying the voltage difference between the outlet of HPLC and the sample targets, ions can be transferred from solution into the gas phase. This method allowed sensitive peptide analysis and have been applied to various peptidomics studies (Schulz-Knappe *et al.* 2001).

Recent advances have been developed to detect lower concentrations of peptides from complex protein mixtures. When ultra low flow LC systems coupled to nanospray sources are used, the flow rates employed for chromatographic separation has been as low as 20nl/min with the internal diameters of columns down to 15μm (Shen *et al.* 2002). However, this method is time consuming and to prevent this, column switching was introduced to separate the sample loading and the actual separation. A whole sample can be analyzed and peptides identified can be selected for further fragmentation in the collision cell of the MS instrument as they passed through the columns (Baggerman *et al.* 2004). When analysing more complex samples, reducing the flow rate during the separation can be performed to increase the time analysis of the mass spectra and thus, more peptides can be fragmented or fragmented longer in the case of LMW peptides.

Another type of peptide analysis is multi-dimensional peptide separation. Reversed-phase LC is frequently used as the second separation stage which enables full separation of all the effluent material from the first step. It can be combined with size exclusion reversed-phase (Oosterkamp *et al.* 1998), reversed phase-reversed phase chromatography (Oosterkamp *et al.* 1998: Wall *et al.* 1999) or ion exchange-reversed phase (Oosterkamp *et al.* 1998; Davis *et al.* 2001). Multidimensional protein identification technology (MudPIT) using a strong cation resin (SCX) and RP columns has been successfully applied for shotgun proteomic research (Hu *et al.* 2009). The individual peptides separated from SCX columns were eluted onto the RP columns before subsequently being sprayed into the ESI MS (Baggerman *et al.* 2004; Hu *et al.* 2009).

Most peptidomes, unlike tryptic peptides, do not carry C-terminal Lys or Arg residues and SCX column separates peptides based on their charges (Hu *et al.* 2009). Reversed phase (RP) on the other hand is pH stable, and by using RP-RP separation, peptides may be better separated as shown by Dowell *et al.* (2008). A total of 56 peptides from known neuropeptides precursors including 17 unidentified peptides, have been successfully separated using this method. A combination of sample ultrafiltration with size-exclusion chromatography prefractionation has been considered as a good method for comprehensive peptidomics analysis (Hu *et al.* 2007). It allows the investigation of HMW peptides and at the same time, identifying the LMW peptides which improved the sensitivity of LMW method of detection. The advances in the MS analysis in detecting HMW peptides have developed with the use of quadrupole TOF MS with collision-induced dissociation (Möhring *et al.* 2005), fourier transformion cyclotron resonance MS using electron capture dissociation and linear quadrupole ion-trap MS wit electron transfer dissociation (Hu *et al.* 2009) which allowed sequencing of large and small peptides.

Another increasingly popular peptidomics analysis is the combination of capillary electrophoresis (CE) with MS. It can perform a separation of peptides from small molecules to large proteins in the same analysis. It has been chosen as a method of analysis due to the low amount of sample used and short analysis time (Baggerman *et al.* 2004). Pre-concentrating of the sample with hydrophobic media can be employed to increase the concentration of the sample.

Quantitative peptidomics has been increasingly important especially in biomarker discovery when it is important to understand the relative difference of each protein/peptide in different conditions (Hu *et al.* 2009; Baggerman *et al.* 2004). Direct profiling with

MALDI/SELDI is most widely used to quantify peptides but another way is the by using a UV detector between the LC system and the MS to compare the peptide relative abundances (Hu *et al.* 2009; Baggerman *et al.* 2004).

Differential peptide display was introduced by Tammen *et al.* (2003) in which the sample was separated by RP-HPLC and each fraction eluted was analysed by MALDI-TOF MS. The mass spectra were transformed into 1D gel-like pattern and the patterns were combined to generate a virtual 2D peptide map. To identify the peptides, individual HPLC fractions were subjected to nESI-qTOF-MS/MS or MALDI-TOF/TOF-MS sequencing.

The SELDI-TOF MS technique is another method using the protein chip array technology with a modified surface target (Huchens & Yip 1993). The target surface can be modified with chemical functionality such as RP, ion exchange chromatography (IEX), immobilized metal affinity chromatography, antibodies, proteins and DNA. This binding activity was considered as a pre-fractionation step to allow easier analysis. Different expressed peptides can be identified from different groups of sample using advanced bioinformatic tools (Issaq *et al.* 2003; 2002).

However, these methods described above (MALDI/SELDI profiling) which were thought to provide semi-quantitative analyses of peptides as ion patterns, could be affected by ionization efficiency, ion suppression effects and sample preparation process (Hu *et al.* 2009). Furthermore, ion intensities in the mass spectrum did not correlate to the relative amounts of the corresponding compounds (Baggerman *et al.* 2004). To overcome this problem, mass labelling was employed in which a known concentration of a 'mass labelled' analogue of the peptide of interest was used as internal reference. A perfect example of mass labelling was by incorporating stable isotopes in which two different samples had the same ionization efficiency but with a slight difference in masses (Hu *et al.* 2009; Baggerman *et al.* 2004).

An iTRAQ approach with amine reactive isobaric tagging and tandem MS to quantify the peptides has been employed in both proteomics and peptidomics analyses. Peptides with the same sequence appeared to be identical in masses but produced as a single ion signal in MS (Hu *et al.* 2009). It may be used in complementary studies with MALDI profiling for both clinical diagnosis and biomarker discovery as performed by Hu *et al.* (2009).

Another isotope labelling method used for cysteine containing peptides/proteins is isotope-coded affinity tags (ICAT). The side chains of cysteinyl residues of protein are derivatized

with an isotopically light form of the ICAT reagent. By comparing their relative signal intensities, the relative quantification of samples in different conditions can be determined (Gygi *et al.* 1999).

In a previous discussion about peptide analysis in bovine mastitis, most of the studies identified proteolytic activities yielding peptides in milk (Wedholm *et al.* 2008; Larsen *et al.* 2010). Many diseases are associated with proteolytic processes (Hu *et al.* 2009). Proteases reflect the response of the host towards the different physiological conditions. Different disease states will lead to different degradation pattern of proteins and these degradation products may be of useful as disease biomarkers (e.g. cancer) as described by Villanueva *et al.* (2006). The activity of proteases in the biological samples can be quantified by the use of activity-based probes (ABPs) coupling to MS. ABP work by targeting specific proteases based on their enzymatic mechanisms such as serine proteases, cysteine proteases, threonine proteases, aspartyl proteases and metalloproteases (Kidd *et al.* 2001; Greenbaum *et al.* 2000, Greenbaum *et al.* 2002, Kessler *et al.* 2001, Chun *et al.* 2004; Saghatelian *et al.* 2004). Affinity chromatography can also be used to characterize proteases as performed by Tian *et al.* (2006).

Peptidomic analysis allows full visualization and characterization of the whole peptidome in given cells, tissues or bodily fluid and even their post-transitional modifications peptides. With the use of nanoscale LC coupled with MS, one can identify peptides at the acid amino sequence level however, the use of tandem MS can only provide partial amino acid sequences of peptides. This can be resolved by using certain bioinformatic programmes to identify peptides from protein database or analogue gene from the genomic database (Choudhary *et al.* 2001). Peptidomics analysis can be applied in complementary with other protein characterization methodologies to discover novel disease biomarkers as well as to identify newly discovered peptides for therapeutic purposes.

4.1.1 Aims of study

Overall, the objective of this chapter is to apply a peptidomic approach to study the presence of polypeptides that may be used to discriminate normal milk samples from milk samples from quarters infected with bacteria causing bovine mastitis. It was hoped that by using this sensitive and high-throughput methodology, significant results can be achieved and lead to the discovery of important and significant mastitis biomarkers. Milk samples

from healthy cows were compared to samples from cows with clinical mastitis caused by either *S. aureus* or *E. coli*. Initially samples were analyzed by CE-MS to detect positive biomarkers and then by LC-MS/MS to sequence putative biomarkers.

4.2 Materials and Methods

4.2.1 Milk Samples and Reagents

Milk samples from clinical mastitic cases were obtained from Vale Veterinary Laboratory, Devon, UK. Bacteriological culture was performed in the Bacteriology Laboratory of Vale Veterinary Laboratory to determine the bacterial cause of bovine mastitis. Milk samples from mastitis caused by either *E. coli* (n=10) or *S. aureus* (n=6) infection were selected for further analysis. Control milk samples (n=10) from healthy cows from the Cochno Farm of School of Veterinary Medicine, University of Glasgow were also used in this study as comparison to those clinical mastitic cases. The healthy milk samples were confirmed by somatic cell counts (<100 000 cells/ml). Prior to peptidomics analysis, SAA and Hp assays (Chapter 2) were performed determine the concentration of both acute phase proteins in each milk sample. Samples were stored at -20°C prior to analysis.

Buffers and reagents for both sample preparation and CE-MS analysis are presented as in Appendix.

4.2.2 Milk sample preparation for CE-MS using PD-10 Desalting Column

4.2.2.1 Preparation of fresh ammonium (NH_4OH) and urea buffer

An aliquot of 25% NH_4OH (400µl) was added into 1 litre of deionised water to make up NH_4OH buffer. The solution was mixed by inversion and pH value was checked (pH 10.5-11.5). Urea buffer was prepared by mixing 5ml of 8M urea, 0.4ml of 5M NaCl, 10µl of 25% (v/v) NH_4OH solution and 100µl of 2% (w/v) SDS solution.

4.2.2.2 Sample Preparation

Milk samples were defrosted at room temperature prior to centrifugation at 14 000 *x* g for 10 minutes at 4ºC to remove the fat. Two aliquots of 1ml milk samples were prepared. An aliquot of 700µl of centrifuged milk was added to 700µl of urea buffer, placed in a centrisart tube (Sartorius, Goettingen, Germany) and mixed well by pipetting up and down.

The filter was put on top of the centrisart tube and centrifuged at 3400 rpm for 80 minutes at 4ºC. During centrifugation, a PD-10 column (GE Healthcare, Sweden) was equilibrated with 25ml of NH_4 solution. After centrifugation, 1.1ml of centrisart filtrate was added into the columns until the column was dry. Next, 1.9ml of NH_4 buffer was added into columns until the column was dry. The column was then placed on 15ml collecting tubes and the sample was eluted with 2ml of NH_4 buffer. The 2ml of flow-through was distributed between three Eppendorf tubes (2 tubes containing 900 µl to be used for CE and LC analysis and 1 tube for protein determination containing 150 µl). Tubes to be used for CE and LC analysis were freeze-dried until further use.

4.2.3 Protein concentration

Protein concentration in milk extracted samples was checked using the BCA (bicinchoninic acid) assay Uptima from Interchim (Montluçon, France), About 25µl of each standard and milk extracted samples were pipetted into microplate wells in duplicates. The BCA assay reagent was then added at 200µl and further mixed. The sample mixtures were incubated at 37°C for 30 minutes or 2 hours at room temperature. Prior to reading the results at the optical absorbance of 562 nm, the microplate was cooled at room temperature. The standard curve was plotted and the protein concentration in the sample was interpolated. Freeze-dried aliquots have to be resuspended in appropriate volume of Milli Q water to reach a concentration of 2µg/µl before running in the CE.

4.2.4 CE-MS Analysis

4.2.4.1 Preparation of buffers for CE-MS analysis

All buffers were freshly prepared every week. 1M sodium hydroxide (NaOH) was prepared in deionised water. Ammonium hydroxide buffer was prepared by adding 3.76 ml of ammonium solution (25%) to deionised water and the volume adjusted to 50 ml with deionised water. Running buffer was prepared by adding 10 ml of acetonitrile and 472 µl of formic acid to a final volume of 50 ml with deionised water. The sheath flow liquid was 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 peptdies : ELMTGELPYSHINNRDQIIFMVGR (2,832 Da), TGSLPYSHIGSRDQIIFMVGR (2,333 Da), GIVLYELMTGELPYSHIN (2,048 Da), REVQSKIGYGRQIIS (1,733 Da).

4.2.4.2 CE-MS Analysis for peptide fingerprint

The capillary was carefully installed into the CE system (Beckman Coulter, Fullerton, USA). The capillary was first conditioned with NaOH (pressure 50 psi) for 15 minutes. The capillary was then washed with NH_4OH solution (50 psi) for 15 minutes and 20 minutes 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. Freeze dried samples were re-suspended to achieve a final concentration 2 µg/µl with HPLC water and centrifuged at 14,000 *x* g for 10 min at 4 ºC 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 volume. Separation was done with +25kV at the injection site for 30 minutes with a capillary set temperature of 35°C. In addition to the +25kV, pressure is applied as followed (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 the following 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 minute after each run followed by a washing step with NH_4OH solution (50 psi) for 3 minutes and lastly by a flushing step with deionised water (50 psi for 3 min). ESI sprayer was grounded to achieve electric potential zero and the electrospray interface potential was set between -4 000 and -5 000 V. The MS spectra were accumulated every 3 s, over a mass-to-charge range from 400 to 3000 m/z for about 60 minutes depending on the analysis requirements. The mass calibration of the MS was performed on a weekly basis using the standard protein/peptide solution (0.5pmol/µl) for CE-MS analysis. CE-MS analysis was performed under supervision of Dr. Bill Mullen and Dr. Amaya Albalat of Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK.

4.2.5 CE-MS Data processing

Mass spectral ion peaks were deconvoluted into single masses using MosaiquesVisu software (www.proteomiques.com). Only signals observed in a minimum of 2 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. MosaiquesVisu uses a probabilistic clustering algorithm and both isotopic distribution and conjugated masses for charge-state determination of the entities.

CE migration time and ion signal intensity were normalized because migration time and ion-counts showed significant variability between samples mainly due to different salt and

peptide concentrations. 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.

4.2.6 Statistical analysis

Only polypeptides that were found in more than 70% of the samples in at least one of the two groups (controls or cases) were considered for biomarker definition. The pre-defined set of polypeptides should be 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). Two models were generated for the classification of the samples depending on their CE-MS profile. The first model was used to classify the milk sample as control (healthy) or bacteria (infected) and the second one to discriminate the infectious microorganism (*E .coli* or *S. aureus*).

4.2.7 LC-MS/MS Analysis for Peptide Sequencing

4.2.7.1 LC-MS/MS analysis

LC-MS analysis was performed using an Ultimate3000 nano-LC pump (Dionex, Sunnyvale, CA, USA) coupled to a LTQ-Velos (Thermo Corp.). Five µL from each sample were loaded onto a Dionex 100µm x 2 cm 5 µm C18 nano trap column at a flowrate of 5 µl/min by a Ultimate 3000 RS autosampler (Dionex, Camberley UK) and separated on a C18 reverse phase column (75 µm ID × 150 mm, 100 Å, Acclaim PepMap RSLC C18, Dionex). Peptides were eluted using a 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 eluant 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 FTMS. The ionisation voltage was 2.5 kV and the capillary temperature was 200 ºC. The mass spectrometer was operated in MS/MS mode scanning from 2=380 to 2000 amu. The top 20 multi charged ions were selected from each full scan for MS/MS analysis, the fragmentation method was HCD at 35% collision energy. The ions selected for MS2 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 MS1 was 60,000 and 7,500 for HCD MS2. LC-MS/MS analysis was performed under supervision of Dr. Bill Mullen and Dr. Amaya Albalat of Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK.

4.2.7.2 LC-MS/MS – Data analysis

Raw spectral data from LC-MS/MS analysis of the samples were uploaded to Thermo Proteome Discoverer 1.2. Peak picking was performed under default settings for Fourier transform mass analyzers (FTMS) analysis i.e. only >+2 peptides were considered with signal to noise ratio higher than 1.5 and belonging to precursor peptides between 700 – 8000 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*, *E. coli* and *S. aureus* entries. This concatenated database was selected to facilitate peptide identification and reduce the probability for false positive identifications upon making searches against 3 differently sized FASTA databases. No enzyme cleavage was selected and oxidation of methionine and proline were chosen as variable modifications. Precursor tolerance was set at 20 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.

4.3 Results

4.3.1 SCC measurement and acute phase proteins concentration

In order to confirm the disease status of milk samples used in this study, SCC measurement and acute phase proteins (SAA and Hp) concentrations were determined. Table 4-1 showed the SCC measurement and APP concentrations in all control milk samples used in this study. In control milk samples, the range of SAA concentration was between 0.079 and

2.96μg/ml. The average of SAA concentration was 0.71μg/ml and the median of SAA concentration was 0.48μg/ml in control milk samples. Meanwhile, the range of Hp concentration was between 1.95 and 32μg/ml. The average of Hp concentration was 12.8μg/ml and the median of Hp concentration was 12.64μg/ml. The range of SCC measurement in control milk samples was between 10 and 96 x 10^3 cells/ml. The average was 45 x 10^3 cells/ml and the median was 46 x 10^3 cells/ml. Table 4-2 (a and b) showed the bacteriological culture results and the concentration of both SAA and Hp for mastitic milk samples caused by *E. coli* and *S. aureus*. For *E. coli* infected group, the range of SAA concentration was between 4.18 and 1500μg/ml. The average of SAA concentration was 626.6μg/ml and the median of SAA concentration was 90.79μg/ml in *E. coli* infected group milk samples. Meanwhile, the range of Hp concentration was between 16.03 and 354.26μg/ml. The average of Hp concentration was 112.5μg/ml and the median of Hp concentration was 98.85μg/ml. For *S. aureus* infected group, the range of SAA concentration was between 16 and 1500μg/ml. The average of SAA concentration was 526.1μg/ml and the median of SAA concentration was 51.54μg/ml in *E. coli* infected group milk samples. Meanwhile, the range of Hp concentration was between 22.15 and 118.83μg/ml. The average of Hp concentration was 71.44μg/ml and the median of Hp concentration was 78.49μg/ml. The Statistical analysis (Mann Whitney) showed that both SAA and Hp were significantly higher ($p=0.00$; $p<0.05$) in the infected group (*E. coli* and *S. aureus*) but not significantly different between two bacterial groups ($p=0.958$; $p>0.05$ for SAA and $p=0.428$; $p>0.05$ for Hp).

Control milk samples

Sample Ids	SAA Conc.(ng/ml)	Hp conc. (ng/ml)	SCC ($x10^3$)
102	0.36	7.78	13
66	0.57	16.38	14
3	0.55	27.98	48
62	0.079	1.95	31
72	1.21	7.24	96
109	0.65	13.21	10
80	0.28	24.61	54
100	0.48	15.36	38
16	0.9	2.86	58
58	0.34	1.95	56
111	0.13	21.94	28
48	0.39	31.95	85
4	2.96	4.17	63
2	1.43	1.95	32
29	0.29	12.64	46
Min	**0.08**	**1.95**	**10**
Max	**2.96**	**31.95**	**96**
Mean	**0.71**	**12.8**	**45**
Median	**0.48**	**12.64**	**46**

Table 4-1: The acute phase proteins (SAA and Hp) concentrations and SCC measurement in all control milk samples used in this study. The range of SAA concentrations was between 0.08 and 2.96 μg/ml with an average of 0.71μg/ml and a median of 0.48μg/ml. The range of Hp concentrations was between 1.95 and 32μg/ml with an average of 12.8μg/ml and a median of 12.64μg/ml. The range of SCC measurement was between 10 and 96 x 10^3 cells/ml with an average of 45 x 10^3 cells/ml and a median of 46 x 10^3 cells/ml.

Mastitic milk samples

Sample Ids	SAA Conc. (ng/ml)	Hp conc. (ng/ml)	Bacteriological results
475	72.13	89.68	*E. coli*
76	1500	115.18	*E. coli*
?	1500	78.51	*E. coli*
814	1500	141.26	*E. coli*
692	9.48	60.27	*E. coli*
697	1500	144.89	*E. coli*
519	109.44	108.02	*E. coli*
453	4.18	16.63	*E. coli*
496	24.31	16.03	*E. coli*
1222	46.02	354.26	*E. coli*
Min	**4.18**	**16.03**	
Max	**1500**	**354.26**	
Mean	**626.56**	**112.47**	
Median	**90.79**	**98.85**	

Table 4-2a: The acute phase proteins (SAA and Hp) concentrations and bacterial causing mastitis in infected milk samples caused by *E. coli* used in this study. The range of SAA concentrations was between 4.18 and 1500µg/ml with an average 626.6µg/ml and a median of 90.79µg/ml. The range of Hp concentrations was between 16.03 and 354.26µg/ml with an average of 112.5µg/ml and a median of 98.85µg/ml.

Mastitic milk samples

Sample Ids	SAA Conc. (ng/ml)	Hp conc. (ng/ml)	Bacteriological results
438	48.12	70.77	*S. aureus*
14	37.21	42.69	*S. aureus*
980	1500	111.83	*S. aureus*
708	54.96	95	*S. aureus*
346	1500	86.2	*S. aureus*
715	15.99	22.15	*S. aureus*
Min	**15.99**	**22.15**	
Max	**1500**	**111.83**	
Mean	**526.05**	**71.44**	
Median	**51.54**	**78.49**	

Table 4-2b: The acute phase proteins (SAA and Hp) concentrations and bacterial causing mastitis in infected milk samples caused by *S. aureus* used in this study. The range of SAA concentrations was between 15.99 and 1500μg/ml with an average of 526.1μg/ml and a median of 51.54μg/ml. The range of Hp concentrations was between 22.15 and 111.8μg/ml with an average of 71.44μg/ml and a median of 78.49μg/ml.

4.3.2 Polypeptide Fingerprinting for control and infected groups

Initially in this work the possibility of differences existing in the milk proteome of healthy cows (n=10) when compared to mastitic cows, regardless of their bacterial cause (n=16) was examined. The polypeptide fingerprints of these groups were very different as shown in Figure 4-1. The y-axis represents the molecular mass (kDa) on a logarithmic scale and the x-axis represents the CE migration time (min) whilst the z-axis represents the mean signal intensity. From the figure, higher molecular mass of peptides appeared to be in higher signal intensity eluting between 25 and 40 minutes in control milk samples compared with infected milk samples. Lower molecular mass of peptides were found to be present in infected milk samples eluting between 50 and 60 minutes although in low signal intensity as compared in control milk samples. Peptides with molecular mass between 1.5 and 4 kDa at 30 to 45 minutes were found to be in higher abundance with higher signal intensity in infected milk samples compared with control milk samples. The next step was to investigate the differences in milk proteome of infected samples caused by two different bacterial pathogens (*E. coli* and *S. aureus*). The polypeptide profiles of these 2 different groups were almost similar in pattern as shown in Figure 4-2 except in their signal intensities. Peptides with molecular mass between 2.5 and 3.0 kDa at 25 to 30 minutes were found to be in higher signal intensity in *E. coli* infected milk samples as compared with *S. aureus* infected milk samples. Peptides with molecular mass between 2.0 and 4 kDa at 35 and 40 minutes were also found to be in higher signal intensity in *E. coli* infected milk samples as compared with *S. aureus* infected milk samples.

Control Infected (all)

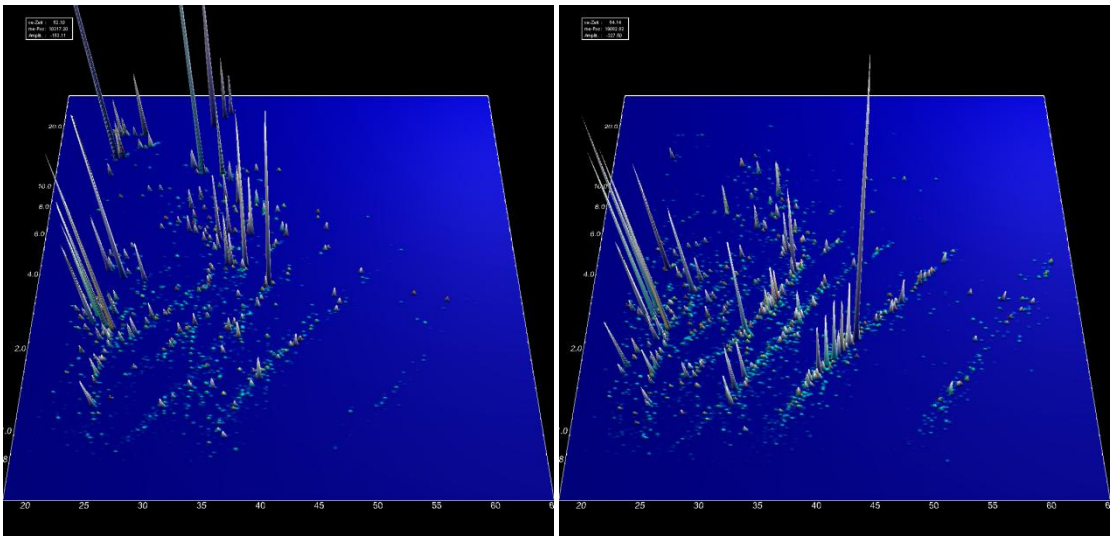


Figure 4-1: The polypeptide fingerprints of both non-infected and infected groups. From these fingerprints, they clearly showed a significant differences in the pattern of polypeptide peaks between non-infected and infected groups. X-axis referred to the CE migration time in minutes whilst y-axis referred to the molecular mass (kDa) on a logarithmic scale and z-axis referred to mean signal intensity.

E. coli *S. aureus*

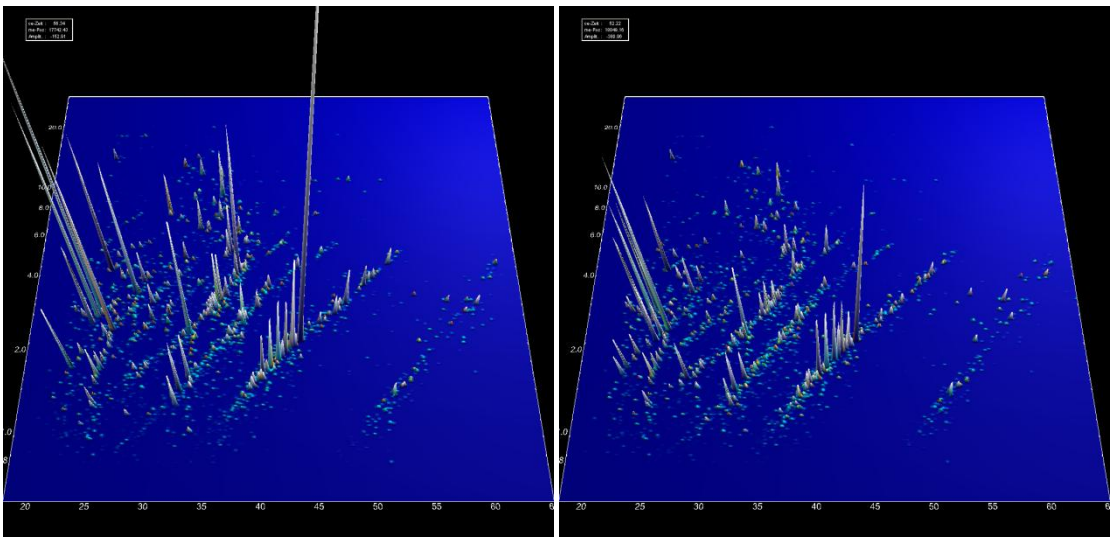


Figure 4-2: The polypeptide fingerprints of both *E. coli* and *S. aureus* groups. From these fingerprints, they clearly showed differences in the pattern of polypeptide peaks between *E. coli and S. aureus* groups.

4.3.3 Model Generation of the polypeptide fingerprints for control and infected groups

Based on the polypeptide fingerprints described above, multiple testing of statistical analysis was performed to identify peaks which discriminate between control and infected groups. In the first model, discriminatory peptides were selected that were able to differentiate between the control and infected groups with the area under curve (AUC) set at more than 0.99, Benjamini-Hochberg (BH) was set at less than 0.05 and Tmax less than 0.05. With these very stringent conditions a group of 31 peaks were identified that can be used to discriminate between control and infected groups. From the statistical analysis, this model possessed 100% value for its sensitivity, specificity and accuracy. The peptides that from this model could be used to discriminate between non-infected and infected group is presented in Figure 4-3.

Based on the polypeptide fingerprints described above, multiple testing of statistical analysis was performed to discriminate peaks between two infected groups caused by *E. coli* and *S. aureus*. There were three models generated with different values of sensitivity, specificity and accuracy. For Model 1 (Figure 4-4), the area under curve (AUC) was set at more than 0.99, Benjamini-Hochberg (BH) was set at less than 0.05 and Tmax at less than 0.05 and 6 peaks were identified that can be used to discriminate between *E. coli* and *S. aureus* groups. The sensitivity value for this model was 100% whilst the specificity value was 91.67% and the accuracy value was 96.3%. For Model 2 (Figure 4-5), with the area under curve (AUC) set at more than 0.8, Benjamini-Hochberg (BH) was set at less than 0.05 and Tmax less than 0.05, 3 peaks were identified that can be used to discriminate between *E. coli* and *S. aureu*s groups. The sensitivity value for this model was 100% whilst the specificity value was 83.3% and the accuracy value was 92.52%. For the Model 3 (Figure 4-6), with the area under curve (AUC) set at more than 0.9, Benjamini-Hochberg (BH) was set at less than 0.1 and Tmax less than 0.05, 13 peaks were identified that can be used to discriminate between *E. coli* and *S. aureu*s groups. The sensitivity value for this model was 100% whilst the specificity value was 83.3% and the accuracy value was 92.52%. These three models with different number of polypeptides were used to discriminate between *E. coli* and *S. aureus* infected groups. All polypeptides generated from all three models were tabulated in Table 4-3. It should be noted that a total of 13 polypeptides appeared in at least one of the models (Table 4-4).

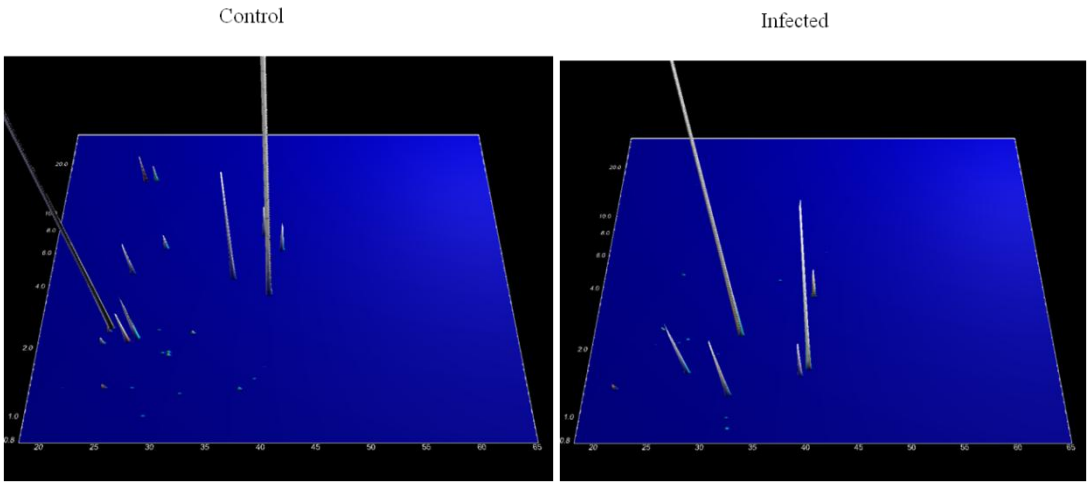


Figure 4-3: The model of polypeptide fingerprint after multiple testing of non-infected and infected groups. This model has successfully identified 31 polypeptide peaks that can be used to discriminate between non-infected and infected groups.

E. coli *S. aureus*

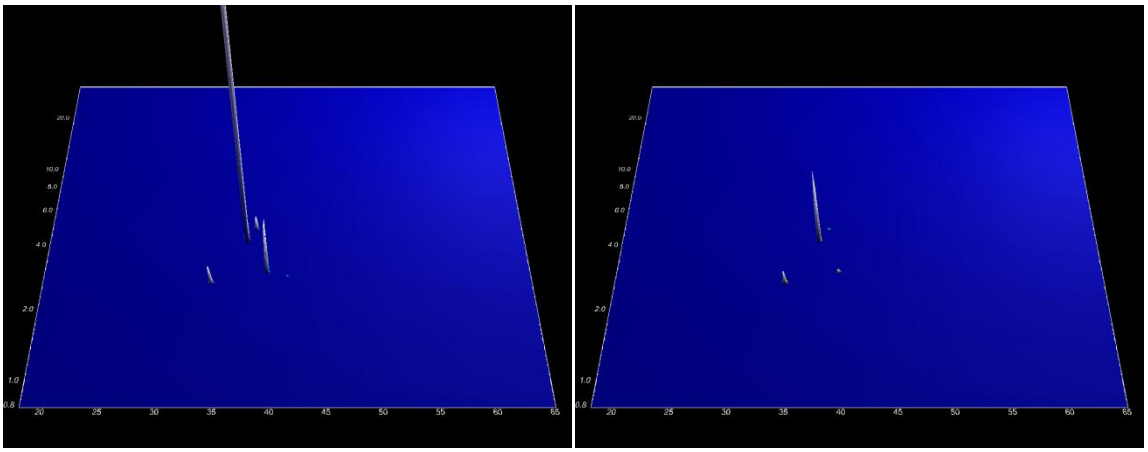


Figure 4-4: The first model of polypeptide fingerprint after multiple testing of *E. coli* and *S. aureus* groups. This model has successfully identified 6 polypeptide peaks that can be used to discriminate between *E. coli* and *S. aureus* groups.

E. coli *S. aureus*

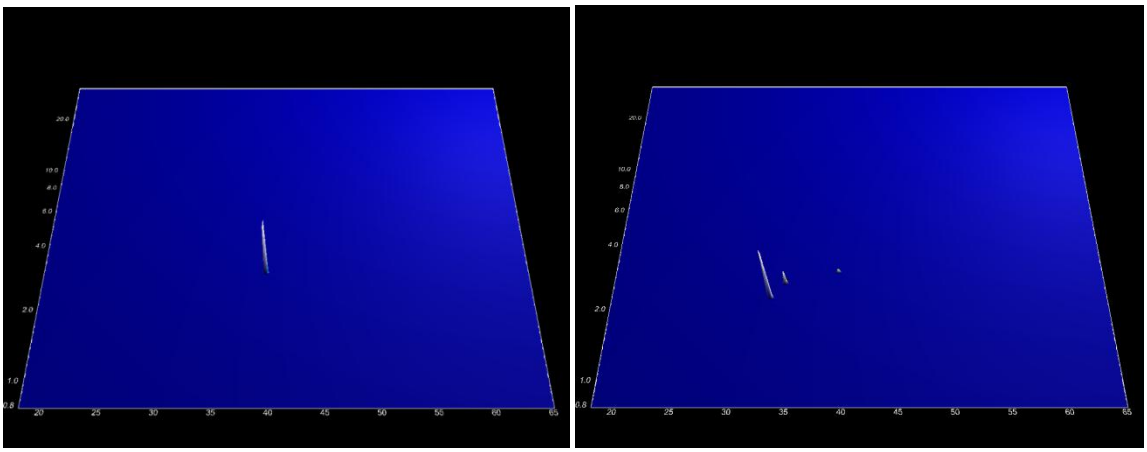


Figure 4-5: The second model of polypeptide fingerprint after multiple testing of *E. coli* and *S. aureus* groups. This model has successfully identified 3 polypeptide peaks that can be used to discriminate between *E. coli* and *S. aureus* groups.

E. coli *S. aureus*

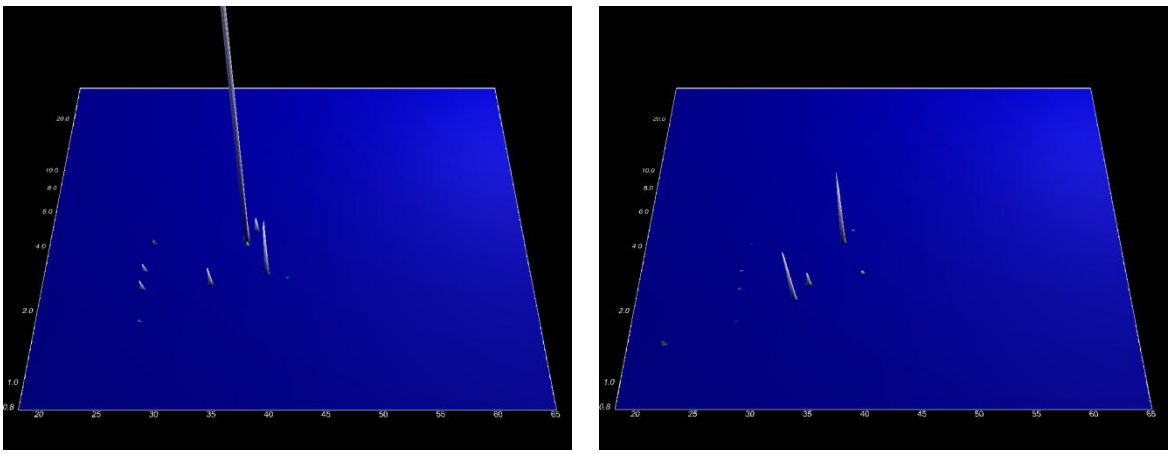


Figure 4-6: The third model of polypeptide fingerprint after multiple testing of *E. coli* and *S. aureus* groups. This model has successfully identified 13 polypeptide peaks that can be used to discriminate between *E. coli* and *S. aureus* groups.

Model 1

Peptide ID
8093
8098
9092
9662
13428
14889

Model 2

Peptide ID
9662
8093
6503

Model 3

Peptide ID
9662
8093
6503
8098
9092
13428
14889
9960
13328
4865
7557
13101
3255

Table 4-3: Polypeptides that were identified from 3 models generated to discriminate *E. coli* from *S. aureus* infected groups. These peptides appeared in at least one of the models

Peptide ID	Models from where the peptide was found
9662	1,2,3
8093	1,2,3
6503	1,2
8098	2,3
9092	2,3
13428	2,3
14889	2,3
9960	3
13328	3
4865	3
7557	3
13101	3
3255	3

Table 4-4: A total of 13 polypeptides generated from 3 models that discriminate *E. coli* from *S. aureus* infected groups. Models from where the peptide was found were also listed.

4.3.4 CE-MS Analysis

A total of 31 discriminatory polypeptides were identified from CE-MS analysis and were significant on the basis of comparison between control and infected groups (*S. aureus* and *E. coli*) and are presented in Table 4-5. Meanwhile, a total of 14 discriminatory polypeptides were identified from CE-MS analysis and were significant on the basis of comparison between two infected groups (*S. aureus* and *E. coli*) and are presented in Table 4-6. A total of 45 discriminatory polypeptides were presented in Table 4-7 based on the sample type comparison (e.g: control vs infected; *E. coli* vs. *S. aureus*). These polypeptides were found in more than 70% of the samples in at least one of the two groups (controls or cases) in order to be considered as disease biomarkers.

Peptide ID	Peptide Mass	CE Migration time
386	914.5	32.3
895	1013.5	32.1
963	1028.6	28.9
2367	1275.7	32.3
2664	1335.7	37.8
2739	1350.7	25.1
2740	1350.7	27.6
2757	1352.8	21.1
2759	1353.7	29
3422	1481.8	39.2
3728	1554.8	39.2
3913	1597.8	28
4226	1667.9	40
5064	1893	30.8
5217	1933.1	30.2
5263	1947.1	30.8
6017	2153.2	24.4
6178	2197.3	26.7
6465	2277.3	27.6
6891	2390.2	33.1
7095	2440.4	24.8
7323	2490.4	29.7
7333	2493.4	24.8
11937	3720	40.5
14110	4498.4	36.9
14701	4780.6	26.4
16632	6376	41.9
16728	6517.8	29.7
17239	7687.5	40
19020	15873.3	26.6
19046	16123.3	27.8

Table 4-5: The discriminatory polypeptides identified from CE-MS analysis that were significant in the basis of comparison between control and infected groups. A total of 31 polypeptides are presented with their mass values and CE migration times.

Peptide ID	Peptide Mass	CE Migration time
3255	1451.8	21.4
4865	1839	27.7
6503	2285.2	33.1
7557	2552.3	27.7
8093	2676.5	34.4
8098	2677.4	34.2
9092	2896.5	41.4
9094	2896.6	59.4
9662	3028.5	39.5
9960	3113.6	27.7
13101	4120.2	37.5
13328	4205.2	28.3
13428	4237.2	37.5
14889	4882.5	38.4

Table 4-6: The discriminatory polypeptides identified from CE-MS analysis that were significant in the basis of comparison between two infected groups (*E. coli* and *S. aureus*). A total of 14 polypeptides are presented with their mass values and CE migration times.

Peptide ID	Peptide Mass	CE Migration Time	Type of comparison
386	914.486	32.3	Control vs Infected
895	1013.55	32.1	Control vs Infected
963	1028.58	28.9	Control vs Infected
2367	1275.67	32.2	Control vs Infected
2664	1335.7	37.8	Control vs Infected
2739	1350.71	25.1	Control vs Infected
2740	1350.71	27.6	Control vs Infected
2757	1352.76	21.1	Control vs Infected
2759	1353.69	29.0	Control vs Infected
3422	1481.8	39.2	Control vs Infected
3728	1554.82	39.2	Control vs Infected
3913	1597.81	28.0	Control vs Infected
4226	1667.9	40.0	Control vs Infected
5064	1893.01	30.8	Control vs Infected
5217	1933.11	30.2	Control vs Infected
5263	1947.07	30.8	Control vs Infected
6017	2153.17	24.4	Control vs Infected
6178	2197.26	26.7	Control vs Infected
6465	2277.28	27.6	Control vs Infected
6891	2390.21	33.1	Control vs Infected
7095	2440.39	24.8	Control vs Infected
7323	2490.36	29.7	Control vs Infected
7333	2493.36	24.8	Control vs Infected
11937	3720.03	40.5	Control vs Infected
14110	4498.43	36.9	Control vs Infected
14701	4780.55	26.4	Control vs Infected
16632	6376.03	41.9	Control vs Infected
16728	6517.75	29.7	Control vs Infected
17239	7687.52	40.0	Control vs Infected
19020	15873.3	26.6	Control vs Infected
19046	16123.3	27.8	Control vs Infected
3255	1451.843	21.4	*E. coli* vs *S. aureus*
4865	1838.965	27.7	*E. coli* vs *S. aureus*
6503	2285.208	33.1	*E. coli* vs *S. aureus*
7557	2552.311	27.7	*E. coli* vs *S. aureus*
8093	2676.482	34.4	*E. coli* vs *S. aureus*
8098	2677.406	34.2	*E. coli* vs *S. aureus*
9092	2896.512	41.4	*E. coli* vs *S. aureus*
9094	2896.564	59.4	*E. coli* vs *S. aureus*
9662	3028.54	39.5	*E. coli* vs *S. aureus*
9960	3113.574	27.7	*E. coli* vs *S. aureus*
13101	4120.18	37.5	*E. coli* vs *S. aureus*
13328	4205.192	28.3	*E. coli* vs *S. aureus*

13428	4237.227	37.5	*E. col*i vs *S. aureus*
14889	4882.521	38.4	*E. col*i vs *S. aureus*

Table 4-7: The discriminatory polypeptides identified from CE-MS analysis that were significant in the basis of both comparison (between control and infected groups as well as between *E. coli* and *S. aureus*). These polypeptides are presented with their mass values and CE migration times.

4.3.5 Peptide sequencing

In order to identify the discriminatory peptides of the models by their sequences, samples were analysed using LC-MS/MS. Twelve of the 45 polypeptides identified with high confidence are listed in Table 4-8. Based on their masses from the CE-MS analysis and the number of basic amino acids in the sequence (H, R, K or histidine, arginine and lysine), these parameters (peptide mass and number of amino acids) were used to link the peptides obtained in both systems (CE-MS and LC-MS). Figure 4-7 showed a scatterplot of polypeptides in all milk samples from CE-MS analysis based on the number of basic amino acids found in the sequence. The y-axis represents the molecular mass (kDa) of milk polypeptides whilst the x-axis represents the CE migration time (min). Based on the molecular mass and the CE migration time of certain polypeptides, the number of amino acids found in the sequence can be determined from Figure 4-7. The number of basic amino acids can be used in the process of determining the peptide sequence for each polypeptide identified from CE-MS analysis. From Thermo Proteome Discoverer software and SEQUEST algorithm, the potential amino acids sequences were obtained according to these polypeptide masses and number of basic amino acid present in the sequence as presented in Table 4-8. In this table, other important parameters are presented such as the amino acid sequence, protein accession, probability/XCorr/scores, charge, mass-charge ratios (m/z), the protonated mass of the peptide (MH +), DM (ppm), LC migration time (RT in minutes) and number of ions matched. As the methionine and proline oxidations were set as variable modifications you, the lower cases were presented (e.g. p and m) instead of their upper cases (e.g. P and M) in the amino acid sequence. The protein accessions obtained from the software programme are the coded name for the protein from which the peptide belongs and this information was used to search for protein identification using protein database (e.g. UniProt or NCBI; see section 4.3.6 below). The probability, XCorr and score values were the statistical values provided by the Proteome Discoverer and SEQUEST algorithm that depict the validity of the identification. Meanwhile, the charge column in the table referred to the charge state in which the peptide was detected by the mass spectrometer and DM (ppm) means the error difference between the theoretical mass value of the peptide and the experimental mass value from the mass spectrometer. Theory mass was the mass deviation of the peptide which was calculated from the sequence whilst ions matched referred to the produced peptide fragments from air collision in the MS and were recorded in a new mass spectrum to produce the amino acid sequence.

It should be noted that not all the identified polypeptides from CE-MS analysis were successfully sequenced. Furthermore, even with the same peptide sequence, the statistical values (probability, XCorr, score values) and other parameters appeared to be different as the respective peptide originated from different type of samples (control, *E. coli* or *S. aureus*) as happened in Peptide ID 2367, 3728, 3913, 4226, 4865, 6017, 6178 and 6465.

4.3.6 Protein identification

As described above, protein identity was determined from a protein database based on their amino acid sequences. The identified and named proteins are presented as in Table 4-9. The UniProt Knowledgebase (UniProtKB) database was used to determine the name of protein by using the protein accession information of each amino acid sequence. These identified proteins will be discussed further in the discussion section.

ID	Peptide Sequence	Protein Accessions	Pb	XC	Sc	C	m/z [Da]	MH+ [Da]	Theory Mass	ΔM [ppm]	RT [min]	Ions Matched	Origins of polypeptides
2367	PFPEVFGKEKV	P02662	2.92	3.00	1.00	2	638.85034	1276.69341	1275.68613	-0.21	64.93	11/20	Found in *S. aureus* sample
	PFPEVFGKEKV	P02662	6.16	3.97	1.00	3	426.23605	1276.69361	1275.68633	-0.05	51.09	17/40	Found in *E. coli* sample
2757	SKVKEAMAPKHK	P02666	23.81	4.33	1.00	3	451.92667	1353.76545	1352.75817	-1.29	8.26	22/44	Found in *S. aureus* sample
3728	YQEPVLGPVRGPFP	P02666;E1BIM4	25.84	3.73	1.00	2	778.41815	1555.82903	1554.82175	1.42	59.97	14/26	Found in *E. coli* sample
	YQEPVLGPVRGPFP	P02666;E1BIM4	32.84	3.07	1.00	2	778.41870	1555.83013	1554.82285	2.13	64.93	12/26	Found in *S. aureus* sample
3913	HKEMPFPKYPVEP	P02666	1.41	3.31	1.00	3	533.60522	1598.80112	1597.79384	-1.57	41.45	16/48	Found in Control sample
	HKEMPFPKYPVEP	P02666;E1BIM4	1.00	4.11	0.20	3	533.60553	1598.80204	1597.79476	-1.00	39.71	17/48	Found in *E. coli* sample
	HKEMPFPKYPVEP	P02666;E1BIM4	1.00	3.93	1.00	3	533.60577	1598.80277	1597.79549	-0.54	38.86	18/48	Found in *S. aureus* sample
	pNpKTpSSKRQVDP	A5D7N6	1.00	3.34		3	533.60590	1598.80313	1597.79586	-6.39	39.22	13/52	Found in *E. coli* sample
4226	YQEPVLGPVRGPFPI	P02666;E1BIM4	18.74	3.76	1.00	2	834.95844	1668.90959	1667.90232	-0.77	64.55	15/28	Found in *E. coli* sample
	YQEPVLGPVRGPFPI	P02666;E1BIM4	4.48	3.16	1.00	2	834.95856	1668.90984	1667.90256	-0.63	66.70	12/28	Found in *S. aureus* sample
	LYQEPVLGPVRGPFP	P02666;E1BIM4	8.54	3.56	1.00	2	834.95856	1668.90984	1667.90256	-0.63	61.65	13/28	Found in *S. aureus* sample
4865	RGPGGAWAAKVISNARET	Q8SQ28;Q56J78	16.53	3.75	1.00	3	614.33032	1840.97641	1839.96914	-0.73	38.82	21/68	Found in *E. coli* sample
	RGPGGAWAAKVISNARET	Q8SQ28;Q56J78	28.50	5.18	1.00	4	460.99957	1840.97646	1839.96919	-0.71	38.01	24/102	Found in *S. aureus* sample
5217	SRQPQSQNPKLPLSILK	P80195	31.69	4.53	1.00	4	484.28540	1934.11977	1933.11250	0.79	42.73	26/96	Found in Control sample
5263	QPQSQNPKLPLSILKEK		1.00	3.54	1.00	4	487.78638	1948.12368	1947.11640	0.52	41.88	22/96	Found in Control sample
6017	RGSKASADESLALGKPGKEPR		10.05	3.87	1.00	5	431.63834	2154.16257	2153.15530	-0.05	15.95	27/160	Found in Control sample
	RGSKASADESLALGKPGKEPR	Q4VS17	9.18	3.77	1.00	5	431.63849	2154.16334	2153.15606	0.30	16.34	24/160	Found in Control sample
	RGSKASADESLALGKPGKEPR		6.92	3.92	1.00	5	431.63867	2154.16425	2153.15698	0.73	15.54	28/160	Found in *E. coli* sample
	RGSKASADESLALGKPGKEPR		9.66	4.66	1.00	5	431.63867	2154.16425	2153.15698	0.73	15.71	27/160	Found in *S. aureus*

													sample
6178	QPQSQNPKLPLSILKEKHL	P80195	22.10	4.59	1.00	5	440.45886	2198.26521	2197.25793	-0.20	44.31	24/144	Found in *E. coli* sample
	QPQSQNPKLPLSILKEKHL	P80195	9.81	4.05	1.00	5	440.45926	2198.26719	2197.25991	0.70	44.51	21/144	Found in Control sample
6465	SSRQPQSQNPKLPLSILKEK	P80195	53.89	6.33	1.00	5	456.46353	2278.28855	2277.28127	0.31	34.79	31/152	Found in *E. coli* sample
	SSRQPQSQNPKLPLSILKEK	P80195	22.40	6.44	1.00	5	456.46353	2278.28855	2277.28127	0.31	35.85	29/152	Found in *S. aureus* sample
	SSRQPQSQNPKLPLSILKEK	P80195	49.13	5.28	1.00	5	456.46356	2278.28870	2277.28143	0.37	36.94	28/152	Found in Control sample
7095	SRQPQSQNPKLPLSILKEKHL	P80195	1.00	3.96	1.00	5	489.08575	2441.39967	2440.39239	0.35	39.30	25/160	Found in Control sample

Table 4-8: The amino acid sequences of potential biomarkers from the LC-MS/MS analysis. Various other parameters are also presented to help better understanding of each of the polypeptides.

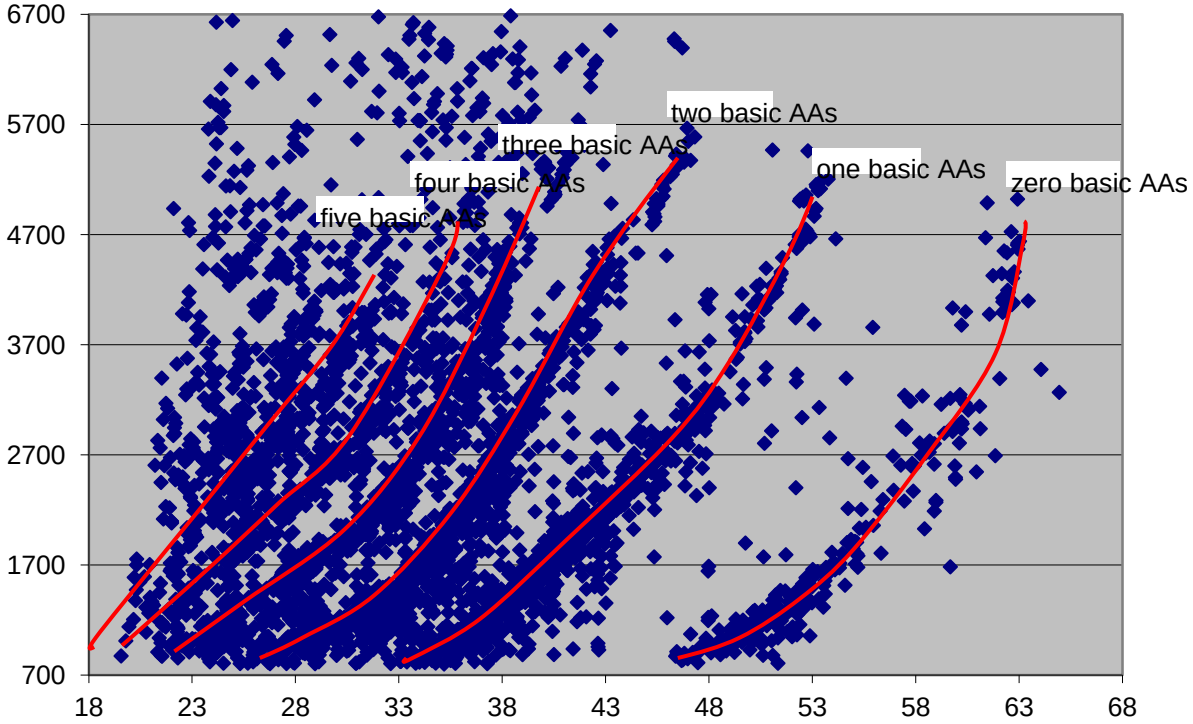


Figure 4-7: Milk polypeptides detected in CE-MS analysis based on the number of basic amino acids found in the amino acid sequence. Y-axis represents the molecular mass (kDa) and x-axis represents the CE migration time (min). The red line is specific for the number of basic amino acids in the sequence. Up to five basic amino acids can be found in certain amino acid sequence. The higher number of basic amino acids found in the sequence, the shorter migration time for the CE-MS analysis. This diagram can be used to match the sequence from LC-MS/MS analysis for each polypeptides identified from CE-MS analysis.

Peptide ID	Sequence	Protein Identity	Fq in control	Fq in infected	Fq in *E. coli*	Fq in *S. aureus*
2367	PFPEVFGKEKV	αS1-casein	0.80	1.00		
2757	SKVKEAMAPKHK	β-casein	0.50	1.00		
3728	YQEPVLGPVRGPFP	β-casein	0.30	1.00		
3913	HKEMPFPKYPVEP	β-casein	0.10	1.00		
4226	YQEPVLGPVRGPFPI	β-casein	0.20	1.00		
4865	RGPGGAWAAKVISNARET	serum amyloid A			1.00	0.58
5217	SRQPQSQNPKLPLSILK	Glycosylation-dependent cell adhesion molecule 1	1.00	0.07		
5263	QPQSQNPKLPLSILKEK	Protein accession not available	1.00	0.48		
6017	RGSKASADESLALGKPGKEPR	Fibroblast growth factor binding protein	1.00	0.59		
6178	QPQSQNPKLPLSILKEKHL	Glycosylation-dependent cell adhesion molecule 1	1.00	0.15		
6465	SSRQPQSQNPKLPLSILKEK	Glycosylation-dependent cell adhesion molecule 1	1.00	0.74		
7095	SRQPQSQNPKLPLSILKEKHL	Glycosylation-dependent cell adhesion molecule 1	1.00	0.07		

Table 4-9: The protein identities obtained from the UniProtKB database (www.uniprot.org/uniprot). The frequency of each of the identified protein was also presented according to the respected groups.

4.4 Discussion

The mastitic milk samples chosen in this study had significantly higher acute phase proteins concentrations for both SAA and Hp as compared with the healthy milk samples ($p<0.05$). However, the concentrations for both SAA and Hp were not significant between *E. coli* and *S. aureus* infectedgroup ($p>0.05$). The SCC measurement for all healthy milk samples was less than 100 000 cells/ml which confirmed the healthy status of the cows (average of 46×10^3 cells/ml).

The chosen peptidomic method of capillary electrophoresis (CE) coupled with the electrospray mass spectrometry is known as a rapid (approximately 45 min per sample), sensitive method using its automated approach (Kolch *et al.* 2005). It was used in this study to detect differences in the milk peptidome between healthy cows (control) and mastitic cows caused by two different bacterial pathogens (*E. coli* and *S. aureus*).

All milk samples were analyzed by CE-MS and only polypeptides present at a frequency >70% in at least one of the two groups (control and cases) were chosen for analysis. The compiled data for all test groups (control, all infected, *E. coli* and *S. aureus*) are shown in Figure 4-1 and 4-2. It was found that the milk polypeptide patterns of infected cows were markedly different from control cows (Figure 4-1). However, the milk polypeptide patterns of both bacterial groups (*E. coli* and *S. aureus*) were more similar (Figure 4-2).

For identification of potential biomarkers for bovine mastitis, milk polypeptides of healthy cows were compared with those of cows with mastitis regardless of its bacterial pathogen. This analysis identified 31 polypeptides of statistical significance after correction for multiple testing ($p < 0.05$ in max T testing) with 100% sensitivity, specificity and accuracy. The distribution of the polypeptides in these groups is shown in Figure 4-3. It is observed that the identified polypeptides significantly higher in the infected than control group were of lower molecular mass (y-axis) and appeared at much later time of migration (x-axis) which is an indicator of containing a lower proportion of basic amino acids.

To identify biomarkers that could discriminate between two mastitic groups caused by *E. coli* and *S. aureus*, the analysis successfully generated three different models with different values of sensitivity, specificity and accuracy. Model 1 appeared to have 100% sensitivity, 91.67% specificity and 96.3% accuracy. Six peaks of discriminatory polypeptides were found in this model. In Model 2, the sensitivity was 100% with the specificity of 83.3% and accuracy of 92.52%. Three peaks of discriminatory polypeptides were found in this

model. Finally, Model 3 appeared to have 100% sensitivity, 83.3% specificity and 92.52% accuracy. 13 peaks of discriminatory polypeptides were found in this model. In conclusion, a total of 13 discriminatory polypeptides were identified from these three models generated (Table 4-4).

The previous step is known as the learning phase in which the 'training set' was used to generate a polypeptide pattern with significant biomarkers (Zürbig *et al.* 2009). To complete biomarker identification exercise, after this training, the model should be able to characterize further unknown data sets so that a correct classification can be confirmed.

There are only a few studies on peptide analysis of milk from cows with bovine mastitis using milk samples (Wedholm *et al.* 2008; Larsen *et al.* 2010). Both studies employed the same method in separating the peptides by RP-HPLC with further protein characterization by MALDI-TOF/MS/MS. These studies were also focused on the result of proteolysis caused by different types of proteolytic enzymes. Wedholm *et al.* (2008) have successfully identified 10 peptides from milk with high SCC (16 000 000 cells/ml) while Larsen *et al.* (2010) on the other hand identified approximately 20 different peptides in milk samples infused with LTA of *S. aureus* as a result of proteolysis.

However, the objective of these studies was to gain better understanding in the milk proteinase activities during mastitis and in comparison, this present study is focused on how the peptide profile of the milk samples can be changed in the presence of bovine mastitis. In addition, the discovery of significant peptide biomarker in bovine mastitis was also one of the important objectives in this present study.

Although further model validation will be needed as discussed above, protein identification of these discriminatory polypeptides should be done to provide an initial insight on the change of peptide profiles in milk samples infected with bovine mastitis. It was interesting to see the presence of peptide ID 9094 as one of the discriminatory polypeptides detected from the generated model that can be used to discriminate *E. coli* from *S. aureus* infected group (Table 4-3 and 4-4). However, this polypeptide was not detected from the CE-MS analysis that discriminate *E. coli* from *S. aureus* infected group (Table 4-6). Although it was not a statistically significant polypeptide that discriminated very well between *E. coli* and *S. aureus* infected group, this shouldn't be a problem because we were interested in determining the peptide sequencing in the LC-MS system. Thus, it would have been a good idea to be able to identify this polypeptide by sequencing in the LC-MS/MS analysis.

A total of 45 discriminatory polypeptides (31 polypeptides which discriminate healthy from mastitis cases and 14 polypeptides which discriminate *E. coli* from *S. aureus* infected cases) from CE-MS analysis were then analysed for their amino acid sequence by LC-MS/MS As the only parameters used to link the peptides obtained in both systems (CE-MS and LC-MS/MS) were peptide mass and the number of basic amino acids, it can lead to mass ambiguities due to the highly complex peptide mixtures which complicate the assignment of sequences obtained from different separation methods. A further factor was the fact that the mass spectrometers used in the two systems were different. For peptide identification, the LC-MS was used but it was not possible with 100% certainty to correlate the results from the CE-MS with the results from the LC-MS/MS data.

Despite having a problem with mass ambiguity, the results from the LC-MS/MS analysis can be used to determine the protein identity for each of the sequences. However, not all of these 45 polypeptides identified by CE-MS were able to be sequenced by the LC-MS/MS. This occurred as the peptide mass calculated from the CE-MS analysis was not matched with the peptide mass calculated from the LC-MS/MS analysis. It should also be noted that the peptide mass calculated from the LC-MS/MS was based on the mass of peptide plus one proton which made them one unit more than the mass calculated from CE-MS analysis.

Due to the problem in sequencing, a total of 12 polypeptides were able to be sequenced by LC-MS/MS (Table 4-8). From the total of 12 successfully sequenced polypeptides, 11 were used to discriminate control milk samples from the infected milk samples. Only 1 sequenced polypeptide in the set was used to discriminate *E. coli* infected milk samples from S. *aureus* infected milk samples. The origin of the milk peptides from which the sequenced polypeptides was found is presented in Table 4-8. Based on their protein accession information, these polypeptides were identified by their amino acid sequences. UniProtKB database was searched for each of the protein identity. Amongst the 12 peptides that were successfully identified were sequences from α S1-casein, β-casein, serum amyloid A protein (SAA), glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) and fibroblast growth factor binding protein (FGFBP) which were mostly on the bovine protein database as showed in Table 4-9.

As the focus was on discriminatory polypeptides, the discussion will be presented based on the polypeptides/proteins that were able to discriminate between control, infected and two different bacterial pathogens groups. Statistically, α S1-casein, β-casein, GlyCAM-1 and

FGFBP were the proteins that can be used to discriminate the infected group (regardless of its causing bacterial pathogen) from the control group.

During mastitis, the proteolytic activity in milk can be increased substantially (Verdi *et al.* 1987). Apart from β-casein, plasmin can also hydrolyses α S2-casein and α S1-casein (Larsen *et al.* 2006) which explain the presence of peptides of α S1-casein in the infected group higher compared with the control group from the hydrolytic activity in milk.

Statistically, peptides from glyCAM 1 and FGFBP were the peptides that can be used to discriminate the control from the infected groups. The presence of glyCAM 1 protein in bovine milk has been reported previously by Smolenski *et al.* (2007) and Boehmer *et al.* (2010). Smolenski *et al.* (2007) have identified it as one of the minor milk protein that present in all peak lactation, colostrums and mastitic whey and MFGM whilst Boehmer *et al.* (2010) considered it as one of the host defence proteins. Again, it was also surprising to observe this minor milk protein which acts in the host defence mechanism to appear in the control group in higher frequency compared with the infected group.

The fibroblast growth factor-binding protein (FGFBP) is a secreted carrier protein that releases fibroblast growth factors (FGFs) from the extracellular matrix storage and thus enhancing the FGF activity (Xie *et al.* 2006). Kawakami *et al.* (2006) concluded that FGFBP may be tightly associated with lactoferrin in bovine milk. Plath *et al.* (1998) successfully examined the expression and localization of FGFBP in the bovine mammary gland and it may be important in the local regulation of the bovine mammary gland.

Peptides from SAA (protein ID 4865) were the only discriminatory peptides that were sequenced that were significantly that can be used to differentiate *E. coli* infected group from *S. aureus* infected group. Serum amyloid A protein was one of the important acute phase protein in bovine mastitis and it can be produced locally in the mammary gland making it a more reliable indicator of bovine mastitis (Eckersall *et al.* 2001, 2006) but not a single previous study that able to determine if SAA can significantly discriminate *E. coli* from *S. aureus* infected milk samples.

No other identified peptides could be used to discriminate *S. aureus* infected group from *E. coli* infected group. Perhaps the use of sample size calculation is needed so that more polypeptides can be sequenced that are able to significantly discriminate between these two groups.

Although several proteins were successfully identified as the precursor of the peptides using the protein database based on their sequences, those proteins cannot be used per se as the potential markers in each group. The reason is that, it is the peptides of specific fragments of the proteins identified as biomarkers. The protein identity only provided with an insight into which protein were affected and played role in the disease mechanism. It is possible that specific peptides resulted from proteases of the cow rather than bacterial proteases. Thus, no ultimate conclusion can be made based on the protein identity that has been identified in each of the group.

The results discussed above can provide an initial insight on how the milk peptide profiles can change as result of bovine mastitis. However, these results can be improved by applying further improvements. First, validation of SVM models is needed to demonstrate that these milk peptidomes really are able to differentiate healthy cows from mastitic cows with different bacterial-causing pathogens. More milk samples without knowing their infection status (blinded samples) should be tested to validate these models. By validating these models, the status of each marker in each group (control, infected, *E. coli* or *S. aureus* infected milk groups) can be confirmed and may eventually be used to determine the presence of bovine mastitis in the cows or even to predict the type of bacterial pathogen that lead to inflammation of the mammary gland.

Second, it was not possible with 100% certainty to correlate the results from the CE-MS with the results from the LC-MS/MS data based on peptide masses, this situation can lead to mass ambiguities due to the highly complex peptide mixtures involved which complicates the assignment of sequences obtained from different separation methods and also due to the fact that the mass spectrometers used after CE-MS and LC-MS/MS were different. Therefore, further work should be conducted in order to resolve this ambiguity issue by running samples with internal standards of known mass in both systems. In this way, an internal mass calibration of each sample can be done that would allow a more correct cross-correlation of results between both systems.

It would also be interesting to propose a time-course of infection study for future work and determine the earliest time that can be applied to diagnose an infected cow based on the proposed polypeptide biomarker of bovine mastitis and if the putative biomarkers would be able to discriminate subclinical mastitis from clinical mastitis and healthy group. More information on peptide sequencing can lead to a better understanding of the

pathophysiology of bovine mastitis and eventually could help in improving the bovine mastitis detection.

Chapter V: Metabolomics Analysis in Bovine Mastitis

5.1 General Metabolomics

Being one of the most newly emerged "-omics" sciences, metabolomics is becoming one of the most significant field of study in system biology which complements the other "-omics" and proving to have unique advantages. It was first proposed by Oliver (1998), and seeks to characterize and quantify the whole collection of both intra and extra-cellular metabolites. The metabolome is defined as the qualitative and quantitative collection of all low molecular weight of molecules that are present in biological fluid, organism or cell under a different set of physiological conditions (Tyagi *et al.* 2010; Banderas *et al.* 2011). These metabolites include a diverse range of endogenous and exogenous chemical molecules such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids and any other chemical that is used, ingested or synthesized by a given cell or organism (Wishart 2008). Some of the metabolites are acquired externally but most are products or intermediates from metabolic reactions (Tomita and Nishioka 2005).

Metabolomic analyses involve two basic methodologies (Nielsen and Jewett 2007). First is metabolite profiling which allows qualitative screening of a large number of metabolites (usually more than 100) and the pattern of metabolites produced via high-throughput detection can be used to find discriminatory elements followed by data deconvolution methods. This method covers metabolic fingerprinting (analysis of endometabolome) and metabolic footprinting (analysis of exometabolome). According to Álvarez-Sáchez *et al.* (2010), global metabolomics profiling involved detection of metabolites by using a single analytical platform or a combination of complementary analytical platforms (GC-MS, LC-MS, CE-MS or NMR). The second method is target analysis which aims to produce absolute or semi-quantitative detection of metabolites. Previously, only small numbers of metabolites were able to be analyzed but with new development on quantitative analysis, more expanded metabolite coverage can be performed.

However, the comprehensive analysis of a wide range of metabolites is only possible with the use of recent technological advancements in separating and identifying these molecules (Wishart 2008). The most widespread analytical platforms include robust, high-resolution mass spectrometry (MS) instruments, nuclear magnetic resonance (NMR) spectrometers,

capillary electrophoresis (CE) and ultra-high pressure liquid chromatography (UPLC and HPLC). Many investigations have used an integration of various analytical methods such as gas chromatography combined with mass spectrometry (GC-MS) and liquid chromatography combined with mass spectrometry (LC-MS) to improve metabolite identification and coverage (Álvarez-Sáchez *et al.* 2010).

There are many advantages to studying the metabolome in comparison with transcriptome and proteome. According to Dunn & Ellis (2005) and Álvarez-Sáchez (2010) metabolomics is often seen more useful because the number of metabolites is smaller than the number of proteins and genes in a cell which reduces the sample complexity. Furthermore, the concentration of metabolites can significantly change during biochemical reaction even though the concentration of enzyme and metabolic flux may not be significantly altered (Kell and Mendes 1999). As the downstream product of gene expression, the metabolome reflects the functional level of the cell more appropriately and is thought to be amplified in physiological changes as compared to proteome and transcriptome. Furthermore, metabolic fluxes are more influenced by environmental stress than gene expression, making the measurement of metabolites more appropriate. Finally, metabolic profiling is less expensive than proteomic and transcriptomic experiments.

Despite having many benefits for the analysis of smaller molecules, one major problem in metabolomics is the lack of standardized methods especially in the analysis of global metabolomics (Álvarez-Sáchez *et al.* 2010). A standard metabolomic experimental approach is needed from sample collection through chemical analysis to data processing.

Metabolomics has been applied to various investigations such as determining the metabolic biomarkers that act as indicators to the presence of a disease or as a response towards drug-based intervention. Other than that, various metabolomic studies have been performed on plants and microbes (Le Gall *et al.* 2003) and on bacterial characterisation (Vaidyanathan *et al.* 2002). For the past few years, metabolomic analyses has gained an interest in food and nutrition fields to give a more comprehensive molecular picture on food composition as well as food consumption.(Wishart 2008).

5.1.1 Metabolites in milk and serum found during bovine mastitis

Metabolomics provide a very important tool to analyse changes in milk metabolome during the presence of bovine mastitis because of its rapid and high throughput characterization of small molecule metabolites found in milk or serum.

There have been many studies on the metabolites in milk and serum in relation to mastitis from the era when metabolism study used traditional methodologies. An early study by Peter *et al.* (1990) investigated the metabolites of prostanoids in milk of heifers after intramammary infusion of *E. coli* organisms. Prostanoids are known to be important mediators in the inflammatory processes associated with endotoxin-induced mastitis. Increases of arichidonic acid metabolites such as thromboxane A2, prostaglandin E2 (PGE2) as well as milk prostacyclin (PCM) were seen in milk from infected quarters. The increased concentrations of these metabolites showed that they have an important role in the pathophysiologic process of coliform mastitis released as the mediators during inflammation.

The presence of change in metabolites during mastitis is also associated with other factor such as dietary selenium (Se). A study by Maddox *et al.* (1991) showed that cows with Se deficiency (-Se) contained significantly higher concentrations of PGE2, 6-keto-prostaglandin F1α and leukotriene B 4 (LTB4) in milk after *E. coli* inoculation as compared with milk from cows with Se sufficiency (+Se). The higher eicosanoid concentration in milk was associated with higher number of bacteria. This is may be due to impaired killing abilities of selenium deficiency polymorphonuclear leukocytes (PMNL) as during non-specific immune response such as in coliform mastitis, the function and activity of PMNL are highly influenced by eicosanoids. Greater concentration of these eicosanoid levels in milk of –Se cows is due to the increase of substrate availability and enzyme activity from the activation of cyclooxygenase and lipoxygenase enzymes in the Se-glutathione peroxidase (Se-GSH-Px) depleted state. The severity of clinical signs seen in –Se cows could be at least partially caused by the higher eicosanoid concentrations in the milk as they have play an important role in the pathogenesis of inflammatory and endotoxin-induced diseases.

Serum trace minerals were also studied (Erskine and Bartlett 1993) during *E. coli*-induced mastitis in which a transient depression in both plasma zinc (Zn) and iron (Fe) was seen during intramammary challenge with *E. coli*. High number of bacteria (*E. coli*) secreted in milk over time was correlated with the severity of clinical signs of infection as well as with decreased plasma Zn and Fe concentrations. These divalent cations are important in the growth of gram-negative bacteria such as *E. coli* and reduced concentrations of both Zn and Fe may be part of defence mechanism to limit the bacterial growth. As for copper (Cu) concentration, this study did not show any increase during the inflammatory process but in fact, it decreased to 75% of pre-challenge levels 24 hours post inoculation. This is not in

accordance with the previous study by Conner *et al.* (1986) in which they found increased ceruloplasmin during the acute phase response in cattle as well as studies by Barber *et al.* (1988) and Etzel *et al.* (1982) where they found increased in serum ceruloplasmin in both rats and hamster. Ceruloplasmin is the Cu-binding protein which plays a role as a scavenger for superoxide anion radicals. The difference might be due to species differences.

Gram negative mastitis in early lactation may also influenced the ovarian and certain endocrine functions and metabolism in dairy cows. Huszenicza *et al.* (1998) found that during lipopolysaccharide (LPS) loading, various events occurred that might impair the ovarian functions such as delay in the resumption of cyclicity or even causing premature luteolysis if sensitive corpus luteum is present simultaneously on the ovary caused by prostaglandin F2α. However, based on individual progesterone (P4) profiles in the blood of the affected cows, gram negative mastitis influenced the ovarian function during the 3rd up to 10th weeks of lactation. During early puerperal (<14 days) mastitis, its prevalence was not influenced by the concentration of acetoacetone, β- OH butyrate (BHB) or non-esterified fatty acids. Cows affected with different forms of early puerperal mastitis did not show any delay in the onset of cyclicity either.

Interesting metabolites that exist in the milk are the reactive oxygen metabolites that include superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and fatty acid radicals (Lindmark-Månsson and Åkesson 2000). These reactive metabolites are released by PMN after phagocytising foreign particles or bacteria and discharging their granules' contents. Although they are harmful to bacteria, these oxidants are known to be cytotoxic and can induce tissue damage by destroying host cells. To control these reactive oxygen radicals, anti-oxidants are also present in the milk during the inflammatory processes which include natural vitamins (vitamin E and C), beta-carotene and enzymatic systems mainly represented by superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx). A study by Hamed *et al.* (2008) investigated the level of these anti-oxidants in association with the bulk milk somatic cell counts (BMSCC). Because oxidants released by PMN induce cellular damage (Weiss 1989; Van Asbeck 1990) and antioxidants are needed to control these reactive oxygen metabolites, the relationship between milk somatic cells and antioxidant activities was studied. SOD activity was found to be unaffected by BMSCC. SOD activity was weakly dependent upon BMSCC and suggested that SOD incorporation did not prevent the cytotoxic effect of the oxygenated radicals and pointed out the importance of other anti-oxidants. As for the catalase, it showed increased

activity in high BMSCC samples probably playing a central role in milk redox control especially during mastitis. It also could be used as an indicator in mastitis. This study also showed a positive correlation between GHSPx activity with the BMSCC especially with the neutrophil content but its role in bovine milk was thought to be limited. However, it was concluded that catalase and GSHPx are the principal enzymatic defence that neutralize the harmful free radicals.

Metabolic activity is able to influence the health of the udder. Near parturition and in the period of early lactation, there is a large nutrient demand in which nutrients are mobilized from tissue reserves. This results in changes in blood concentration of both hormones and metabolites (Ingvartsen and Friggens, 2005). In general, primiparous cows have a greater prevalence of mastitis as several important immune factors are impaired around calving. Thus, one study by Nyman *et al.* (2008) revealed that blood concentration of *β*-hydroxybutyrate (BHBA), nonesterified fatty acids (NEFA), glucose and α-tocopherol around calving were associated with the SCC at first test-milking of primiparous cows. Immune variables such as the collectins which consist of collectin-43 (CL-43) and conglutinin as well as haptoglobin (Hp) were also seen to be correlated with Box-Cox transformed SCC (bcSCC) and these correlations must be based from a direct breed effect. It is also known that age and breed factors were significantly associated with several metabolites and immunological variables. For example, heifers calving at an older age had greater concentrations of serum BHBA and NEFA but lower glucose, insulin and urea nitrogen values compared with heifers calving at a younger age (Nyman *et al.* 2008). This has shown that older heifers were in more severe negative energy balance in the period around calving and in early lactation than younger heifers.

Another study that investigated various metabolites in serum and their variation during the transition period in dairy cows was performed by Moyes *et al.* (2009). This study is in agreement with Nyman *et al.* (2008) in which there was an increased NEFA concentration in cows that developed clinical mastitis at calving and during early lactation. Thus, higher circulating NEFA was shown to inhibit immune responses that lead to the development of mastitis especially during early lactation. Clinical mastitic cows also showed higher circulating BHBA concentrations during the transition period. This result was in contrast with that of Nyman *et al.* (2008) in which high BHBA concentrations were associated with lower bcSCC at the first test-milking during early lactation. According to Nyman *et al.* (2008), this increase may be associated with microbial butyrate production from rumen carbohydrate fermentation, which is then converted to BHBA in the rumen epithelium. As

for glucose levels, the results were inconclusive. Higher glucose levels were seen during pre-partum period in cows that developed clinical mastitis as compared to cows that did not developed mastitis during early lactation period. This can be explained by the lack of difference in insulin concentration in clinical mastitic cows and this might suggested a lower efficiency of glucose clearance from circulation for these cows compared with subclinical mastitic and healthy cows. This study also suggested that aspartate aminotransferase (ASAT) has no association with the development of clinical and subclinical mastitis during the first week of lactation making it a less useful marker to evaluate the risk of mastitis due to low specificity of ASAT in lactation. Overall, NEFA was the most consistent with least conflicting positive association with the development of mastitis and making it the most useful marker for the risk of mastitis during early lactation. The use of metabolites as the risk factors of mastitis is promising but further investigations are needed.

Melendez *et al.* (2009) studied a relationship between NEFA at calving with the incidence of many periparturient diseases including mastitis in Holstein dairy cattle. The authors agreed that increased in NEFA concentrations in serum have been associated with impaired immunity which predisposed cows to periparturient diseases. Thus the NEFA values at calving can be used to monitor the nutritional management as well as to be used as indicators in the risk factors in many periparturient diseases including mastitis.

Metabolites can also be produced by the mastitis-causing pathogens. These metabolites have been used to determine the pathogen. This is based on the fact that all microorganisms produced their own range of often volatile metabolites. Hettinga *et al.* (2008) identified a wide range of volatile metabolites that were produced by different types of microorganisms using a solid-phase microextraction (SPME) coupled to gas chromatography/mass spectrometry (GC/MS). In this study, they found that volatile metabolites found in milk from uninfected quarters differed significantly from those found in milk from infected quarters. Lower numbers and amounts of metabolites were found in the samples from uninfected quarters compared to the samples from mastitis quarters. For example, *S. aureus* and *E. coli* both formed a large amount of ethyl acetate and acetic acid. It was confirmed that different microorganisms produced different types of metabolites which means that the presence of certain volatile metabolites in infected milk samples could be used to detect and identify mastitis-causing pathogens.

A further study on volatile metabolites in mastitis milk samples by the same authors investigated the origin of these components (Hettinga *et al.* 2009). Although it is known

that pathogens are responsible to the presence of volatile metabolites in mastitic milk samples, some of these detected metabolites may also be formed by enzymes, present in the blood of the animals but which travel into the milk due to the disruption of blood-milk barrier during inflammatory process. Around 20% of metabolites identified in this study were found to be different between inoculated milk (milk from healthy animals inoculated with the same bacteria) and mastitic milk samples. Nevertheless, most of the volatile metabolites detected in mastitis samples are mainly formed by the pathogens except for ethyl esters of free fatty acids (FFA) which were absent or significantly lower in inoculated samples. These metabolites are formed by the esterase enzymes found in cow's blood. During mastitis, due to increase leaking of the barrier between blood and udder, esterase enzymes can be found in the mastitic milk (Raulo *et al.* 2002). Thus, during mastitis, this enzyme can probably transfer from the blood to the milk due to disruption of the blood-milk barrier. Furthermore, dimethyl sulphide may be useful for *E. coli* identification as it was found that dimethyl sulphide concentrations is increased in samples inoculated with *E. coli.*

Another type of metabolite which is seen to have an effect in milk production are the metabolites of nitric oxide (NO). Bouchard *et al.* (1999) have investigated the nitric oxide production during endotoxin-LPS induced bovine mastitis. In this study, a significant three- to five-fold of increase in nitrate and nirite contents (metabolites of nitric oxide) were formed in milk samples upon LPS infusion. Nitric oxide is produced from L-arginine by the enzymes termed as nitric oxide synthase (NOS). Inducible isoforms of NOS (iNOS) are also thought to be cause overproduction of nitric oxide. It is known that somatic cells are responsible for the production of nitric oxide although in this study, it did not establish of which cell type(s) that is (are) involved. They have also studied the effect of co-infusion of LPS with an inhibitor of iNOS; aminoguanidine which successfully prevented the nitric oxide production upon LPS infusion. These results confirmed the activity of iNOS during the endotoxin-induced inflammatory reaction.

During lactation, nitric oxide synthase plays a role in generating nitric oxide contained in the mammary gland tissues (Lacasse *et al.* 1996). Nitrate and nitrite are the end products of nitric oxide oxidation in aqueous solutions (Moncada *et al.* 1991). Milk also contains S-nitrosothiols (RSNO) which are also metabolites of short-lived NO. Because milk contains lactoperoxidase, nitrite can be oxidized to toxic nitrogen dioxide (Silanikove *et al.* 2005). A study by Titov *et al.* (2010) revealed by using an enzyme sensor method that in healthy milk and colostrums, no nitrite compounds were found. Thus, its presence in milk can be

an indicator of mastitis. However, the occurrence of these compounds was accompanied with a lower content of RSNO which possibly occurs under the effect of reactive oxygen species. Nitrite also can be produced by leukocytes. As said earlier, the oxidation of nitrite by lactoperoxidase can lead to the production of toxic nitrogen dioxide, a powerful oxidant that could cause the oxidative breakdown of milk lipoprotein. To avoid this toxic effect, the presence of high concentrations of other compounds that are able to compete with nitrite for lactoperoxidase is important (thiocyanate and ascorbate).

5.1.2 Aims of Study

In conclusion, the aim of this study is to apply metabolomic study by the use of LC-MS/MS analysis to study the presence of important metabolites that can be used to discriminate normal milk samples from milk samples from quarters infected with bacteria causing bovine mastitis. It was hoped that by using this sensitive and high-throughput methodology, significant results could be achieved and lead to the discovery of important and significant mastitis biomarkers and that differences may be found in samples of milk from infected quarters caused by different pathogens.

5.2 Materials and Methods

5.2.1 Animals and Samples

Milk samples from clinical mastitic cases were sent from Vale Veterinary Laboratory, Devon, UK and bacteriological culture was performed in the Bacteriology Laboratory of Vale Veterinary Laboratory to determine the bacterial cause of bovine mastitis. Milk samples from mastitis caused with either *E. coli* (n=10) or *S. aureus* (n=6) infection were collected for further analysis. Control milk samples (n=10) from healthy cows from the Cochno Farm of School of Veterinary Medicine, University of Glasgow were also used in this study as comparison to those clinical mastitic cases. The healthy milk samples were confirmed by somatic cell counts (<100 000 cells/ml). Prior to metabolomic analysis, SAA and Hp assays (Chapter 2) were performed determine the concentration of both acute phase proteins in each milk sample for further confirmation of disease status. Samples were stored at -20°C prior to analysis.

5.2.2 Chloroform and methanol Extraction

5.2.2.1 Reagent preparation

A mixture (1:3) of chloroform: methanol was prepared by adding 20ml of chloroform into 60ml methanol.

5.2.2.2 Extraction method

An aliquot of 500µl of each milk sample was added to 2ml of 1:3 chloroform: methanol solution and mixed vigorously in the cold room at 4ºC for 2 hours. Next, the sample was centrifuged at 13 000 x g for 3 minutes at 4ºC. The supernatant was aspirated and was then stored at -80ºC until analysis by LC-MS.

5.2.3 LC-MS Analysis

LC/MS was carried out using a Dionex UltiMate 1 coupled to an Orbitrap Exactive. The separation of metabolites was performed using a 4.6 x 150mm ZIC-HILIC column at 300µl/min. The gradient ran from 80% acetonitrile (ACN) to 20% ACN in 30 minutes, with a step to 5% ACN for 9 minutes. Re-equilibration was then performed for 6 minutes at 80% ACN. 10µl of each sample was used per run. The MS acquisition was performed in both negative and positive ionisation modes with full scan modes. The MS was set at 50,000 resolution with the scan range from 70-1400 amu. The LC-MS analysis was performed under supervision of Dr. Karl Burgess of Sir Henry Wellcome Functional Genomic Functional, University of Glasgow, UK.

5.2.4 Data Acquisition

The LC/MS data was analyzed using MzMine software (version 2.0) (http://mzmine.sourceforge.net/index.shtml; Finland and Japan). For the peak detection, a noise threshold of 5000 and m/z tolerance of 0.0005 Da was performed. An alignment with a mass tolerance of 0.0005 and a retention time tolerance was 15 seconds.

5.2.5 Analysis of Individual Metabolites

Output from the LC/MS for both positive and negative modes was obtained as spreadsheet of mass-to-charge ratio peaks for each sample and with m/z values providing molecular formula by comparison to metabolite database and a putative identification was based on the formula. Not all m/z peaks gave identified metabolites. For each peak, the mean and SD of the m/z intensities for all samples in the group (control; *E. coli*; *S. aureus*) were automatically determined. Inspection of the results and manipulation of the spreadsheet allowed analysis in a variety of criteria.

For both positive and negative modes, the metabolites were sorted to identify those with the 20 metabolites with highest m/z intensities in each of the 3 groups.

Numerous metabolites were not present in the control group but were present in either or both the *E. coli* and *S. aureus* groups. These were identified and the 20 metabolites with largest difference in mean m/z intensities determined by substraction.

Numerous metabolites had large difference between the mean of control group and the mean of either *E. coli* or *S. aureus* groups. They were determined by calculation of the total increase or decrease by dividing the mean of the m/z intensities of the infected groups by the mean of the control group and the 15 metabolites with greatest fold increase or decrease were identified.

Metabolites showing significant difference between the *E. coli* and *S. aureus* groups were identified by calculation of the fold change by dividing the median of the m/z intensities of the *S. aureus* group by the median m/z intensities of the *E. coli* group. The 15 metabolites with the highest fold change between groups increased in either *S. aureus* or in *E. coli* were identified and the Mann Whitney test for significant differences applied for non-parametric data. The metabolites were listed in order of increasing probability of the significance of a difference (P-value).

5.2.6 Metabolites Analysis – Pathway Analysis

Bioinformatic analysis of the metabolomics data performed using tools provided by the Scottish Metabolomics Facility. An in-house web facility, known as PATHOS (http://motif.gla.ac.uk/Pathos/index.html), has the ability to display metabolites identified based on the metabolic pathways in which they occur. By listing areas of metabolism and by generating a Kyoto Encyclopaedia of Genes and Genomes (KEGG) map, the data of experimental m/z values or refined lists of metabolites generated by the MS data by other means was analysed. PATHOS also provides quantitative data for the metabolites identified and these changes are indicated by colour-coding on KEGG map.

For preliminary analyses, changes in metabolites were grouped based on different metabolism; lipid, protein and carbohydrate metabolisms. In each group, the analysis was focussed on six metabolic pathways showing most changes for both positive and negative ion modes.

5.3 Results

5.3.1 SCC measurement and acute phase proteins concentration

In order to confirm the disease status of milk samples used in this study, SCC measurement and acute phase proteins (SAA and Hp) concentrations were determined. Table 5-1 showed the SCC measurement and APP concentrations in all control milk samples used in this study. The results were as the same as in Chapter 4, Section 4.3.1. Table 5-2 (a and b) showed the bacteriological culture results and the concentration of both SAA and Hp for mastitic milk samples caused by *E. coli* and *S. aureus*. The results were also as the same as in Chapter 4, Section 4.3.1. The Statistical analysis (Mann Whitney) showed that both SAA and Hp were significantly higher ($p=0.00$; $p<0.05$) in the infected group (*E. coli* and *S. aureus*) but not significantly different between two bacterial groups ($p=0.958$; $p>0.05$ for SAA and $p=0.428$; $p>0.05$ for Hp).

Control milk samples

Sample Ids	SAA Conc.(ng/ml)	Hp conc. (ng/ml)	SCC (x10^3)
102	0.36	7.78	13
66	0.57	16.38	14
3	0.55	27.98	48
62	0.079	1.95	31
72	1.21	7.24	96
109	0.65	13.21	10
80	0.28	24.61	54
100	0.48	15.36	38
16	0.9	2.86	58
58	0.34	1.95	56
111	0.13	21.94	28
48	0.39	31.95	85
4	2.96	4.17	63
2	1.43	1.95	32
29	0.29	12.64	46
Min	**0.08**	**1.95**	**10**
Max	**2.96**	**31.95**	**96**
Mean	**0.71**	**12.8**	**45**
Median	**0.48**	**12.64**	**46**

Table 5-1: The acute phase proteins (SAA and Hp) concentrations and SCC measurement in all control milk samples used in this study. The range of SAA concentrations was between 0.08 and 2.96 μg/ml with an average of 0.71μg/ml and a median of 0.48μg/ml. The range of Hp concentrations was between 1.95 and 32μg/ml with an average of 12.8μg/ml and a median of 12.64μg/ml. The range of SCC measurement was between 10 and 96 x 10^3 cells/ml with an average of 45 x 10^3 cells/ml and a median of 46 x 10^3 cells/ml.

Mastitic milk samples

Sample Ids	SAA Conc. (ng/ml)	Hp conc. (ng/ml)	Bacteriological results
475	72.13	89.68	*E. coli*
76	1500	115.18	*E. coli*
?	1500	78.51	*E. coli*
814	1500	141.26	*E. coli*
692	9.48	60.27	*E. coli*
697	1500	144.89	*E. coli*
519	109.44	108.02	*E. coli*
453	4.18	16.63	*E. coli*
496	24.31	16.03	*E. coli*
1222	46.02	354.26	*E. coli*
Min	**4.18**	**16.03**	
Max	**1500**	**354.26**	
Mean	**626.56**	**112.47**	
Median	**90.79**	**98.85**	

Table 5-2a: The acute phase proteins (SAA and Hp) concentrations and bacterial causing mastitis in infected milk samples caused by *E. coli* used in this study. The range of SAA concentrations was between 4.18 and 1500µg/ml with an average 626.6µg/ml and a median of 90.79µg/ml. The range of Hp concentrations was between 16.03 and 354.26µg/ml with an average of 112.5µg/ml and a median of 98.85µg/ml.

Mastitic milk samples

Sample Ids	SAA Conc. (ng/ml)	Hp conc. (ng/ml)	Bacteriological results
438	48.12	70.77	*S. aureus*
14	37.21	42.69	*S. aureus*
980	1500	111.83	*S. aureus*
708	54.96	95	*S. aureus*
346	1500	86.2	*S. aureus*
715	15.99	22.15	*S. aureus*
Min	**15.99**	**22.15**	
Max	**1500**	**111.83**	
Mean	**526.05**	**71.44**	
Median	**51.54**	**78.49**	

Table 5-2b: The acute phase proteins (SAA and Hp) concentrations and bacterial causing mastitis in infected milk samples caused by *S. aureus* used in this study. The range of SAA concentrations was between 15.99 and 1500µg/ml with an average of 526.1µg/ml and a median of 51.54µg/ml. The range of Hp concentrations was between 22.15 and 111.8µg/ml with an average of 71.44µg/ml and a median of 78.49µg/ml.

5.3.2 Metabolites found in milk

Upon LC-MS analysis, the identified metabolites were analyzed by MzMine software, the data was presented based on the individual metabolites found according to their m/z values, retention times, numbers of detected peaks, molecular names and their abundances in each of the milk samples. The data was divided into positive and negative ionisation modes based on the MS acquisition. A total of 9904 were detected in the milk samples using positive mode detection of which 814 were identified using the MzMine software. For the negative ion detection the equivalent figures were a total of 5551 metabolites detected with 512 being identified. Numerous metabolites were present with both ion detection systems and inspection of the data showed that 221 metabolites were in both the positive and negative mode so that a total 1105 metabolites were detected across the milk samples analysed (Table 5-3). The mean values of m/z intensities of metabolites in each group were then determined. The full excel files with the identified and non-identified metabolites are presented in the supplementary information. The following examination of the metabolites and their variation between milk from healthy cows and that from udder quarters with mastitis caused by *E. coli* or *S. aureus* are focused on the metabolites that were identified in the samples by LC-MS-MS and the MzMine software programme.

With the large data set generated by the study, the results were examined by scrutiny of the individual metabolites and their variation in mean m/z intensity between control and infected groups. In order to reduce the complexity of the interpretation, the metabolites are discussed in relation to the m/z intensity and the mean of the peaks obtained with the discussion focused on the metabolites with the highest intensity or on those with the greatest relative value between bacterially infected groups. Thereafter the major possible metabolic pathway alterations that produce the identified changes in metabolites are described.

	Positive Mode	Negative Mode	Total of metabolites
Number of metabolites	9904	5551	15455
Number of identified metabolites	814	512	1326
Number of unidentified metabolites	9090	5039	14129
Number of common identified metabolites			221
Number of individual identified metabolites			1105

Table 5-3: The total number of metabolites, identified and unidentified in both positive and negative ionisation modes. The number of common and individual identified metabolites was also presented. A total of 15 455 metabolites were identified from the LC-MS/MS analysis. However, only 1326 metabolites were successfully identified by the MzMine software. About 221 of common metabolites and 1105 individual metabolites were identified from this software.

5.3.3 Effect of mastitis on the metabolites of milk

The first 20 metabolites with highest m/z intensities in each three groups of milk samples; control, *E. coli* and *S. aureus* groups were tabulated in each positive and negative ionisation modes as presented in Table 5-4 and 5-5. From the positive mode MS, it was found that the metabolites with greatest intensity differed between the groups although most of the highest metabolites in the control were still within the top group. The 3 metabolites with highest intensity in the control group (Table 5-6) were L-acetylcarnitine, choline and sn-glycero-3-phosphocholine. The fourth metabolite was lactose, the primary carbohydrate of milk. However, the Pathos analysis programme identified this as 1-alpha-D-Galactosyl-myo-inositol which has an identical molecular formula of C12H22O11. On tables, the form given is maintained with lactose identified in bracket. In the group with *E. coli* infection (Table 5-7) choline was the metabolite with highest m/z intensity but L-carnitine and the amino acid, L-valine were also at the top of the metabolites detected while lactose had the fourth highest intensity. In the group with *S. aureus* infection (Table 5-8), L-valine had the highest intensity and it was noticeable that 11 of the top 20 were amino acids, di-peptides or tri-peptides, compared to 6 in the *E. coli* group and only one, glutamate in the milk samples from the control group. Lactose was detected in the *S. aureus* group but was tenth of the twenty metabolites with the highest m/z intensity.

With the negative ion detection, all three groups had the same metabolites with the two highest m/z intensities, being citrate (though incorrectly identified as 5-dehydro-4deoxy-D-glucarate) and lactose (Tables 5-9 to 5-11), which reflect their high concentration in milk. In the samples for both the *E. coli* and *S. aureus* infections the third highest m/z intensity was lactate, which was not in the top 20 in the control group and its presence in the samples from mastitic milk would indicate the alteration in metabolism caused by the bacteria. Also noticeable was that that glucose/galactose (identified as myo-inositol) was detectable in milk from the control group and that from the *E. coli* infected but not the *S. aureus* infected groups. It was noticeable that the metabolites in the top 20 of the negative ion detection were mostly small molecular weight acidic metabolites while those in the positive ion detection had a higher proportion of basic metabolites.

Metabolites which were absent in control milk samples but present in both infected groups (*E. coli* and *S. aureus*) were examined in both positive and negative ion modes. Twenty metabolites with the largest difference between intensities in *E. coli* and *S. aureus* infected groups were tabulated in Table 5-12, 5-13, 5-14, 5-15. From the positive mode analysis, of the 20 metabolites which were higher in the *S. aureus* group than *E. coli* 18 were

tripeptides, with Leu-Val-Ser being the metabolite with greatest difference from that in the *E. coli* group (Table 5-12). In contrast ten different tripeptides had higher intensities in the samples from the *E. coli* group compared to the *S. aureus* group (Table 5-13). However also detected in this group were other metabolites such as calligonine, harmolol (both are alkaloids) and lincomycin. From the negative mode analysis, of the 20 metabolites which were higher in the *S. aureus* group than *E. coli*, 12 were tripeptides with Leu-Gln-Ser being the metabolites with greatest difference from that in the *E. coli* group (Table 5-14). In contrast, eight different tripeptides had higher intensities in the samples from the *E. coli* group compared to the *S. aureus* group (Table 5-15).

Name	**Control**	**Name**	***E. coli***	**Name**	***S. aureus***
L-Acetylcarnitine	5.3E+08	Choline	6.9E+08	L-Valine	4.7E+08
Choline	4.6E+08	L-Carnitine	3.3E+08	Choline	4.6E+08
sn-glycero-3-Phosphocholine	4.6E+08	L-Valine	3.2E+08	L-Phenylalanine	4.2E+08
1-alpha-D-Galactosyl-myo-inositol (lactose)	4.1E+08	1-alpha-D-Galactosyl-myo-inositol (lactose)	3E+08	L-Acetylcarnitine	3.5E+08
L-Carnitine	4E+08	L-Acetylcarnitine	3E+08	Piperidine	3.2E+08
[FA methyl(4:2/18:2)] methyl 9-butylperoxy-10_12-octadecadienoate	2.3E+08	L-Phenylalanine	2.2E+08	Leucyl-leucine	3E+08
Methyl acetyl ricinoleate	1.9E+08	[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	2.1E+08	L-Carnitine	2.8E+08
Methacholine	1.7E+08	Piperidine	1.9E+08	[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	2.7E+08
L-Glutamate	1.5E+08	[FA methyl(4:2/18:2)] methyl 9-butylperoxy-10_12-octadecadienoate	1.8E+08	L-Proline	2.5E+08
N-Acetyl-D-glucosamine	1.5E+08	Methyl acetyl ricinoleate	1.6E+08	1-alpha-D-Galactosyl-myo-inositol (lactose)	2.5E+08
Glycogen	1.5E+08	Methacholine	1.4E+08	L-Glutamate	2.4E+08
[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	1.4E+08	Glycogen	1.3E+08	sn-glycero-3-Phosphocholine	2.3E+08
1-O-(1Z-Tetradecenyl)-2-(9Z-octadecenoyl)-sn-glycerol	1.2E+08	L-Tyrosine	1.2E+08	L-Tyrosine	2.1E+08
[FA oxo(19:0)] 10-oxo-nonadecanoic acid	1.1E+08	Leu-Tyr	1.1E+08	[FA oxo(19:0)] 10-oxo-nonadecanoic acid	1.6E+08

[FA methoxy_hydroxy(18:2)] 8-methoxy-13-hydroxy-9_11-octadecadienoic acid	1.1E+08	L-Proline	1.1E+08	Leu-Phe	1.5E+08
O-Propanoylcarnitine	9.3E+07	[FA oxo(19:0)] 10-oxo-nonadecanoic acid	1.1E+08	Leu-Tyr	1.5E+08
[FA (17:1/2:0)] 8E-Heptadecenedioic acid	8.8E+07	L-Glutamate	1E+08	Leu-Val-Ser	1.5E+08
Mercaptoethanol	7.6E+07	[FA methoxy_hydroxy(18:2)] 8-methoxy-13-hydroxy-9_11-octadecadienoic acid	9.7E+07	Leu-Leu-Val	1.4E+08
1_3_8-Naphthalenertriol	7E+07	sn-glycero-3-Phosphocholine	8.7E+07	Leu-Thr	1.3E+08
O-Butanoylcarnitine	6.6E+07	[FA (17:1/2:0)] 8E-Heptadecenedioic acid	7.8E+07	Methacholine	1.3E+08

Table 5-4: The first 20 of metabolites with the highest m/z intensity of positive ionisation mode from the total of 814 metabolites identified using the MzMine software in control, *E. coli* infected and *S. aureus* infected groups.

Name	**Control**	**Name**	***E. coli***	**Name**	***S. aureus***
5-Dehydro-4-deoxy-D-glucarate (citrate)	1.8E+09	5-Dehydro-4-deoxy-D-glucarate (citrate)	1.4E+09	5-Dehydro-4-deoxy-D-glucarate (citrate)	1.7E+09
1-alpha-D-Galactosyl-myo-inositol (lactate)	1.5E+09	1-alpha-D-Galactosyl-myo-inositol (lactate)	1.2E+09	1-alpha-D-Galactosyl-myo-inositol (lactose)	1.2E+09
N-Acetyl-D-glucosamine 6-phosphate	2.6E+08	(R)-Lactate	8.1E+08	(R)-Lactate	9.2E+08
cis-Aconitate	1.7E+08	myo-Inositol (glucose/galactose)	1.8E+08	L-Glutamate	1.6E+08
myo-Inositol (glucose/galactose)	1.1E+08	Succinate	1.8E+08	cis-Aconitate	1.2E+08
Hippurate	9.9E+07	cis-Aconitate	1.1E+08	myo-Inositol (glucose/galactose)	1.2E+08
Urate	9E+07	2-C-Methyl-D-erythritol 4-phosphate	9.2E+07	(S)-Malate	1E+08
2_5-Dioxopentanoate	8.4E+07	2-Dehydro-3-deoxy-L-arabinonate	8.6E+07	Urate	9.4E+07
Miserotoxin	7.9E+07	Pyruvate	8.2E+07	2-C-Methyl-D-erythritol 4-phosphate	8.5E+07
2-C-Methyl-D-erythritol 4-phosphate	7.8E+07	Creatine	7.3E+07	L-Erythrulose	7.5E+07
L-Erythrulose	7.5E+07	Hippurate	7.2E+07	N-Acetyl-D-glucosamine	7.4E+07
N-Acetyl-D-glucosamine	7.4E+07	(S)-Malate	7.1E+07	Miserotoxin	7.1E+07
Creatine	7.4E+07	2-C-Methyl-D-erythritol 4-phosphate	6.3E+07	2_5-Dioxopentanoate	6.9E+07
(S)-Malate	7.4E+07	Urate	6.1E+07	Taurine	6.2E+07
L-Glutamate	7.4E+07	L-Glutamate	6E+07	(R)-3-Hydroxybutanoate	6.2E+07
sn-glycero-3-Phospho-1-inositol	6.6E+07	2_5-Dioxopentanoate	6E+07	Pyruvate	5.8E+07
D-Glucose 6-phosphate	5.2E+07	N-Acetyl-D-glucosamine 6-phosphate	5.9E+07	Creatine	5.7E+07
2-Dehydro-3-deoxy-L-arabinonate	4.4E+07	Glycerol 2-phosphate	5.2E+07	Hippurate	5.4E+07
2-Butyne-1_4-diol	4.4E+07	2-Oxoglutarate	4.8E+07	2-Dehydro-3-deoxy-L-arabinonate	4.5E+07
Pantothenate	4.3E+07	(2S)-2-Isopropylmalate	4.6E+07	Mesaconate	4.3E+07

Table 5-5 : The first 20 of metabolites with the highest m/z intensity of negative ionisation mode from the total of 512 metabolites identified using the MzMine software in control, E. *coli* infected and S. *aureus* infected groups.

Controls			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
204.12288	C9H17NO4	L-Acetylcarnitine	532069651.6
104.10717	C5H14NO	Choline	459631082.1
258.10994	C8H21NO6P	sn-glycero-3-Phosphocholine	455789062.4
343.12334	C12H22O11	1-alpha-D-Galactosyl-myo-inositol (lactose)	410172949.4
162.11236	C7H16NO3	L-Carnitine	404013964.3
383.31543	C23H42O4	[FA methyl(4:2/18:2)] methyl 9-butylperoxy-10_12-octadecadienoate	233919160.6
355.28412	C21H38O4	Methyl acetyl ricinoleate	190471829.2
160.1331	C8H18NO2	Methacholine	165776723.1
148.06034	C5H9NO4	L-Glutamate	151091045.9
222.09705	C8H15NO6	N-Acetyl-D-glucosamine	148793168.2
667.22894	C24H42O21	Glycogen	146868998.2
369.35138	C27H44	[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	137976616.2
551.50347	C35H66O4	1-O-(1Z-Tetradecenyl)-2-(9Z-octadecenoyl)-sn-glycerol	121462416.9
313.27353	C19H36O3	[FA oxo(19:0)] 10-oxo-nonadecanoic acid	112684801.4
327.25284	C19H34O4	[FA methoxy_hydroxy(18:2)] 8-methoxy-13-hydroxy-9_11-octadecadienoic acid	108737293.9
218.13858	C10H19NO4	O-Propanoylcarnitine	93016510.12
299.22155	C17H30O4	[FA (17:1/2:0)] 8E-Heptadecenedioic acid	87738450.94
79.02126	C2H6OS	Mercaptoethanol	75530497.29
177.05456	C10H8O3	1_3_8-Naphthalenertriol	69725634.82
232.15421	C11H21NO4	O-Butanoylcarnitine	66011561.24

Table 5-6: The top 20 metabolites by m/z intensity of positive ionisation mode analysis in control group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

E. coli			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
104.1071682	C5H14NO	Choline	688544094.3
162.1123612	C7H16NO3	L-Carnitine	328583661.3
118.0865562	C5H11NO2	L-Valine	323402732.5
343.1233364	C12H22O11	1-alpha-D-Galactosyl-myo-inositol (lactose)	302781524.5
204.1228806	C9H17NO4	L-Acetylcarnitine	301572172.6
166.0861809	C9H11NO2	L-Phenylalanine	220244923.1
369.3513767	C27H44	[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	208831576.3
86.09644175	C5H11N	Piperidine	191708498.1
383.315431	C23H42O4	[FA methyl(4:2/18:2)] methyl 9-butylperoxy-10_12-octadecadienoate	179797044.2
355.2841233	C21H38O4	Methyl acetyl ricinoleate	156590246.4
160.1331034	C8H18NO2	Methacholine	144540343
667.2289361	C24H42O21	Glycogen	125929094.7
182.08111	C9H11NO3	L-Tyrosine	119735739.5
295.16499	C15H22N2O4	Leu-Tyr	113966956.8
116.070917	C5H9NO2	L-Proline	113953650.9
313.2735285	C19H36O3	[FA oxo(19:0)] 10-oxo-nonadecanoic acid	106969595.2
148.0603401	C5H9NO4	L-Glutamate	99580277.45
327.2528422	C19H34O4	[FA methoxy_hydroxy(18:2)] 8-methoxy-13-hydroxy-9_11-octadecadienoic acid	97136487.65
258.1099366	C8H21NO6P	sn-glycero-3-Phosphocholine	86502311.46
299.2215456	C17H30O4	[FA (17:1/2:0)] 8E-Heptadecenedioic acid	77637189.42

Table 5-7: The top 20 metabolites by m/z intensity of positive ionisation mode analysis in *E. coli* group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

S. aureus			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
118.09	C5H11NO2	L-Valine	474193023
104.11	C5H14NO	Choline	459760192
166.09	C9H11NO2	L-Phenylalanine	420899486
204.12	C9H17NO4	L-Acetylcarnitine	353171406
86.10	C5H11N	Piperidine	317818082
245.19	C12H24N2O3	Leucyl-leucine	304379656
162.11	C7H16NO3	L-Carnitine	275815803
369.35	C27H44	[ST] (5Z_7E)-9_10-seco-5_7_10(19)-cholestatriene	266427751
116.07	C5H9NO2	L-Proline	253743805
343.12	C12H22O11	1-alpha-D-Galactosyl-myo-inositol	248665608
148.06	C5H9NO4	L-Glutamate	244795489
258.11	C8H21NO6P	sn-glycero-3-Phosphocholine	231299639
182.08	C9H11NO3	L-Tyrosine	214276582
313.27	C19H36O3	[FA oxo(19:0)] 10-oxo-nonadecanoic acid	161755798
279.17	C15H22N2O3	Leu-Phe	154149870
295.16	C15H22N2O4	Leu-Tyr	153060034
318.20	C14H27N3O5	Leu-Val-Ser	151748149
344.25	C17H33N3O4	Leu-Leu-Val	139384457
233.15	C10H20N2O4	Leu-Thr	132935813
160.13	C8H18NO2	Methacholine	131531174

Table 5-8: The top 20 metabolites by m/z intensity of positive ionisation mode analysis in *S. aureus* group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

Control			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
191.02	C6H8O7	5-Dehydro-4-deoxy-D-glucarate (citrate)	1750330040
341.11	C12H22O11	1-alpha-D-Galactosyl-myo-inositol (lactose)	1513754934
300.05	C8H16NO9P	N-Acetyl-D-glucosamine 6-phosphate	261652267
173.01	C6H6O6	cis-Aconitate	166893330
179.06	C6H12O6	myo-Inositol	106110746
178.05	C9H9NO3	Hippurate	98680533
167.02	C5H4N4O3	Urate	89863389
129.02	C5H6O4	2_5-Dioxopentanoate	84341727
266.09	C9H17NO8	Miserotoxin	78910119
215.03	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	78482788
119.03	C4H8O4	L-Erythrulose	75351299
220.08	C8H15NO6	N-Acetyl-D-glucosamine	74489145
130.06	C4H9N3O2	Creatine	74407132
133.01	C4H6O5	(S)-Malate	73918031
146.05	C5H9NO4	L-Glutamate	73661846
333.06	C9H19O11P	sn-glycero-3-Phospho-1-inositol	66333040
259.02	C6H13O9P	D-Glucose 6-phosphate	52176193
147.03	C5H8O5	2-Dehydro-3-deoxy-L-arabinonate	44090003
85.03	C4H6O2	2-Butyne-1_4-diol	43558519
218.10	C9H17NO5	Pantothenate	43488200

Table 5-9: The top 20 metabolites by m/z intensity of negative ionisation mode analysis in control group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

E. coli			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
191.02	C6H8O7	5-Dehydro-4-deoxy-D-glucarate (citrate)	1375581553
341.11	C12H22O11	1-alpha-D-Galactosyl-myo-inositol (lactose)	1163878111
89.02	C3H6O3	(R)-Lactate	812425519
179.06	C6H12O6	myo-Inositol	182735001
117.02	C4H6O4	Succinate	179764837
173.01	C6H6O6	cis-Aconitate	105428739
215.03	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	91564904
147.03	C5H8O5	2-Dehydro-3-deoxy-L-arabinonate	86395991
87.01	C3H4O3	Pyruvate	81948401
130.06	C4H9N3O2	Creatine	73380634
178.05	C9H9NO3	Hippurate	72131854
133.01	C4H6O5	(S)-Malate	71188741
215.03	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	63327089
167.02	C5H4N4O3	Urate	60860379
146.05	C5H9NO4	L-Glutamate	60367636
129.02	C5H6O4	2_5-Dioxopentanoate	59941285
300.05	C8H16NO9P	N-Acetyl-D-glucosamine 6-phosphate	59306858
171.01	C3H9O6P	Glycerol 2-phosphate	51930002
145.01	C5H6O5	2-Oxoglutarate	47839083
175.06	C7H12O5	(2S)-2-Isopropylmalate	46199319

Table 5-10: The top 20 metabolites by m/z intensity of negative ionisation mode analysis in *E. coli* group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

S. aureus			
row m/z	**Molecular formula**	**Name**	**m/z intensity**
191.02	C6H8O7	5-Dehydro-4-deoxy-D-glucarate (citrate)	1713079641
341.11	C12H22O11	1-alpha-D-Galactosyl-myo-inositol (lactose)	1246746397
89.02	C3H6O3	(R)-Lactate	921868860
146.05	C5H9NO4	L-Glutamate	155669416
173.01	C6H6O6	cis-Aconitate	124042799
179.06	C6H12O6	myo-Inositol (glucose/galactose)	116014627
133.01	C4H6O5	(S)-Malate	100377602
167.02	C5H4N4O3	Urate	94020844
215.03	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	85004997
119.03	C4H8O4	L-Erythrulose	74942006
220.08	C8H15NO6	N-Acetyl-D-glucosamine	73840064
266.09	C9H17NO8	Miserotoxin	70788430
129.02	C5H6O4	2_5-Dioxopentanoate	69476176
124.01	C2H7NO3S	Taurine	62376397
103.04	C4H8O3	(R)-3-Hydroxybutanoate	62310337
87.01	C3H4O3	Pyruvate	58442163
130.06	C4H9N3O2	Creatine	56614851
178.05	C9H9NO3	Hippurate	53708833
147.03	C5H8O5	2-Dehydro-3-deoxy-L-arabinonate	45261272
129.02	C5H6O4	Mesaconate	42718964

Table 5-11: The top 20 metabolites by m/z intensity of negative ionisation mode analysis in *S. aureus* group. The mass-to-charge ratio, formula and the m/z intensity of each named metabolites are presented as well.

row m/z	Molecular formula	Name	m/z intensity (*E. coli;* EC)	m/z intensity (*S. aureus;* SA)	Difference in m/z intensity, SA - EC
318.20	C14H27N3O5	Leu-Val-Ser	7169464	1.52E+08	144578685
392.25	C21H33N3O4	Leu-Leu-Phe	2515770	62511479	59995709
347.19	C14H26N4O6	Leu-Gln-Ser	66451.19	37865683	37799232
378.20	C19H27N3O5	Val-Pro-Tyr	77005.78	20353927	20276921
373.24	C17H32N4O5	Leu-Leu-Gln	68299.4	15413856	15345557
321.14	C11H20N4O7	Gln-Ser-Ser	24894.88	15198151	15173256
253.12	C12H16N2O4	Ala-Tyr	7303190	22111654	14808464
333.21	C14H28N4O5	Lys-Val-Ser	262397	13470687	13208290
496.22	C25H29N5O6	Trp-Gln-Tyr	381281.3	4664604	4283322
300.19	C14H25N3O4	Leu-Ala-Pro	139108.5	3942298	3803190
332.18	C14H25N3O6	Val-Val-Asp	1028657	4747132	3718476
318.17	C13H23N3O6	Leu-Ala-Asp	6637.732	3344439	3337801
423.22	C20H30N4O6	Leu-Gln-Tyr	338654.2	3321623	2982969
361.24	C16H32N4O5	Leu-Lys-Thr	5150.919	2567885	2562734
294.14	C14H19N3O4	Phe-Ala-Gly	1376626	3563815	2187189
409.21	C19H28N4O6	Leu-Asn-Tyr	3618.938	2079692	2076073
333.16	C16H20N4O4	Trp-Ala-Gly	115248	1737862	1622614
303.18	C11H22N6O4	Ala-Gly-Arg	213576.7	1810903	1597326
287.24	C14H30N4O2	N1_N12-Diacetylspermine	3925092	5423238	1498145
290.17	C12H23N3O5	Leu-Ala-Ser	17054.07	944656.8	927603

Table 5-12: Metabolites identified from positive ionisation mode analysis which were absent in control group but found greater in *S. aureus* infected group by means of m/z intensity than *E. coli* infected group. The m/z intensity for each of metabolites identified in both infected groups and the difference in m/z intensity were as presented. The difference in m/z intensity was equal to the m/z intensity of *S. aureus* infected group minus the m/z intensity of *E. coli* infected group

row m/z	Molecular formula	Name	m/z intensity (*E. coli;* EC)	m/z intensity (*S. aureus;* SA)	Difference in m/z intensity, SA - EC
187.12	C12H14N2	Calligonine	7135922	2485802	4650121
376.22	C20H29N3O4	Leu-Phe-Pro	15054435	11700662	3353774
181.07	C7H8N4O2	Theophylline	4655026	1465804	3189222
306.17	C12H23N3O6	Leu-Ser-Ser	1107760	283639	824122
407.22	C18H34N2O6S	Lincomycin	1197998	434791	763207
199.09	C12H10N2O	Harmalol	591018	108776	482242
408.25	C21H33N3O5	Leu-leu-tyr	1228049	841475	386574
350.21	C18H27N3O4	Leu-Phe-Ala	490555	130928	359627
468.34	C23H50NO6P	[PC methyl(14:2)] 2-methyl-3-tetradecyl-sn-glycero-1-phosphocholine	731979	412006	319973
295.18	C19H22N2O	Eburnamonine	597554	286885	310669
334.16	C13H23N3O7	Leu-Asp-Ser	380049	88191	291859
286.18	C13H23N3O4	Leu-Gly-Pro	324809	36711	288099
165.07	C8H8N2O2	Ricinine	243149	4239	238910
458.25	C22H31N7O4	Trp-Pro-Arg	641744	451644	190100
140.03	C6H5NO3	4-Nitrophenol	171175	5689	165486
366.17	C17H23N3O6	Pro-Ser-Tyr	163275	14072	149204
296.12	C13H17N3O5	Asn-Tyr	655710	512128	143582
451.16	C20H26N4O6S	Met-Trp-Asp	126010	1722	124289
393.14	C14H24N4O7S	Met-Asp-Gln	164428	40184	124244
407.22	C18H34N2O6S	Lincomycin	201509	80887	120622

Table 5-13: Metabolites identified from positive ionisation mode analysis which were absent in control group but found greater in *E. coli* infected group by means of m/z intensity than *S. aureus* infected group. The m/z intensity for each of metabolites identified in both infected groups

and the difference in m/z intensity were as presented. The difference in m/z intensity was equal to the m/z intensity of *S. aureus* infected group minus the m/z intensity of *E. coli* infected group.

row m/z	Molecular formula	Name	m/z intensity (*E. coli;* EC)	m/z intensity (*S. aureus;* SA)	Difference in m/z intensity, SA - EC
345.18	C14H26N4O6	Leu-Gln-Ser	46782	18858881	18812099
331.20	C14H28N4O5	Lys-Val-Ser	640021	18012351	17372329
231.14	C10H20N2O4	Leu-Thr	189543	14873123	14683580
390.24	C21H33N3O4	Leu-Leu-Phe	258374	6241107	5982733
359.23	C16H32N4O5	Leu-Lys-Thr	34118	5490747	5456629
244.13	C10H19N3O4	Leu-Asn	145972	5592910	5446938
354.18	C15H25N5O5	Thr-Val-His	337	4768081	4767744
342.17	C15H25N3O6	Leu-Asp-Pro	50023	4469062	4419038
201.12	C9H18N2O3	Leu-Ala	303533	3822614	3519082
247.09	C9H16N2O6	Glu-Thr	618714	3901821	3283106
329.18	C14H26N4O5	Leu-Ala-Gln	11706	3261910	3250204
251.10	C12H16N2O4	Ala-Tyr	1199633	4388952	3189320
373.17	C15H26N4O7	Leu-Asp-Gln	27874	2290843	2262968
217.12	C9H18N2O4	Leu-Ser	1781005	3651567	1870562
311.14	C18H20N2O3	Phe-Phe	1020478	2422191	1401713
364.19	C18H27N3O5	Leu-Ala-Tyr	105095	1312711	1207616
345.21	C15H30N4O5	Leu-Lys-Ser	207570	1389000	1181430
205.08	C7H14N2O5	Thr-Ser	14626	1171204	1156578
350.17	C17H25N3O5	Leu-Gly-Tyr	147416	1294531	1147114
301.16	C11H22N6O4	Ala-Gly-Arg	204285	1220662	1016377

Table 5-14: Metabolites identified from negative ionisation mode analysis which were absent in control group but found greater in *S. aureus* infected group by means of m/z intensity than *E. coli* infected group. The m/z intensity for each of metabolites identified in both infected groups and the difference in m/z intensity were as presented. The difference in m/z intensity was equal to the m/z intensity of *S. aureus* infected group minus the m/z intensity of *E. coli* infected group.

row m/z	Molecular formula	Name	m/z intensity (*E. coli;* EC)	m/z intensity (*S. aureus;* SA)	Difference in m/z intensity, SA - EC
270.15	C12H21N3O4	Val-Gly-Pro	1214191	445816	768374
304.15	C12H23N3O6	Leu-Ser-Ser	399260	61590	337670
197.07	C7H10N4O3	5-Acetylamino-6-amino-3-methyluracil	340796	54974	285821
277.12	C14H18N2O4	Pro-Tyr	520743	281163	239580
374.21	C20H29N3O4	Leu-Phe-Pro	1167418	987379	180040
147.01	C5H8O3S	4-Methylthio-2-oxobutanoic acid	154641	1714	152927
294.11	C13H17N3O5	Asn-Tyr	469728	328468	141260
355.20	C16H28N4O5	Leu-Gln-Pro	160771	42431	118340
281.11	C13H18N2O5	Thr-Tyr	408989	315714	93275
373.21	C16H30N4O6	Leu-Lys-Asp	903396	851149	52247
91.06	C7H8	Toluene	52963	996	51967
221.09	C11H14N2O3	Phe-Gly	95534	44609	50925
344.18	C15H27N3O6	Leu-Val-Asp	47648	16611	31037
217.12	C9H18N2O4	Leu-Ser	33732	11357	22375
395.20	C17H28N6O5	Leu-Gln-His	19559	3891	15667
284.16	C13H23N3O4	Leu-Gly-Pro	28598	17983	10615

Table 5-15: Metabolites identified from negative ionisation mode analysis which were absent in control group but found greater in *E. coli* infected group by means of m/z intensity than *S. aureus* infected group. The m/z intensity for each of metabolites identified in both infected groups and the difference in m/z intensity were as presented. The difference in m/z intensity was equal to the m/z intensity of *S. aureus* infected group minus the m/z intensity of *E. coli* infected group.

5.3.4 Relative changes

In order to identify the metabolites in both modes that showed differences between bacterial infection groups and control group, the median of the m/z intensities were calculated for each metabolite in each group. Fifteen metabolites that were either increased or decreased in both bacterial infection groups from control group in both modes are presented in Table 5-16, 5-17, 5-18 and 5-19. Most of the metabolites that increased in both bacterial infection groups were tripeptides or dipeptides (Val-Pro-Pro, Leu-Leu-Ser, Lys-Ala-Pro, Leu-Ala-Gln, Val-Arg, Leu-Val, etc).

The relative change in intensity for each identified metabolite between the sample groups from the infections was also calculated by dividing the median m/z intensity from the *S. aureus* samples by the median m/z intensity determined for the *E. coli* group. Fifteen metabolites that either increased or decreased when comparing between bacterial infection groups (Table 5-20 and 5-21). As the majority of the group data was non-parametric, the Mann-Witney test used to test for the significance of the difference between the bacterial groups. The tables (5-20 and 5-21) are arranged in increasing value of P as determined by the Mann-Witney test which shows the change observed in m/z intensity for the metabolites with the most significant difference in each of the categories. (Positive mode: significantly increased in *S. aureus* or increased in *E. coli*; Negative mode; significantly increased in *S. aureus* or in *E. coli*.) In positive mode, the p-values increased from 0 to 0.0439 in group of metabolites increased in *E. coli* and the p-values increased from 0.0001 to 0.037 in group of metabolites increased in *S. aureus.* In negative mode, the p-values increased from 0 to 0.0419 in group of metabolites increased in *E. coli* and the p-values increased from 0 to 0.0489 in group of metabolites increased in *S. aureus*.

Reduced in *.E coli*					
row m/z	**Molecular formula**	**Name**	**m/z intensity (Control)**	**m/z intensity (*E. coli*)**	**Fold increase/decrease**
221.09	C11H12N2O3	5-Hydroxy-L-tryptophan	396487.6	0	0
261.06	C14H12O3S	Suprofen	235737.3	0	0
345.18	C14H24N4O6	Thr-Gln-Pro	405720.5	0	0
223.10	C12H14O4	Dillapiole	14979735	23468.75	0.002
177.05	C10H8O3	1_3_8-Naphthalenertriol	69725635	211373.6	0.003
419.32	C26H42O4	Diisononyl phthalate	14710838	45524.96	0.003
75.09	C3H10N2	1_3-Diaminopropane	469562.7	2367.125	0.005
79.02	C2H6OS	Mercaptoethanol	75530497	1880468	0.025
160.10	C7H13NO3	(-)-Betonicine	1187418	31450.85	0.026
126.13	C8H15N	gamma-Coniceine	284401.3	9156.271	0.032
208.10	C11H13NO3	Cantleyine	246641.8	8702.787	0.035
85.10	C6H12	Cyclohexane	1094320	42105.6	0.038
165.09	C10H12O2	Isoeugenol	661742.4	39724.09	0.060
268.10	C10H13N5O4	Deoxyguanosine	7124045	462562	0.065
142.03	C2H8NO4P	Ethanolamine phosphate	505132.3	33286.07	0.066
Increased in *E. coli*					
329.15	C18H20N2O4	Phe-Tyr	71	3960112	55932
283.13	C13H18N2O5	Thr-Tyr	48	1374349	28406
312.19	C15H25N3O4	Val-Pro-Pro	235	3696719	15739
188.18	C9H21N3O	N1-Acetylspermidine	171	2211589	12931
313.15	C18H20N2O3	Phe-Phe	744	8893432	11959

274.19	C11H23N5O3	Val-Arg	855	9403370	10995
332.22	C15H29N3O5	Leu-Leu-Ser	78	753642	9678
315.20	C14H26N4O4	Lys-Ala-Pro	35	304693	8789
249.13	C10H20N2O3S	Met-Val	103	867833	8393
217.12	C9H16N2O4	Thr-Pro	91	651287	7184
264.10	C9H17N3O4S	Ala-Ala-Cys	115	492733	4274
366.20	C18H27N3O5	Leu-Ala-Tyr	227	801312	3524
272.16	C12H21N3O4	Val-Gly-Pro	2911	8509340	2923
175.11	C7H14N2O3	N-Acetylornithine	2353	5549482	2359
262.14	C10H19N3O5	Lys-Asp	25	55845	2267

Table 5-16: A relative change of metabolites in positive ionisation mode analysis in *E. coli* infected group in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups was calculated by dividing the mean m/z intensity from the *E. coli* infected samples by the mean m/z intensity determined for the control group.

Reduced in *S. aureus*					
row m/z	**Molecular formula**	**Name**	**m/z intensity (Control)**	**m/z intensity (*S.aureus*)**	**Fold increase/decrease**
221.09	C11H12N2O3	5-Hydroxy-L-tryptophan	396487.6	0	n/a
261.06	C14H12O3S	Suprofen	235737.3	0	n/a
232.17	C15H21NO	Metazocine	230323.2	0	n/a
142.03	C2H8NO4P	Ethanolamine phosphate	505132.3	0	n/a
212.04	C4H10N3O5P	Phosphocreatine	20806449	0	n/a
152.06	C5H5N5O	8-Hydroxyadenine	7285295	0	n/a
183.09	C6H14O6	Mannitol	257290.9	0	n/a
174.02	C6H7NO3S	2-Aminobenzenesulfonate	6894.951	0	n/a
240.11	C9H13N5O3	Dihydrobiopterin	298827	0	n/a
223.10	C12H14O4	Dillapiole	14979735	25325.15	0.002
268.10	C10H13N5O4	Deoxyguanosine	7124045	17460.44	0.002
75.09	C3H10N2	1_3-Diaminopropane	469562.7	1188.912	0.003
131.13	C5H14N4	Agmatine	238399.8	813.1191	0.003
160.10	C7H13NO3	(-)-Betonicine	1187418	4261.909	0.004
177.05	C10H8O3	1_3_8-Naphthalenertriol	69725635	273655.9	0.004
Increased in *S .aureus*					
329.15	C18H20N2O4	Phe-Tyr	71	6899649	97449
344.18	C15H25N3O6	Leu-Asp-Pro	370	24766005	66964
366.20	C18H27N3O5	Leu-Ala-Tyr	227	8501998	37387

331.20	C14H26N4O5	Leu-Ala-Gln	599	22347998	37289
274.19	C11H23N5O3	Val-Arg	855	30879290	36106
313.15	C18H20N2O3	Phe-Phe	744	25542108	34345
332.22	C15H29N3O5	Leu-Leu-Ser	78	2561617	32895
246.14	C10H19N3O4	Leu-Asn	290	8706973	30007
249.13	C10H20N2O3S	Met-Val	103	2856985	27630
292.13	C14H17N3O4	Trp-Ser	188	4827665	25732
312.19	C15H25N3O4	Val-Pro-Pro	235	5247047	22340
302.21	C14H27N3O4	Leu-Leu-Gly	92	2001246	21736
283.13	C13H18N2O5	Thr-Tyr	48	1023145	21147
401.29	C18H36N6O4	Leu-Leu-Arg	213	3979172	18697
288.20	C12H25N5O3	Leu-Arg	1262	21946148	17391

Table 5-17: A relative change of metabolites in positive ionisation mode analysis in *S. aureus* infected group in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups was calculated by dividing the mean m/z intensity from the *S. aureus* infected samples by the mean m/z intensity determined for the control group.

Reduced in *E. coli*					
row m/z	**Molecular formula**	**Name**	**m/z intensity (Control)**	**m/z intensity (*E. coli*)**	**Fold increase/decrease**
219.01	C7H8O8	4-Carboxy-4-hydroxy-2-oxoadipate	528160	2379	0.005
141.06	C7H10O3	4-Oxocyclohexanecarboxylate	129673	1436	0.011
112.99	C4H2O4	Acetylenedicarboxylate	143871	2380	0.017
275.02	C13H9N2O3Cl	GW 9662	324140	12136	0.037
95.01	C5H4O2	Protoanemonin	59718	2432	0.041
155.03	C7H8O4	2-Hydroxy-6-keto-2_4-heptadienoate	231762	10357	0.045
210.03	C4H10N3O5P	Phosphocreatine	1659260	89840	0.054
373.10	C18H18N2O7	PortulacaxanthinII	27781	1508	0.054
188.99	C6H6O5S	3-Sulfocatechol	88004	5368	0.061
113.02	C5H6O3	2-Hydroxy-2_4-pentadienoate	11126302	756437	0.068
153.02	C7H6O4	3_4-Dihydroxybenzoate	965175	99451	0.103
194.05	C9H9NO4	Salicyluric acid	1409008	163048	0.116
104.04	C3H7NO3	L-Serine	161750	19405	0.120
266.12	C17H17NO2	(-)-Caaverine	87469	10795	0.123
140.01	C2H8NO4P	Ethanolamine phosphate	1079475	135707	0.126
Increased in *E. coli*					
187.11	C8H16N2O3	N6-Acetyl-L-lysine	45	2013370	44736
259.13	C11H20N2O5	Glu-Leu	398	2881723	7249
327.14	C18H20N2O4	Phe-Tyr	37	263896	7187
195.05	C7H8N4O3	1-3-Dimethyluricacid	92	634581	6865
272.17	C11H23N5O3	Val-Arg	857	5143175	6004
339.09	C12H20O11	3-Ketosucrose	92	343018	3746

277.16	C15H22N2O3	Leu-Phe	132	392424	2962
466.33	C23H50NO6P	[PC methyl(14:2)] 2-methyl-3-tetradecyl-sn-glycero-1-phosphocholine	255	730878	2861
229.16	C11H22N2O3	Leu-Val	341	743738	2183
313.19	C14H26N4O4	Lys-Ala-Pro	151	310677	2060
181.04	C6H6N4O3	1-Methyluric acid	1359	2723161	2004
203.08	C11H12N2O2	L-Tryptophan	187	320534	1714
261.12	C14H18N2O3	Phe-Pro	525	855177	1628
243.10	C10H16N2O5	Glu-Pro	364	556272	1527
175.06	C7H12O5	(2S)-2-Isopropylmalate	31248	46199319	1478

Table 5-18: A relative change of metabolites in negative ionisation mode analysis in *E. coli* infected group in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups was calculated by dividing the mean m/z intensity from the *E. coli* infected samples by the mean m/z intensity determined for the control group.

Reduced in *S. aureus*					
row m/z	**Molecular formula**	**Name**	**m/z intensity (Control)**	**m/z intensity (*S.aureus*)**	**Fold increase/decrease**
210.03	C4H10N3O5P	Phosphocreatine	1659260	0	n/a
150.04	C5H5N5O	8-Hydroxyadenine	216939	0	n/a
219.01	C7H8O8	4-Carboxy-4-hydroxy-2-oxoadipate	528160	1524	0.003
140.01	C2H8NO4P	Ethanolamine phosphate	1079475	7703	0.007
179.03	C9H8O4	3-(4-Hydroxyphenyl)pyruvate	53543	477	0.009
229.01	C5H11O8P	D-Ribose 5-phosphate	655172	10906	0.017
155.03	C7H8O4	2-Hydroxy-6-keto-2_4-heptadienoate	231762	6764	0.029
259.02	C6H13O9P	D-Glucose 6-phosphate	52176193	1588423	0.030
171.01	C3H9O6P	Glycerol 2-phosphate	15741286	539478	0.034
141.06	C7H10O3	4-Oxocyclohexanecarboxylate	129673	5551	0.043
112.99	C4H2O4	Acetylenedicarboxylate	143871	6429	0.045
104.04	C3H7NO3	L-Serine	161750	9620	0.059
289.03	C7H15O10P	D-Sedoheptulose 7-phosphate	1368021	81751	0.060
175.02	C6H8O6	D-Glucuronolactone	5130855	368321	0.072
298.07	C9H18NO8P	D-4'-Phosphopantothenate	1604867	128723	0.080
Increased in *S. aureus*					
187.11	C8H16N2O3	N6-Acetyl-L-lysine	45	8264384	183632
277.16	C15H22N2O3	Leu-Phe	132	13157998	99319

259.13	C11H20N2O5	Glu-Leu	398	9024354	22699
327.14	C18H20N2O4	Phe-Tyr	37	594526	16192
286.19	C12H25N5O3	Leu-Arg	304	4349308	14315
272.17	C11H23N5O3	Val-Arg	857	10020592	11697
243.17	C12H24N2O3	Leucyl-leucine	2334	23052455	9879
229.16	C11H22N2O3	Leu-Val	341	2683365	7875
233.08	C8H14N2O6	Glu-Ser	233	1236984	5305
203.08	C11H12N2O2	L-Tryptophan	187	786619	4207
241.09	C9H14N4O4	Ser-His	311	1043698	3360
321.24	C20H34O3	2alpha-(Hydroxymethyl)-5alpha-androstane-3beta_17beta-diol	496	1658760	3343
345.24	C22H34O3	Taxa-4(20)_11(12)-dien-5alpha-acetoxy-10beta-ol	561	1687503	3010
232.13	C9H19N3O4	Lys-Ser	305	800527	2627
466.33	C23H50NO6P	[PC methyl(14:2)] 2-methyl-3-tetradecyl-sn-glycero-1-phosphocholine	255	669343	2620

Table 5-19: A relative change of metabolites in negative ionisation mode analysis in *S. aureus* infected group in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups was calculated by dividing the mean m/z intensity from the *S. aureus* infected samples by the mean m/z intensity determined for the control group.

Row m/z	Molecular formula	Name	Control	*E. coli* (EC)	*S. aureus* (SA)	SA/EC	P value for EC.>SA
E. coli* > *S. aureus							
268.10	C10H13N5O4	Deoxyguanosine	7393670	246102	2422	0.010	0
360.17	C17H21N5O4	Phe-Gly-His	74624	147797	19243	0.130	0
131.13	C5H14N4	Agmatine	7274	25157	678	0.027	0.0001
262.13	C11H19NO6	Mycosporine	2215241	1490476	73639	0.049	0.0002
662.48	C35H68NO8P	[PC (11:0/16:0)] 1-(10E-undecenoyl)-2-hexadecanoyl-sn-glycero-3-phosphocholine	807946	1369890	82201	0.060	0.0004
395.32	C24H42O4	[ST (4:0)] 5alpha-Cholane-3alpha_7alpha_12alpha_24-tetrol	675090	237003	28238	0.119	0.0007
347.17	C14H26N4O4S	Lys-Cys-Pro	0	14053	2309	0.164	0.0008
302.15	C16H19N3O3	Trp-Pro	3722	60648	6427	0.106	0.001
690.51	C37H72NO8P	[PC (18:0/11:0)] 1-octadecanoyl-2-(10E-undecenoyl)-sn-glycero-3-phosphocholine	6104185	6834848	1086403	0.159	0.0012
122.10	C8H11N	Phenethylamine	332351	366314	28392	0.078	0.0013
136.06	C5H5N5	Adenine	15149	25308	2037	0.080	0.0015
170.08	C8H11NO3	Pyridoxine	10690	43702	4123	0.094	0.0035
292.10	C11H17NO8	2_7-Anhydro-alpha-N-acetylneuraminic acid	392316	242233	30159	0.125	0.0061
341.27	C20H36O4	[FA (20:2)] 15S-hydroperoxy-11Z_13E-eicosadienoic acid	3284897	1757064	278653	0.159	0.0077
415.23	C22H30N4O4	Tentoxin	4205	873662	59028	0.068	0.0185
247.11	C10H18N2O3S	Met-Pro	83543	3674935	453681	0.123	0.0307
291.12	C11H18N2O7	N-Succinyl-LL-2_6-diaminoheptanedioate	132015	45106	6421	0.142	0.0371
452.28	C21H42NO7P	LysoPE(0	22523	429336	70584	0.164	0.0439

Row m/z	Molecular formula	Name	Control	*E. coli* (EC)	*S. aureus* (SA)	SA/EC	P value for EC.>SA
S. aureus* > *E. coli							
288.20	C12H25N5O3	Leu-Arg	882	106405	2643318	24.8	0.0001
295.18	C19H22N2O	Eburnamonine	0	7322	911037	124.4	0.0001
204.13	C8H17N3O3	Lys-Gly	0	4545	105894	23.3	0.0002
275.17	C11H22N4O4	Lys-Ala-Gly	0	7408	259091	35.0	0.0007
288.22	C15H29NO4	L-Octanoylcarnitine	32223	21165	882738	41.7	0.0011
217.13	C8H16N4O3	N-a-Acetyl-L-arginine	17622	6593	251098	38.1	0.0012
316.25	C17H33NO4	O-Decanoyl-L-carnitine	6766	11711	528729	45.1	0.0012
233.15	C10H20N2O4	Leu-Thr	1694	599153	13755161	23.0	0.0042
93.05	C3H8O3	Glycerol	948	2760	301366	109.2	0.0049
262.10	C9H15N3O6	Ala-Asp-Gly	0	2511	527607	210.1	0.0062
243.11	C9H14N4O4	Ser-His	0	3427	111584	32.6	0.0067
246.16	C9H19N5O3	beta-Alanyl-L-arginine	0	595	21148	35.5	0.0086
304.16	C11H21N5O5	Glu-Arg	0	1208	57459	47.6	0.0102
246.14	C10H19N3O4	Leu-Asn	0	6124	1226699	200.3	0.0139
220.09	C7H13N3O5	1D-1-Guanidino-1-deoxy-3-dehydro-scyllo-inositol	0	401	116297	290.2	0.0162
400.34	C23H45NO4	L-Palmitoylcarnitine	0	49632	1440263	29.0	0.0166
300.29	C18H37NO2	Sphingosine	10283	11327	975206	86.1	0.0185

428.37	C25H49NO4	Stearoylcarnitine	3663	31932	870431	27.3	0.0203
241.03	C6H12N2O4S2	L-Cystine	233951	15219	1219633	80.1	0.0228
320.12	C15H17N3O5	Trp-Asp	0	1689	186229	110.2	0.037

Table 5-20: A relative change of metabolites in positive ionisation mode analysis in *E.coli* and *S. aureus* infected groups in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups from the infections was also calculated by dividing the median m/z intensity from the *S. aureus* samples by the median m/z intensity determined for the *E. coli* group.

Row m/z	Molecular formula	Name		Control	*E. coli* (EC)	*S. aureus* (SA)	P- value SA/EC
E. coli* > *S. aureus							
377.11	C13H22N4O7S	Met-Asn-Asp	6890577.34	8802326.18	214344.33	0.024	0
421.22	C23H34O7	Picrasin C	14239.19	41027.42	3517.35	0.086	0
181.05	C9H10O4	3-Methoxy-4-hydroxyphenylglycolaldehyde	62009.99	529282.74	39401.15	0.074	0.0001
149.02	C8H6O3	2-Carboxybenzaldehyde	1776.99	19169.73	3885.44	0.203	0.0002
208.06	C7H15NO4S	MOPS	463757.13	674283.55	55062.11	0.082	0.0003
413.22	C22H30N4O4	Tentoxin	0.00	9914.58	684.99	0.069	0.0006
227.14	C11H20N2O3	Leu-Pro	0.00	586049.23	103807.18	0.177	0.0012
175.06	C7H12O5	(2S)-2-Isopropylmalate	28034.31	484537.27	16910.05	0.035	0.0013
185.09	C8H14N2O3	Ala-Pro	0.00	110493.93	4949.62	0.045	0.0015
225.05	C9H10N2O5	3-Nitrotyrosine	16030.61	64844.81	8919.53	0.138	0.0015
191.06	C7H12O6	Valiolone	459049.45	836759.32	142111.11	0.170	0.0022
464.28	C22H44NO7P	[PC (14:0)] 1-(9Z-tetradecenoyl)-sn-glycero-3-phosphocholine	6635.16	61018.85	9555.26	0.157	0.0026
240.05	C10H11NO6	N-(2_3-Dihydroxybenzoyl)-L-serine	4933.71	12066.26	1245.97	0.103	0.0031
241.01	C6H11O8P	D-myo-Inositol 1_2-cyclic phosphate	126772.78	268187.98	18729.54	0.070	0.0037
138.02	C6H5NO3	4-Nitrophenol	2779.26	266216.76	18730.86	0.070	0.0068
157.05	C7H10O4	2-Isopropylmaleate	9008.42	120032.46	18651.52	0.155	0.0077
163.04	C9H8O3	Phenylpyruvate	4799.75	188677.58	36508.59	0.193	0.0077
304.15	C12H23N3O6	Leu-Ser-Ser	0.00	77330.97	9092.19	0.118	0.0108
166.04	C5H5N5O2	8-Hydroxyguanine	284694.89	163094.08	17370.75	0.107	0.0166
181.04	C6H6N4O3	1-Methyluric acid	1341.04	237648.75	1724.51	0.007	0.0302
171.01	C3H9O6P	Glycerol 2-phosphate	12866412.16	3984791.07	350936.16	0.088	0.0339
91.06	C7H8	Toluene	0.00	3206.91	574.50	0.179	0.0419

Row m/z	Molecular formula	Name		Control	*E. coli* (EC)	*S. aureus* (SA)	P-value SA/EC
S. aureus* > *E. coli							
111.01	C5H4O3	3-Furoicacid	144280.02	37867.85	1270896.79	33.6	0
241.09	C9H14N4O4	Ser-His	0.00	5983.68	404950.12	67.7	0.0007
315.20	C14H28N4O4	Leu-Lys-Gly	0.00	2223.93	50059.68	22.5	0.0015
237.09	C11H14N2O4	Gly-Tyr	0.00	7716.76	145858.86	18.9	0.0024
339.25	C20H36O4	[FA (20:2)] 15S-hydroperoxy-11Z_13E-eicosadienoic acid	5925.74	7410.93	161970.79	21.9	0.0025
302.15	C11H21N5O5	Glu-Arg	0.00	1449.54	51160.10	35.3	0.0028
246.15	C10H21N3O4	Lys-Thr	203.59	72670.00	2556646.63	35.2	0.0042
260.09	C9H15N3O6	Ala-Asp-Gly	0.00	4508.99	1183200.27	262.4	0.0042
227.20	C14H28O2	Tetradecanoic acid	1803.83	7008.93	176840.89	25.2	0.0048
360.14	C14H23N3O8	Leu-Asp-Asp	0.00	1015.08	78304.48	77.1	0.0094
255.23	C16H32O2	Hexadecanoic acid	24943.22	57428.79	1014884.73	17.7	0.0108
203.07	C7H12N2O5	Ala-Asp	0.00	10488.46	522376.69	49.8	0.0145
239.02	C6H12N2O4S2	L-Cystine	85659.22	2122.87	560196.97	263.9	0.0161
260.13	C10H19N3O5	Lys-Asp	0.00	1026.23	56647.44	55.2	0.0185
301.24	C17H34O4	MG(0	3153.57	4636.33	93332.57	20.1	0.0278
278.05	C8H14N3O6P	Cidofovir	655109.72	136903.56	2662709.08	19.4	0.0283
231.10	C9H16N2O5	N2-Succinyl-L-ornithine	0.00	1513.78	46034.74	30.4	0.0293
233.08	C8H14N2O6	Glu-Ser	0.00	7358.12	491377.73	66.8	0.0303
253.03	C7H14N2O4S2	L-Djenkolic acid	0.00	762.58	15942.83	20.9	0.0324
218.08	C7H13N3O5	1D-1-Guanidino-1-deoxy-3-dehydro-scyllo-inositol	0.00	738.01	101885.03	138.1	0.038
353.31	C22H42O3	[FA oxo(22:0)] 10-oxo-docosanoic acid	1592.50	6321.76	113698.29	18.0	0.0489

201.12	C9H18N2O3	Leu-Ala	0.00	32485.10	869131.79	26.8	0.0644
258.15	C11H21N3O4	Leu-Ala-Gly	0.00	38433.25	666891.94	17.4	0.083
205.08	C7H14N2O5	Thr-Ser	0.00	3997.60	199953.30	50.0	0.0963
300.05	C8H16NO9P	N-Acetyl-D-glucosamine 6-phosphate	250003647.30	373727.48	19134245.55	51.2	0.2508

Table 5-21: A relative change of metabolites in negative ionisation mode analysis in *E.coli* and *S. aureus* infected group in comparison to control group. The relative change in intensity for each identified metabolite between the sample groups from the infections was also calculated by dividing the median m/z intensity from the *S. aureus* samples by the median m/z intensity determined for the *E. coli* group.

5.3.5 Metabolic Pathways Altered by Bovine Mastitis

Pathos web facility provided by the Scottish Metabolomics Facility was used to further analyze the metabolites identified in each group of milk samples. The file was downloaded and this website (http://motif.gla.ac.uk/Pathos/index.html) allowed an investigation of the metabolites found in certain group of samples according to corresponded metabolic pathways. The pathways were listed starting from the greatest changes in the metabolites abundances in mastitic samples to the least changes in the metabolites abundances in both groups of mastitic as presented in Figure 5-1 and 5-2. The colour-coding scale indicator at each of the metabolites showed their abundances from increase to decrease in the milk samples. According to the website, light blue colour is an indication of decreased of metabolites in mastitic milk samples whilst the dark red colour is an indication of increased of metabolite in mastitic milk samples.

On each of the metabolic pathway, all metabolites involved in the particular metabolic pathway can be analyzed with the presented histogram showing the abundances in three different groups of milk samples as shown in Figure 5-3. A KEGG map was also be generated to highlight the potential metabolites involved in a particular metabolic pathway as showing in Figure 5-4 for the arachidonic acid pathway.

The next step involved was dividing the metabolic pathways identified into three different groups; lipid, protein and carbohydrate metabolisms. Six metabolic pathways with the most apparent changes in each metabolism from both positive and negative ionisation modes were chosen based on the abundances values provided by the Pathos website. For the lipid metabolism (Table 5-22), the arachidonic acid metabolic pathway was the pathway with most apparent changes with icosatetraenoic acid, prostaglandin F2 alpha, prostaglandin A2, leukotrienes A4 and B4 were increased in mastitic samples whilst no metabolites related to this pathway was found decreased in mastitis. Linoleic acid and α-linolenic acid metabolic pathways were the next pathways with the most changes with increased in icosatetraenoic and stearidonic acids in mastitic samples. Hexadecanoic acid was also found to increase in mastitic milk which derived from fatty acid-related metabolic pathways. The majority of these metabolites were the main precursors of eicosanoids involved in inflammatory activity in a host. Thus, it is not surprising to see these metabolites increased in mastitic samples.

For carbohydrate metabolism (Table 5-23), galactose, pentose and glucuronate interconversions, fructose and mannose, starch and sucrose metabolic pathways were the pathways with the most apparent changes in carbohydrate metabolism apart from glycolysis/gluconeogenesis pathway. Glycerol, glycerone phosphate, fructose and glucose were the metabolites found increased in mastitic samples, whilst fructose-6-phosphate, mannose-6-phosphate and glucose-6-phosphate were among the metabolites decreased in mastitic samples.

Protein metabolism was analyzed and the metabolic pathways with the most apparent changes were arginine, tryptophan, tyrosine, phenylalanine, cysteine and methionine, as well as glycine, serine and threonine metabolic pathways (Table 5-24). The metabolites found to be increased in mastitic samples were tryptophan, ornithine, methionine and tyrosine. Glutamine, phosphocreatine, acetoacetate, propanoate and serine were among the protein metabolites decreased in mastitic milk samples.

Di- and tri-peptides were also found to increase in both groups of mastitic samples. A table of peptides identified by Pathos programme is presented in Table 5-25 and includes Asn-His, Asp-Arg, Gln-Ser-Ser and Leu- Gly- Ser.

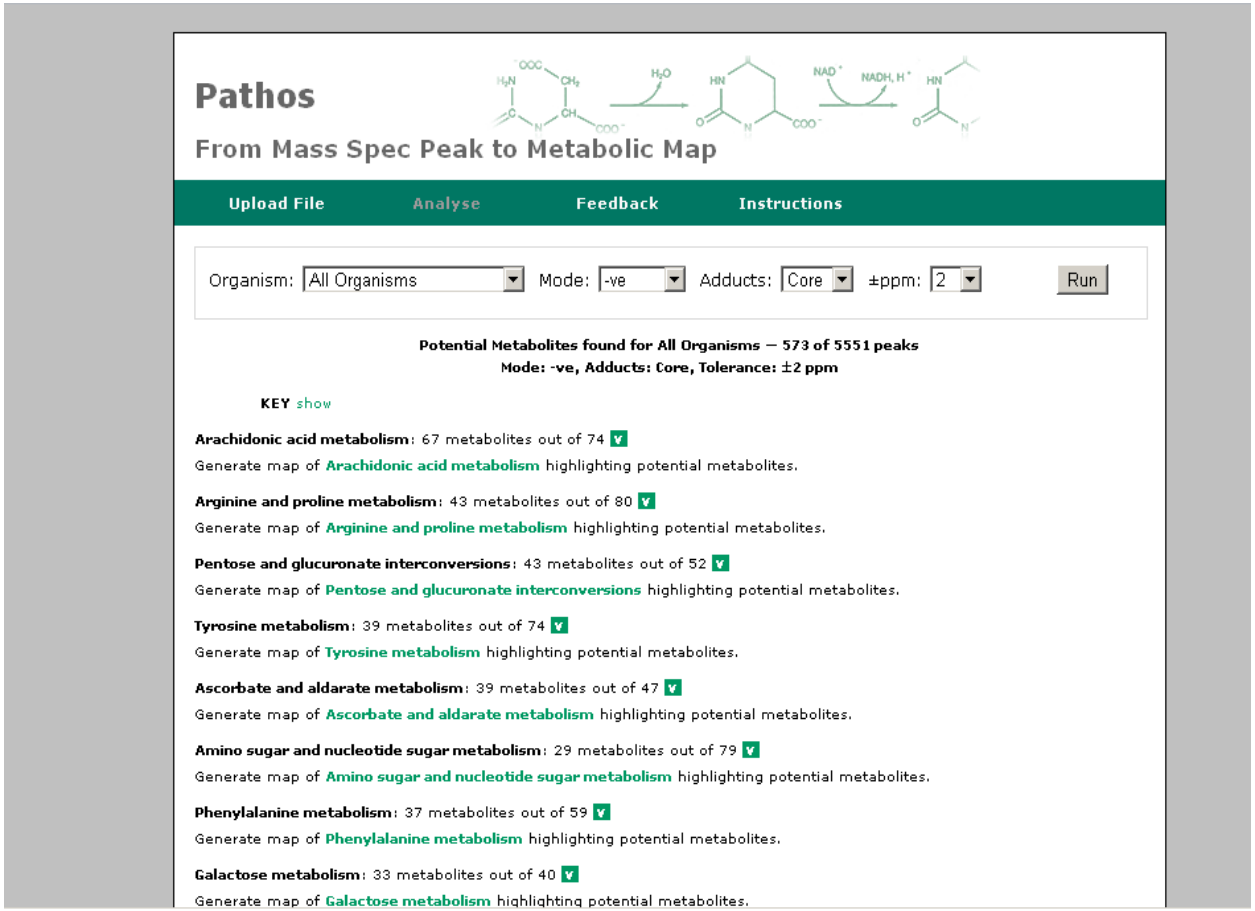
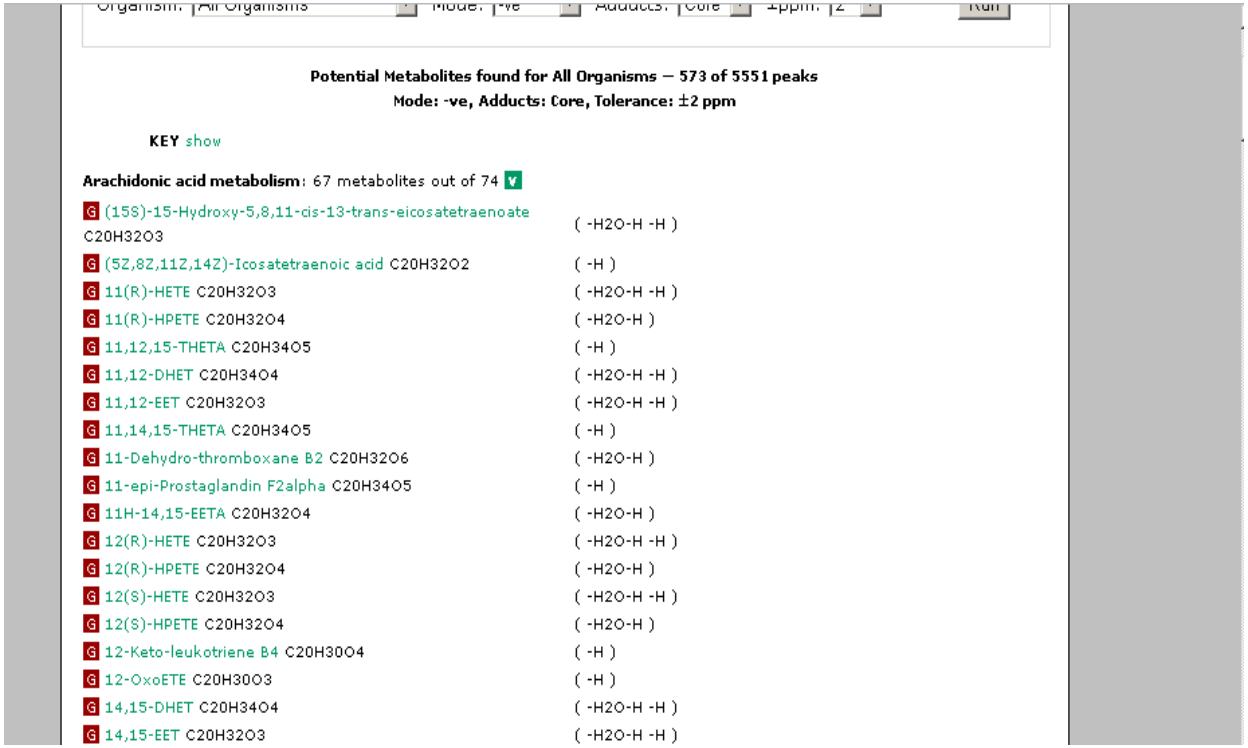


Figure 5-1: An example of various metabolic pathways generated by PATHOS website. In this example, the data from negative ionisation mode was used and the metabolic pathway was listed starting with the most apparent changes of metabolites to the least apparent changes of metabolites.



Organism: All Organisms Mode: -ve Adducts: Core 1ppm: 2 Run

Potential Metabolites found for All Organisms – 573 of 5551 peaks
Mode: -ve, Adducts: Core, Tolerance: ±2 ppm

KEY show

Arachidonic acid metabolism: 67 metabolites out of 74

(15S)-15-Hydroxy-5,8,11-cis-13-trans-eicosatetraenoate C20H32O3	(-H2O-H -H)
(5Z,8Z,11Z,14Z)-Icosatetraenoic acid C20H32O2	(-H)
11(R)-HETE C20H32O3	(-H2O-H -H)
11(R)-HPETE C20H32O4	(-H2O-H)
11,12,15-THETA C20H34O5	(-H)
11,12-DHET C20H34O4	(-H2O-H -H)
11,12-EET C20H32O3	(-H2O-H -H)
11,14,15-THETA C20H34O5	(-H)
11-Dehydro-thromboxane B2 C20H32O6	(-H2O-H)
11-epi-Prostaglandin F2alpha C20H34O5	(-H)
11H-14,15-EETA C20H32O4	(-H2O-H)
12(R)-HETE C20H32O3	(-H2O-H -H)
12(R)-HPETE C20H32O4	(-H2O-H)
12(S)-HETE C20H32O3	(-H2O-H -H)
12(S)-HPETE C20H32O4	(-H2O-H)
12-Keto-leukotriene B4 C20H30O4	(-H)
12-OxoETE C20H30O3	(-H)
14,15-DHET C20H34O4	(-H2O-H -H)
14,15-EET C20H32O3	(-H2O-H -H)

Figure 5-2: An example of metabolic pathway (Arachidonic acid metabolism) which listed down the metabolites with greatest changes of abundances (dark red colour) to the least changes of abundances in both groups of mastitis (light blue colour; not showing).

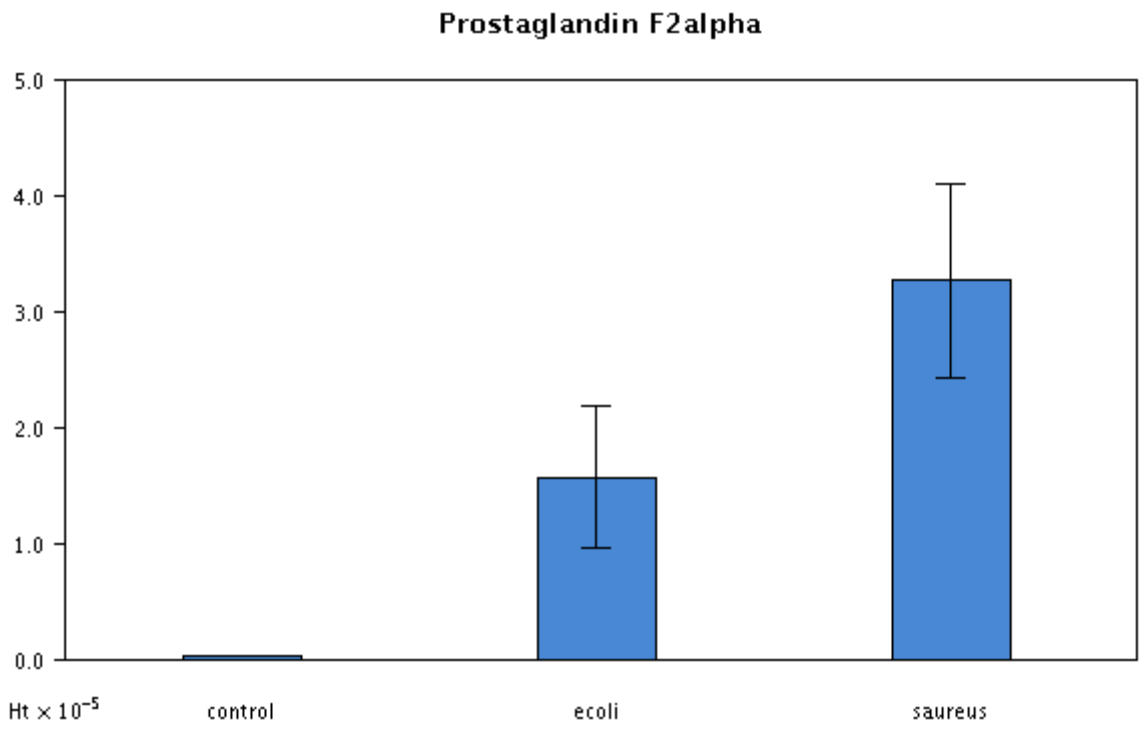


Figure 5-3: An example of histogram of prostaglandin F2 alpha showing abundances in control, *E. coli* infected and *S. aureus* infected groups. It shows here that prostaglandin F2 alpha increased in abundances in both mastitis groups but present in the lowest abundances in control group.

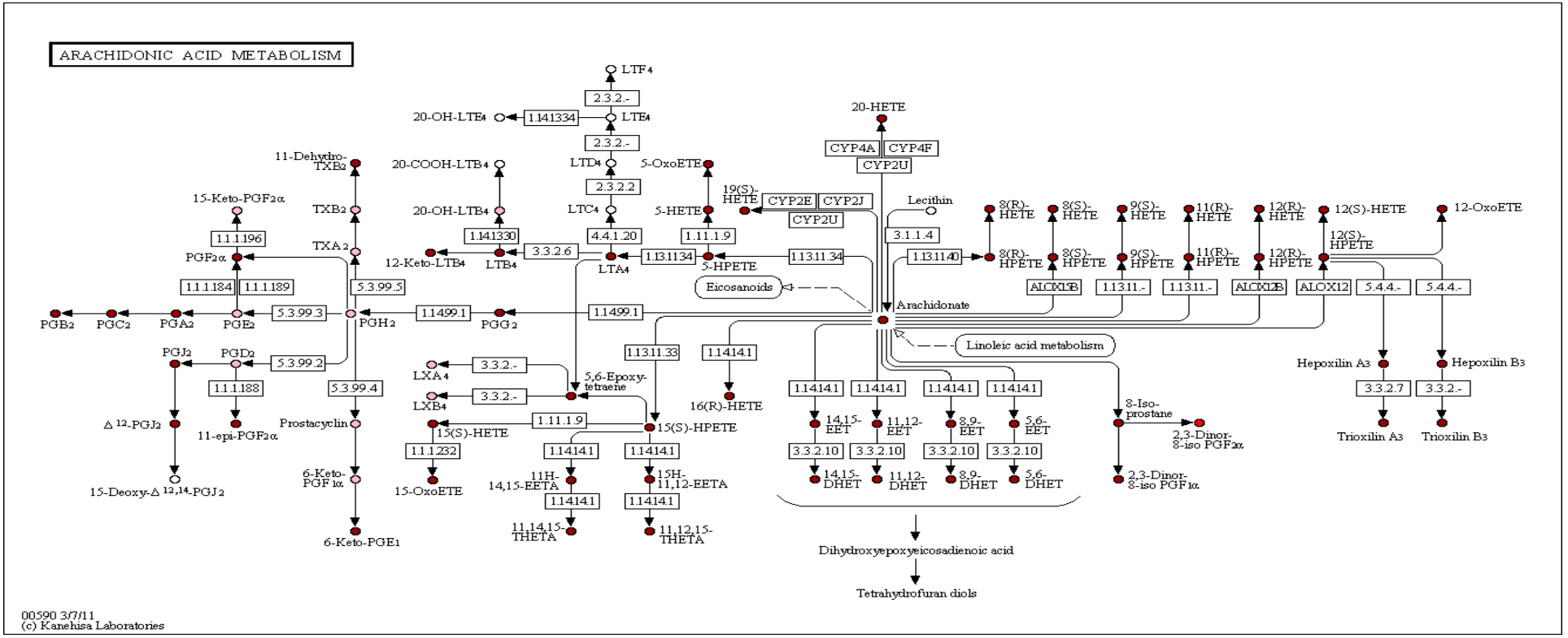


Figure 5-4: An example of KEGG map generated from arachidonic acid metabolism to highlight the potential metabolites involved in a particular metabolic pathway. A dark red dot shows metabolite which was increased in mastitic milk samples. Further click on the dot revealed a histogram as showed in Figure 5.2.

Lipid Metabolic Pathways	Metabolites Increased in Mastitis	Metabolites Decreased in Mastitis
Arachidonic acid Met.	Icosatetraenoic acid, Prostaglandin F2α, Leukotriene A4, Leukotriene B4, Prostagladin A2	None
Linoleic acid Met.	Icosatetraenoic acid,	Linoleate
α-linolenic acid Met.	Stearidonic acid	Heptadecatrienal
Biosynthesis of unsaturated fatty acid	Hexadecanoic acid	Icosatetraenoic acid
Fatty acid biosynthesis	Hexadecanoic acid	None
Fatty acid Met.	Hexadecanoic acid	None

Table 5-22: Six metabolic pathways in lipid metabolism with the most apparent changes of metabolites from both positive and negative ionisation mode analyses. The abundances values were provided by the Pathos website.

Carbohydrate Metabolic Pathways	Metabolites Increased in Mastitis	Metabolites Decreased in Mastitis
Galactose Met.	Glycerol	D-Glucose-1-Phosphate, α-D-Galactose 1-phosphate
Pentose & Glucuronate interconversions	Glycerone phosphate, D-Xylonolactone	D-Fructose-6-Phosphate
Fructose & Mannose Met	D-Fructose	D-Mannose-6-Phosphate
Starch & Sucrose Met.	Reduced acceptor D-Glucose	D-Glucose-6-phosphate
Glycolysis/Gluconeogenesis	None	Acetate

Table 5-23: Six metabolic pathways in carbohydrate metabolism with the most apparent changes of metabolites from both positive and negative ionisation mode analyses. The abundances values were provided by the Pathos website.

Protein Metabolic Pathways	Metabolites Increased in Mastitis	Metabolites Decreased in Mastitis
ArginineMet.	N-Acetylornithine, L-Ornithine	L-Glutamine, Phosphocreatine
Tryptophan Met.	L-Tryptophan	Anthranilate, 2-Oxoadipate
Tyrosine Met.	L-Tyrosine, 4-Hydroxyphenylacetate	Acetoacetate
Phenylalanine Met.	L-Phenylalanine, Succinate	Propanoate, 2-Hydroxy-2,4-pentadienoate
Cysteine and Methionine Met.	L-Methionine	2-Oxobutanoate, L-Serine
Glycine, Serine and Threonine Met.	L-Tryptophan	L-Serine,D-serine

Table 5-24: Six metabolic pathways in protein metabolism with the most apparent changes of metabolites from both positive and negative ionisation mode analyses. The abundances values were provided by the Pathos website.

Amino acid/Peptides	Examples
Di-peptides	Asn-His, Asp-Arg, Lys-Asn, Leu-Met, Ala-Tyr, Trp-Gly
Tri-peptides	Gln-Ser-Ser, Leu-Gly-Ser, Met-Ala-Ser, Ala-Gly-Arg, Leu-Asp-Gly, Leu-Asn-Gly

Table 5-25: Examples of di- and tri-peptides which were found increased in both mastitic groups from both positive and negative ionisation mode analyses. The abundances values were provided by the Pathos website.

5.4 Discussion

From positive ion mode analysis, the 3 metabolites with highest intensity in the control group (Table 5-4) were L-acetylcarnitine, choline and sn-glycero-3-phosphocholine which are related to lipid metabolism and the choline content of phopsholipids in the milk fat globule membrane. Citrate, lactose, glucose and galactose were found high in intensity from negative ion mode in both control and *E. coli* infected group but the monosaccharides were not at the top in *S. aureus* infected group, possibly indicative that these monosaccharides are more rapidly absorbed for use in metabolism by the latter bacterium

Identification of the metabolites which show the most significant differences between the infections caused by the bacteria provide valuable indications for demonstration of differences in the metabolic effects of the infections and also reveal potential targets for biomarkers to identify the type of bacteria causing the mastitis. Notable in the evaluation of metabolite changes on infection are the increase in identification of di-and tripeptides in in both infections with particular tripeptides being associated with both of the bacteria, though with *S. aureus* infection causing more of these peptides to be present. The source of the peptides in the infected samples is likely to be breakdown products from milk proteins caused by bacterial or immune cell protease activity. The finding that there are differences between bacterial causative agent could be either due to differences in the specifity of action of the bacterial proteases or that the bacteria have differing efficiencies in absorbing the peptides after they are released by hydrolysis. As *S. aureus* infection caused the greater degree of di and tripeptide in the metabolite pool, the bacteria either have more active proteolytic enzymes or are less efficient at absorbing the peptides for their own metabolism.

An attempt was made to find the origin of the tripeptides by comparison to the bovine genome of those found in either infection group. Although 40% of the tripeptides present in the mastitis milk could be identified in the amino acid sequence of the milk proteins: caseins, α-lactalbumin, β-lactoglobin, albumin and lactoferrin it was found by scanning the bovine protein database, that the tri-peptides were identified in over 5000 proteins in the bovine proteome so that no definitive conclusion could be made on the origin of the peptides.

Of the 100 metabolites investigated, with the largest differences in m/z intensity between *S. aureus* and *E. coli*, there was a mix of metabolites, with lipid, nucleotide and carbohydrate related metabolites being identified. There were also non-host and non-

pathogen metabolites identified such as tentoxin, ricinine, quinate, toluene and others. When these occurred in the infected groups and not in the control group it is possible that the metabolites are derived from therapeutic reagents. A number had metabolite database hits as either drugs (for infection or inflammation) or of compounds used in herbal or chinese medicine. As mentioned above the identification by the computer database of a number of metabolites were questionable, and while a number of these were identified and corrected (1-alpha-D-galactosyl-myo-inositol =lactose; myo-inositol = glucose or galactose) it is possible that the molecules are incorrectly named and these identified agents may be erroneously named. Confirmation of molecular identity would be a major task in following up this initial investigation.

5.4.1 Lipid Metabolism

As part of the analysis, the effect of mastitis on lipid metabolism was assessed by examination of the metabolites in the 6 metabolic pathways with the most apparent changes from both positive and negative ion modes. From these six metabolic pathways, the 6 metabolites greater in both diseased and control groups were selected for discussion.

The method reported in this chapter revealed increases in arachidonic acid metabolites in both two mastitic group caused by *E. coli* and *S. aureus*. Examples of increased metabolites are icosatetraenoic acid, prostaglandin F2α (PGF2α), prostaglandin E2 (PGE2), leukotriene A_4 (LKA4), leukotriene B_4 (LKB4), thromboxane A_2 (TXA2), thromboxane B_2 (TXB2) and others.

These metabolites are the main precursors of eicosanoids which are the fatty acid-derived mediators involved in a wide-range of inflammatory activity in the host cell (Bannerman 2009). Thromboxanes and prostaglandins are formed by the catabolism of arachidonic acid mediated by cyclooxygenase while the formation of leukotrienes is mediated by lipoxygenase (Kuehl Jr. and Egan 1980; Williams and Higgs 1988). Each eicosanoids has a differing role in inflammatory processes and may have antagonizing effects to other eicosanoids. Thromboxane A2 for example is most potent of the eicosanoids in contracting aortic tissue and can trigger platelet aggregation whereas other prostaglandins (e.g, prostacyclin) and PGI2 cause vasodilation and inhibit platelet aggregation (Kuehl Jr. and Egan 1980; Ball *et al.* 1986; Feuerstein and Hallenbeck 1987). Meanwhile, leukotrienes are involved in inducing leukocyte recruitment and promoting leukocyte respiratory burst activity and cytokine production (Zipser and Laffi 1985; Williams and Higgs 1988; Bottoms and Adams 1992). According to Bannermann (2008), leukotriene B4 is the most

potent endogenous chemoattractant. Thus, eicosanoids are responsible in mediating inflammatory response with a wide-range of activities on the immune response towards infection and injury.

With their important role as mediators in inflammatory responses, it is not surprising that increases in concentrations of these eicosanoids happen in bovine mastitis. These results were in accordance with Giri *et al.* (1984) in which they also found a greater concentration of both PGF1α and PGF2α in milk after 50μg infusion of LPS. However, they did not find any increase in other eicosanoids such as TXB2 or LKB4. Anderson *et al.* (1985) on the other hand, revealed only an increase of TXB2 milk level after 10μg of LPS infusion. According to Anderson *et al.* (1985), the reason of an absence of PGF2α in the milk samples following infusion in their study is because of the reduced concentration of LPS infused into the quarters as compared to the former study.

In this present study, both of the different pathogen-causing mastitis; *E. coli* and *S. aureus* are able to induce the release of these eicosanoids. One experimental intrammary infection study on *E. coli* by Peter *et al.* (1990) showed increases in prostacyclin (PCM), PGE2 and TXB2. However, no PCM was detected in this present study. However, Anderson et al (1985) also failed to show any increased in both PGF2α and TXB2 in experimental study of *E.Coli*. These differences are due to large biological and individual variability observed in milk eicosanoids concentrations in cows with acute coliform mastitis.

Furthermore, studies by Atroshi *et al.* (1986, 1987) which involved naturally occurring cases of mastitis caused by *S. uberis, S. aureus*, *S. dysgalactiae* or *Micrococcus species* revealed increases in PGE2, PGF2α and TXB2. In this present study, milk samples infected with *S. aureus* showed greater concentrations of both TXA2 and TXB2 than milk samples infected with *E. coli*. Similarly, study by Boutet *et al.* (2003) on chronic mastitis caused by coagulase-negative staphylococci such as *S. uberis, S. agalactiae* or *S. aureus* showed increases in LTB4. Based on these results, it is indicated that different types of pathogen elicit increases in releasing eicosanoids during bovine mastitis.

Apart from increased level of eicosanoids in all mastitic milk samples, there was also an increased in fatty acids metabolites seen in both groups of mastitic samples such as hexadecanoic acid, icosatetraenoic acid, linoleate, octadecenoic acid, octadecanoic acid, tetradecanoic acid etc.

Fatty acids in the blood play an important role in modulating inflammatory reactions (Calder 2008; Serhan *et al.* 2008). Stored fatty acids are important as energy substrate

especially during negative energy balance (NEB) period. During this period, in a lactating cow the majority of available glucose is directed to the mammary gland for lactose synthesis (Herdt 2000). Thus, lipid mobilization from body reserve occurs and as a consequence, there is a release of non-esterified fatty acids (NEFA) into the blood stream. However an excess in the levels of circulating NEFAs could contribute to increase in disease susceptibility dairy cows especially in periparturient inflammatory-based diseases including mastitis, ketosis and metritis (Bernabucci *et al.* 2005; Goff 2006; Douglas *et al.* 2007).

Fatty acids can either provide benefit or have detrimental effects on the host through its regulating mechanisms in inflammatory reactions (Sordillo *et al.* 2009). For example, saturated fatty acids including lauric, palmitic and oleic acids can activate the toll-like receptor 4 (TLR4) through activation of NK-κB which leads to activation of innate immune response of the host. Thus, the presence of hexadecanoic acid or palmitic acid; a saturated fatty acid in both groups of mastitic samples could play an important role in activation of host innate immune response which provide the initial protection against invading pathogens before the acquired immune response is activated.

During inflammation of the udder, milk composition is known to change and this includes the fat content in the milk. Previous studies showed that the levels of free fatty acids (FFA) is increased during mastitis (Randolph & Erwin, 1979; Salih & Anderson 1979; Needs & Anderson 1984; Atroshi *et al.* 1989). These events were contributed to a high lipase activity which leads to lipolysis and increase in the permeability of the mammary gland to blood lipids (Randolph & Erwin 1979). According to Salih & Anderson (1979), lipoprotein lipase (LPL) is an enzyme that responsible for lipolysis in milk. Furthermore, the transfer of an increased amount of LPL activator from blood to milk may contribute to partly caused rise of the FFA in mastitic milk.

However, a study by Fitz-Gerald *et al.* (1981) did not agree on the role of LPL in increasing the level of FFA in mastitic milk. It might be explained by other mastitis-influenced factors (e.g; presence of blood-serum activators or changes in substrate susceptibility). Lower LPL activity in mastitic milk is due to impaired biosynthetic capability of mammary gland through permanent tissue damage or the action of proteolytic enzymes which are also present in the milk during mastitis. This was also supported by Anderson and Needs (1983) who observed high levels of FFA from lipolysis in mastitic milk but these increases were not necessarily due to LPL activators. Blood serum or heparin might give a stimulatory effect on lipolysis as discussed by Salih & Anderson

(1979) previously. According to Murphy *et al.* (1989), increase in lipolysis was contributed from the susceptibility of the milk fat substrate to lipolysis in mastitic milk and not from the high level of lipase acitivity.

A number of factors can influence the level of FFA in milk such as above-normal FFA levels on secretion, LPL activator, cell lipase, proteolytic enzymes, anions, heparin-like substances and fat globule membrane (Salih & Anderson 1979; Fitz-Gerald *et al.* 1981). Other than that, the variation occurred in individual cows on the levels of FFA in milk are also influenced by factors such as diet, oestrus and stage of lactation (Anderson & Needs 1983).

In this present study, both linoleic and α-linolenic acid metabolism were increased in both mastitic groups. This is with an agreement with Hogan *et al.* (1987) in which they observed high content of unsaturated linoleic and linolenic acid in mastitic milk which might be important in the killing of bacteria. Linoleic acid is a precursor of archidonic acid and both could be released from the cell membrane phospholipids by phospholipase-like enzymes. Futhermore, according to Atroshi *et al.* (1989), α-linolenic acid was positively correlated with PGE2 and PGF2α which were also found in higher levels in mastitic milks in this present study than in healthy milk.

In this present study, high levels of long-chain fatty acids were observed in both groups of mastitic milk. However, it is not an agreement with studies by Kisza & Batura (1969), Randolph & Erwin (1974) and Karijord *et al.* (1982) only reported the highest content of 18:0 and C 18:1 in milk during early lactation but not during mastitis.

In contrast, other previous studies argued with the high fat content observed in mastitic milk (Bartsch *et al.* 1981; Massart-Leen *et al.* 1994). They assumed that during mastitis, the low level in milk yield could lead to reduce synthesis of the total amount amount of milk fat as the mammary gland epithelium does not retain its normal integrity for synthesis of major milk components.

5.4.2 Carbohydrate Metabolism

Carbohydrates are one of the most important sources of energy in all living organisms. In cows, carbohydrate is fermented by microorganisms in the rumen to produce volatile fatty acids such as acetic acid, butyric acid and propionic acid. Gluconeogenesis in the cows occurs in the liver and glucose is produced from metabolites such as propionate, valerate, amino acids, lactate and glycerol (Nafikov & Beitz 2007).

The mammary gland takes up glucose from the liver via circulatory system to be used in lactose synthesis. Glucose is also important for lipid metabolism as it serves as the backbone of milk fat synthesis in which glucose is converted to glycerol. Glycerol is then being attached to the fatty acids produced by acetate and ß-hydroxybutyrate. About 70% of net uptake of glucose from blood by lactating bovine udders is used to synthesize lactose and the remainder is oxidized in pentose and TCA cycles (Qiao *et al.* 2005).

Following mass spectrometry of metabolomics, six carbohydrate metabolic pathways were chosen and in each metabolic pathway, metabolites that increased or decreased in mastitis in relation to control animals were assigned in both positive and negative ion modes.

It was apparent that some carbohydrate metabolites were less evident in both groups of mastitis compared to control animals. Among these metabolites are glycerol, D-fructose, and others, while D-glucose or its isomer D-galactose and identified by the PATHOS programme as myo-inositol was still in the top 4 in *E. coli* and top 6 in *S. aureus* groups. Marschke & Kitchen (1984) agreed that levels of glucose were negatively correlated with both log somatic cell counts (logSCC) and log N-acetyl-beta-D-glucosaminidase (logNAGase). However, the reason for low levels of glucose found in mastitic milk is unknown. Several hypotheses have been made on how this event occurred. According to Linzell & Peaker (1971), during mastitis, reduced blood flow to the udder result in reduced in the supply of blood glucose to the secretory cells of mammary gland. It is also possible that glucose would move out of milk during mastitis by a paracellular pathway as Na^+ and Cl^- increased in milk. This would to help in maintaining the osmolarity between extracellular fluid and milk (Marschke & Kitchen 1984).

A study by Cecil *et al.* (1965) revealed that glycogen concentrations were greatly increased in mastitic milk compared with normal milk. As leukocytes contain high glycogen levels, there is a possibility that high glucose levels in mastitic milk are due to leukocyte infiltration during the inflammatory processes. However, the results in this present study showed that the end-products of glycogen breakdown such as glucose-1-phosphate and glucose-6-phosphate are lower in mastitic milk than in control milk. It seems that the influence of somatic cells in mastitis milk samples used in the present study was not great enough to release glycogen in the milk. Futhermore, because glycogen is only available from the liver as glucose for it to become accessible to other organs, reduced blood flow to the mammary gland during mastitis was the reason why glycogen levels are reduced in mastitic milk.

Giesecke & Heever (1981) observed a fluctuation in glucose levels in milk from artificially-induced mastitis by human strains Group B *Streptococci* intracisternally. In certain conditions, low levels of glucose may occur relative to its supply and demand even in normal udder quarters. Reduced concentration of glucose in mastitic milk can affect the abilities of PMN-leukocytes by affecting the efficiency of phagocytosis and the killing of bacteria (Giesecke & Heever 1981).

Low levels of glucose contribute to cessation of milk secretion implying the importance of glucose in milk secretion (Rook *et al.* 1965).Thus, a reduced blood perfusion to the mammary gland and less glucose uptake by the mammary cells may occur in mastitis. In turn, depressed milk yield is seen which could explain one of the important clinical sign in mastitis.

5.4.3 Protein Metabolism

Ruminant animals require two types of digestible proteins; the one which escapes rumen degradation which is digested in the small intestine to be used by the animals itself and the one is a degradable protein in the rumen used by the micro-organisms to synthesize microbial protein. Proteins are important sources for maintenance of vital functions, reproduction, growth and lactation. Within the mammary gland, large amounts of amino acids are needed to synthesize milk proteins. Amino acids can also be converted into other non-essential amino acids or oxidized into energy in the mammary gland (Wattiaux and Karg 2004).

In this study, the six protein metabolic pathways with greatest change were chosen for discussion and in each metabolic pathways, six metabolites were identified that increased or decreased in mastitis compared to control animals. However, breakdown of milk proteins such as casein is most likely to be responsible for much of the amino acid and di-, tri- peptides formed in this study.

Amongst these metabolites, it is apparent that methionine (Met) is increased in both mastitic groups. As mentioned previously, amino acids in milk are absorbed from blood but also synthesized and degraded in the mammary gland (Bequette *et al.* 1998). According to Pinotti *et al.* (2002), healthy dairy ruminants produce large amounts of methionine or a combination of Met and lysine (Lys) (Schwab *et al.* 1992). But in the present study, no increase in free Lys was observed in mastitic groups. These amino acids are the first limiting amino acids for milk protein synthesis and Met particularly is required for transmethylation reactions and milk protein synthesis and also able to stimulate both

milk and milk fat productions. However, the level of amino acids in milk is greatly influenced by the quality and quantity of amino acids in basal diets (Klein *et al.* 2010).

Glycine (Gly) level is increased in milk from both mastitic groups in this present study and this increased has been observed during the early lactation due to increased Gly metabolism during this period (Klein *et al.* 2010). Phosphocholine levels showed a positive correlation (r=0.81) with the levels of Gly which is also increased during early stage of lactation (Klein *et al.* 2010). In this present study, phosphocholine is also increased in mastitic milk samples. Phosphocholine acts as a precursor of Gly and serves as its storage form within the cytosol. It is therefore possible that the increased levels of both Gly and phosphocholine found in this study are due to mastitic milk collected at an early stage of lactation and that the ability of mammary gland to synthesize these amino acids are not impaired by udder infection.

High levels of both tyrosine and isoleucine amino acids are seen in the present study in mastitic milk samples. These amino acids are seen to increase in organic milk compared to conventional whole milk as studied by Boudonck *et al.* (2009). High tyrosine levels in mastitic milks can be explained by the extensive proteolysis in milk. Proteolysis can result in accumulation of small peptides which contribute to the bitterness in the milk. According to Murphy *et al.* (1989), casein proteolysis products can be measured by the determination of tyrosine level. Thus, these authors agreed that high values on tyrosine during infection period are due to high milk protease activity during mastitis.

Bacterial fermentation in the milk can produced milk products which contained considerably amounts of D-amino acids (Man and Bada 1987) and bacteria can be regarded as a biological source of D-amino acids (D-AA). Study by Pohn *et al.* (2009) revealed greater increases of free amino acids are seen in milk with different scores of CMT especially Ile, Ala, Asp, Pro and Leu. This is in accordance with the present study in which Ile, Pro and Leu were increased in mastitic milk samples. Study by Pohn *et al.* (2009) also observed high free D-AA as CMT score increased for all nine free D-AA (D-Asp, D-Ser, D-Glu, D-Pro, D-Ala, D-Val, D-allo-Ile, D-Leu and D-Lys). In this present study, D-Pro and D-Cys did increase in mastitic milk samples. Thus, it can be concluded the presence of D-AA content is associated with mastitis and related to the bacterial activity in the udder. However, the MS analysis cannot differentiate D- from L- amino aicd as they have identified m/z and retention time, so that identification as D- amino acid is not definitive.

It can be concluded that increase in many amino acids in mastitic milk samples is due to proteolytic activity caused by proteases from pathogens and also from immune cells such as plasmin caused by the presence of microorganisms during udder infection. End products of major milk proteins degradation by proteases contribute to a wide-range of amino acids present in the mastitic milk samples. Thus, certain amino acids can be used to indicate the activity of pathogenic bacteria which lead to inflammation of mammary gland.

In conclusion, there was an increase in identification of di-and tripeptides in both infections with particular tripeptides being associated with either of the bacteria, though with *S. aureus* infection causing more of these peptides to be released. Differences that present between bacterial causative agents could be either due to differences in the specificity of action of the bacterial proteases or that the bacteria have differing efficiencies in absorbing the peptides after they are released by hydrolysis. Amongst other metabolites changes in lipid, nucleotide and carbohydrate metabolism have been identified, there were also non-host and non-pathogen metabolites identified such as tentoxin, ricinine, quinate, toluene and others and it was possible that the metabolites are derived from therapeutic reagents though mis-classification is also possible.

To differentiate between *E. coli* and *S. aureus* infected milk samples, certain metabolites may have potential as biomarkers such as pyridoxine, N-acetyl-L-glutamate, isopropylmalate and pyruvate. These metabolites were seen in *E. coli* infected milk samples as compared with *S. aureus i*nfected milk samples. The majority of increased metabolites in *S. aureus* infected milk samples were comprised of di- or tri-peptides such as Gln-Ser-Ser, Leu-Asn-Tyr, Leu-Thr and others.

Mastitis had an effect on lipid, protein and carbohydrate metabolic pathways. In lipid metabolic pathway, there was an increased in arachidonic acid metabolites in mastitis. As for protein metabolic pathway, arginine metabolites were seen increased in mastitis whilst galactose metabolites from carbohydrate metabolic pathway were increased in mastitic milk samples.

From this study, it can be concluded that metabolomic analysis has successfully studied the presence of important metabolites or even determined the changes of metabolites that occurred in milk samples of dairy cows during the course of intramammary inflammation. Further analysis is needed with more milk samples being studied to confirm the status of these important metabolites and also to assess their appearance in subclinical as well as the clinical mastitic samples studied in this investigation.

Chapter VI. General Discussion

The aims of this thesis were outlined in chapter 1 and the relevant conclusions have been detailed in the appropriate chapters. This chapter will summarise the important and significant findings and highlight the areas which have been recognised as worthy of future research.

Despite the numerous researches that have been undertaken to control bovine mastitis, this disease still poses one of the most prevalent and costly diseases in dairy cows. The negative impacts of bovine mastitis include reduction in milk production and quality because of changes in milk composition which in turn affects the economy of dairy industry. Other factors such as costs of treatment, veterinary services, diagnostics and culling were also contribute to the detrimental effect on dairy industry worldwide (Halasa *et al.* 2007).

In chapter 1, various traditional and common bovine mastitis diagnostic methods have been discussed. Somatic cell counts (SCC) is still widely used as one of the most useful diagnostic techniques to detect the level and occurrence of bovine mastitis especially for bovine subclinical mastitis (Dohoo & Meek 1982; Schukken *et al.* 2003). However, in order to avoid false-positive or false-negative results, several factors should be taken into consideration before any conclusion can be made. These factors include mastitis causing pathogens, number of infected quarters, previous mastitis history, age, stage of lactation, milking frequency as well as the type of samples taken for SCC analysis (quarter, composite or bulk tank).

Other important diagnostic methods that have been discussed in detecting the presence of bovine mastitis are California Mastitis Test (CMT), enzyme levels, electrical conductivity (EC) and infrared thermography (IRT). CMT which was developed by Schalm & Noorlander (1957) is the main cow-side test used to diagnose bovine mastitis and is based on the formation of gel formation which reflects the cell count of the milk (Barnum & Newbould 1961). Previous studies have been shown the relationship of CMT results with the leukocyte content of milk, the level of subclinical mastitis as well as bacteriological analyses (Barnum & Newbould 1961; Daniel *et al.* 1966; Wesen *et al.* 1967).

Several types of enzymes may be useful as mastitis indicators but one enzyme that is considered reliable and sensitive is N-acetyl-β-D-glucosaminidase (NAGase) (Kitchen 1976). The use of both SCC and NAGase provides valuable interpretation for the diagnosis

of bovine mastitis so that the level and extent of infection can be controlled (Pyörälä & Pyörälä 1997). Other important enzymes include β-glucuronidase, milk antitrypsin, L-lactate dehydrogenase (LDH) and myeloperoxidase (MPO).

The use of EC has been one of the most popular methods in diagnosing bovine mastitis. The concentration of NaCl is often measured as milk electrical conductivity and many studies have been performed to assess the ability of EC in diagnosing bovine mastitis particularly subclinical mastitis (Linzell *et al.* 1974; Fernando *et al.* 1982, 1985).

Finally, another popular bovine mastitis diagnostic method is IRT. It has been shown that IRT has strong correlation with CMT and a high predictive diagnostic ability making it a sensitive tool in detecting early onset of subclinical mastitis (Berry *et al.* 2003; Polat *et al.* 2010) although several cow factors should be consider before analysing the reliability of IRT in diagnosing bovine mastitis.

However, these traditional methods of bovine mastitis diagnosis may have their technical drawbacks such as less sensitive, less specific, lacking in speed, very labour-intensive and expensive. Their results can also be mis-interpreted which then lead to false positive or negative (Chagunda *et al.* 2006).

Recently, much research interest has been focused on analysing the presence of bovine mastitis biomarkers that can be used to predict the progress and outcome of a disease. Biomarkers are important analytes which can be used to predict changes in any biological and pathological processes so that any disease states can be revealed (Biomarkers Definitions Working Group 2001). Because of the limitations in traditional methods of diagnosing bovine mastitis that had been discussed previously (Chagunda *et al.* 2006; Le Roux *et al.* 2003; Labohm *et al.* 1998; Nielen *et al.* 1995), the discovery of specific mastitis biomarkers is needed for an early diagnosis of bovine mastitis. Thus, several bovine mastitis biomarkers have been proposed including the use of combinations of several bovine mastitis indicators such as enzymes, SCC, prostanoids, bacteriological culture and others (Mattila *et al.* 1986; Zank & Schlatterer 1998; Barta *et al.* 1990; Baeker *et al.* 2002; Bannikov *et al.* 2010).

Two important APPs that are known to increase in response to disease are SAA and Hp (Conner *et al.* 1986; Eckersall *et al.* 2001). Hp and SAA are also known to rise substantially in serum or milk as indicators of bovine mastitis (Pyörälä *et al.* 2011). In chapter 2, the reliability of both SAA and Hp was assessed by validating and optimising these APPs assays with the use of bovine milk samples.

The aim of chapter 2 was to develop sandwich ELISA by using rabbit anti-bovine antibody to measure Hp level in bovine milk and used commercialized Phase^{TM} Range SAA Assay to quantify the level of milk SAA so that the concentrations of the APPs in different types of milk samples could be compared statistically. To group milk samples into healthy, subclinical and clinical mastitis, the estimation of SCC and bacteriological culture were used before APP assays were performed in order to confirm the disease status. It was shown in this chapter that Hp concentrations showed to be statistically increased in clinical mastitic samples when compared between healthy and clinical mastitic cases as well as between subclinical and clinical mastitic cases. However, Hp showed no significant difference between healthy and subclinical mastitic milk samples ($p>0.05$). Several factors were discussed which may contribute to this reason which include different types of cytokines produced during bovine mastitis, less Hp production during subclinical mastitis, insensitive immunoassay to detect a slight elevation of Hp concentrations in subclinical mastitic milk samples as well as different types of pathogen virulence and host responses.

Milk serum amyloid A3 (M-SAA3) has also been shown to have an important role as biomarker in bovine mastitis (Eckersall *et al.* 2001; Grönlund *et al.* 2003, 2005; Nielsen *et al.* 2004; Pyörälä *et al.* 2011). This study successfully confirmed the use of M-SAA3 in diagnosing bovine mastitis in which M-SAA3 concentrations were significantly different between healthy, subclinical and clinical mastitic milk samples ($p<0.05$).

SAA levels showed a relatively strong relationship with Hp levels further confirming that SAA and Hp can be used together as good mastitis indicator. It has also been shown that these APP measurements can be performed using milk samples instead of serum/plasma samples for an ease of sampling and sample preparation.

Somatic cell counts (SCC) were measured in both healthy and subclinical mastitic milk samples and the relationship between SCC values and APP measurements was determined. SCC measurement was significantly different between healthy and subclinical mastitic samples ($p<0.05$) which proved the reliability of SCC to diagnose subclinical mastitis. The correlation between SCC and APP showed positive relationship although the relationship was not strong. The correlation between SCC and SAA gave a medium relationship with the value of r was 0.438 while here was no correlation between SCC and Hp as the value of r was 0.08. The reason for this difference could be that these correlations were calculated based on all milk samples regardless of the type of milk samples (control vs. infected) and various cut-off values for SCC have been suggested depending on several factors (countries, breeds, types of milk samples) (Mattila *et al.* 1986; Holdaway *et al.*

1996). The use of log transformed SCC can be considered when analysing their relationship to achieve good correlation between APP and SCC.

The application of advances in proteomics has been a major recent area of research in studying the protein changes in bovine milk during mastitis. In chapter 3, several proteomics methods were applied, such as 1DE, 2DE, DiGE and MALDI-MS analyses, to investigate the changes of milk proteome during the presence of intramammary inflammation. Milk samples from subclinical and clinical mastitic cases were used in comparison with healthy milk samples as comparison. It was hoped that by applying these methods, discovery of new biomarkers of bovine mastitis can be achieved.

Prior to the use of proteomic methods, SCC and APP measurements were used to confirm the disease status of the milk samples investigated. In initial studies rennet coagulation was performed to remove the majority of milk casein in order to examine changes in low abundance proteins. In the first phase of this study, subclinical mastitic milk samples were analysed in comparison with healthy milk samples using 1D gel electrophoresis and the protein bands developed on the gel were analysed by the MALDI-MS for protein identification. Unfortunately, no clear difference was seen on the gel although lower intensity bands but with high molecular weight were not successfully identified by the MALDI-MS analysis.

Because no clear difference was observed on the gel pattern of 1D gel electrophoresis, 2D gel electrophoresis was applied to similar samples. Unfortunately, there was again no observable difference in low abundance proteins between pooled subclinical mastitic and pooled healthy milk samples. However, it was evident that albumin was not present in healthy milk samples which further supported the idea that albumin concentrations was increased during bovine mastitis as a result of either an increase in leakage from serum into the mammary gland or in albumin expression in the mammary gland.

Due to these limitations, clinical mastitic milk samples were analysed by using DiGE which enables the visualization of multiple protein samples in one 2D gel. However, the only findings observed were increases in albumin concentration and decreased whey protein concentrations (β-LG and α-LA) in clinical mastitic milk samples as compared with healthy milk samples. Due to lack of significant findings in this chapter, non-gel proteomic approaches were recommended to continue the investigation of bovine mastitis biomarkers.

Peptidomic approach was applied next to investigate the presence of polypeptides in bovine milk that may be used to discriminate normal from bovine mastitic milk samples and also to determine if peptide biomarkers could discriminate between mastitis caused by two different types of bacterial pathogens. It was hoped that by using this sensitive and high-throughput methodology, significant results can be achieved and lead to the discovery of important and significant bovine mastitis biomarkers by employing CE-MS and LC-MS/MS analyses.

Milk samples used in this study were from clinical mastitic cases, caused by two common bacterial pathogens which were *E. coli* and *S. aureus*, and were assessed in comparison to healthy milk samples. Two different types of comparison were analysed to determine the ability of peptidomics to compare between healthy and clinically infected milk samples as well as the ability of peptidomics to compare between *E. coli* infected and *S. aureus* infected milk samples. SCC and APP measurements were used as previously to confirm the disease status of the milk samples used.

From CE-MS analysis, polypeptide fingerprinting was performed for different groups of milk samples to generate disease models. However, these models need to be validated so that the status of each marker in each group (control, infected, *E. coli* or *S. aureus*) can be confirmed and may eventually used to determine the presence of bovine mastitis or even predict the type of bacterial pathogens that caused bovine mastitis.

A total of 45 discriminatory polypeptides were identified by CE-MS analysis with 31 polypeptides used to discriminate healthy from mastitis cases and 14 polypeptides discriminating *E. coli* from *S. aureus* infected cases. These polypeptides were then further analysed for their amino acid sequence by LC-MS/MS, although not all polypeptides were successfully sequenced. The reason was because the only parameters used to link the peptides obtained in both systems (CE-MS and LC-MS/MS) were peptide mass and the number of basic amino acids. Thus, mass ambiguities occurred which complicate the assignment of sequences obtained from these two different methods.

However, a total of 12 sequenced polypeptides were identified using UniProtKB database. Amongst the proteins identified from the peptide sequences were α S1-casein, β-casein, serum amyloid A protein (SAA), glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) and fibroblast growth factor binding protein (FGFBP). Although GlyCAM-1 was characterized as one of the host defence mechanism proteins (Boehmer *et al.* 2010; Smolenski *et al.* 2007), it was interesting to see the presence of these peptides from GlyCAM-1 in healthy milk samples. Peptides from SAA were also seen in both *E. coli* and

S. aureus infected milk samples but they were not able to discriminate *E. coli* infected milk samples from *S. aureus* infected milk samples. The results from this chapter provided an initial insight on how milk peptide profiles can change as a result of bovine mastitis. Further improvements have been discussed but it was confirmed that peptidomic methodology able to identify significant biomarkers in bovine mastitis.

Finally, a metabolomic approach was applied to further investigate the presence of important metabolites that can be used to discriminate normal from mastitic milk samples by the use of LC-MS/MS analysis. As previously, SCC and APP measurements were performed to confirm the disease status of the samples used. Individual metabolites were analysed in both positive and negative ionisation modes in each different types of milk sample. Metabolites which were absent in control milk samples but present in both infected groups (*E. coli* and *S. aureus*) were also examined in both ionisation modes. Among the many metabolites detected, a variety of tri-peptides were seen with higher intensities in *S. aureus* group compared with *E. coli* group regardless of the type of ionisation modes. The relative change in intensity for each identified metabolites between two infected groups was determined and Mann-Whitney test was performed to show the significant difference in each of the categories. Amongst the identified metabolites which were significantly increased in the *E. coli* infected group regardless of the type of ionisation modes were 3-nitrotyrosine, deoxyguanosine and 3-methoxy-4-hydroxylphenyl. Whilst Leu-Arg, Ser-His and Lys-Ala-Gly were amongst the identified metabolites found significantly increased in *S. aureus* infected group.

Metabolic pathways were also analysed to allow an investigation of the metabolites found in different group of milk samples according to their corresponding metabolic pathways. In lipid metabolic pathway, arachidonic acid metabolites were seen increased in mastitis while in protein metabolic pathway, arginine metabolites were found to be increased in mastitis. Finally, galactose metabolites from carbohydrate metabolic pathways were increased in mastitic milk samples.

In chapter 4 and 5, several significant findings were found which could be proposed as bovine mastitis biomarkers. It was also apparent that some of the milk indicators could be applied to differentiate clinical mastitis caused by different type of bacterial pathogens (*E. coli* and *S. aureus*). This would be of especial value in allowing the appropriate antibacterial treatment. However, further experiments with more milk samples should be performed to validate and confirm these results so that they can be used to diagnose bovine mastitis. Furthermore it is important to investigate the differentiation of subclinical and

clinical bovine mastitis, so that subclinical mastitic milk samples should be used in both peptidomic and metabolomics methodologies in comparison with clinical mastitic and healthy milk samples as it was apparent that the use of proteomic methodology in this study was not able to differentiate between subclinical and clinical bovine mastitis. It is also hoped that changes in bovine milk proteome, peptidome or metabolome can be perceived efficiently.

In summary of this thesis, with a prior knowledge of known acute phase proteins during bovine mastitis, various '-omics' studies such as proteomics, peptidomics and metabolomics can be applied to study the presence of other lower abundance proteins, peptides and metabolites which may serve as important mastitis biomarkers.

Appendices

Appendix Chapter 3

1. Preparation of 12% PAGE gels

The monomer solution was prepared by mixin APS. The mixture was degassed for 15 minutes.

Percent Gel	DDI water (ml)	30% Degassed Acrylamide/Bis (ml)	• Gel buffer (ml)	10% w/v SDS (ml)
12%	3.4	4.0	2.5	0.1

- Resolving Gel Buffer – 1.5M Tris-HCl, pH 8.8
- Stacking Gel Buffer – 0.5M Tris-HCl, pH 6.8

Immediately prior to pouring the gel, for 10 ml monomer solution, these solutions were added:

Resolving Gel: 50µl 10% APS and 5µl TEMED

Stacking Gel: 50µl 10% APS and 10µl TEMED

The mixed solution was gently swirled to initiate polymerization

2. Bradford Assay

The Bradford reagent which consists of Brillian Blue G in phosphoric acid and methanol as well as Protein Standard (BSA) Solution (2mg/ml) were purchased from Sigma-Aldrich.Inc.

a) Protein Standard preparation

Bovine serum albumin at 2mg/ml was used as the protein standard in performing Bradford assay. The standard was further diluted using MilliQ water into 1.5mg/ml, 1.0mg/ml and 0.5mg/ml to produce a standard curve.

b) Milk samples preparation

Milk samples were used from both healthy and mastitic cows. All samples were kept at -20°C before analysis. After thawed, all samples were further diluted in 1:2, 1:4, 1:6 and 1:8 using MilliQ water. This dilution procedure was not necessary to perform in all assays.

c) Assay

250µl of Bradford reagent was added into 5µl of each standard and sample at different dilutions. The sample was incubated for a few minutes at room temperature and the absorbance was measure at 595nm using [name].

d) Protein Concentration Analysis

The net absorbance values against the protein concentration of each standard were plotted automatically after the measurement. The protein concentration of each sample was determined by comparing the Net A_{595} values against the standard curve.

3. Lysis buffer

6M urea

2M thiourea

4% CHAPS

25mM tris base

4. Equilibration buffer I

pH8.8, 1.5M Tris HCl

216.21 g urea

180ml glycerol

12g SDS

100mg DTT

5. Equilibration buffer II

pH8.8, 1.5M Tris HCl

216.21 g urea

180ml glycerol

12g SDS

250mg iodoacetamide

6. DiGE Lysis Buffer

7M urea

2M Thiourea

4% CHAPS

25mM Tris HCl

Appendix Chapter 4

1. Preparation of Standards for microplate assay

Standard	BSA standard	Water or buffer	Final protein concentration
Standard A	75 µL stock (2 mg/ml)	225 µl	500 µg/ml
Standard B	200 µL of A	200 µl	250 µg/ml
Standard C	200 µL of B	200 µl	125 µg/ml
Standard D	80 µL of C	120 µl	50 µg/ml
Standard E	60 µL of D	140 µl	15 µg/ml
Standard F	60 µL of E	120 µl	5 µg/ml
Standard G	80 µL of F	120 µl	2 µg/ml
Blank	0	200 µl	0 µg/ml

2. Preparation of BC Assay reagent

Prepare the required amount of BC Assay reagent by adding:

1 part of reagent B to 50 parts of reagent A.

3. Components for sample preparation

1. Disposable de-salting PD10-columns (GE Healthcare Biosciences AB, Bedford, USA).
2. Centristart ultrafilter tubes 20 kD, 0.62 cm^2 tubes (Sartorius Stedim Limited, UK).
3. BCA protein quantification kit (Uptima, Interchim, Montluçon, France).
4. Lyophilisation Christ Speed-Vac RVC 2-18/Alpha 1-2 (Christ, Osterode am Harz, Germany).

4. Components for CE-MS analysis

1. P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Crea, CA, USA)
2. Micro-TOF (time-of-flight) mass spectrometer (Bruker Daltonics, Bremen, Germany).
3. Electro-ionisation sprayer (Agilent Technologies, Santa Clara, USA) (*see* **Note 3**).
4. Capillary 90 cm, 50 µm I.D. fused silica capillary non-coated (New Objective, Woburn, USA)
5. Cole-Parmer single-syringe infusion pump (Cole-Parmer Instrument Company, USA).
6. Hamilton syringe 2.5 ml

5. Software for Data processing and Statistical Analysis

a) Software for peak detection: MosaiquesVisu (Biomosaiques, Hannover, Germany).

b) Commercial statistical packages: SAS (www.sas.com) and MedCal (www.medcalc.be).

c) Multitest R-package available at www.bioconductor.org

d) SVM-based program for multiparametric data classification: MosaCluster (Biomosaiques, Hannover, Germany).

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