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Title	Investigation of the presence and activity of the innate immune component, Complement, in bovine milk
Authors	Maye, Susan
Publication date	2016
Original Citation	Maye, S. 2016. Investigation of the presence and activity of the innate immune component, Complement, in bovine milk. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-10-30 08:48:39
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Investigation of the presence and activity of the innate immune component, Complement, in bovine milk

A Thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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April 2016

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DECLARATION

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Student Number: 105011299

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ABSTRACT

The occurrence of Complement in human milk indicates the presence of innate immune components of maternal origin and their putative defensive role in the neonatal gut. In order to ascertain the status of Complement in bovine milk, it was necessary to validate the suitability of the Complement-sensitive bacterial sequestration assay used to monitor human milk. The relative bacteriostatic effects, expressed as a differential between the initial and final assay counts following inhibition of the *E. coli* O111 marker strain were, 6.20 and 6.06 log CFU/ml for raw bovine and human milks, respectively. Lower levels of Complement activity measured in pasteurised and low-fat milks were observed during the course of heatinactivation studies, while gravity separation of cream over a 24 h period confirmed a greater degree of attachment by Complement to the rising cream layer. The *E. coli* O111 sequestration assay strain had restricted growth of 7.5 and 8.2 CFU/ml in the higher and lower gravity-separated fractions, respectively. An animal health effect was observed, with Complement appearing to be more active in milk samples from cows with a higher somatic cell counts (SCC). Inducing sub-clinical mastitis in the healthy quarter of a lactating cow confirmed that increased Complement activity effect was local to the affected quarter and not manifested systemically.

Complement activity levels varied in the milks of 7 commercially important prominent dairy breeds and crossbreeds surveyed. Both the Norwegian Red and Kerry breed cows stood out as having higher Complement activity (6.25 and 6.92 CFU/ml, respectively); these two breeds are known to have stronger immunity and a better capacity to resist mastitic infection.

The findings of this study have implications for both milk production and processing disciplines. In order to retain the antimicrobial efficacy associated with Complement in raw bovine milk, milder process treatments than hitherto practised in industry should be adopted.

PUBLICATIONS

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research*, *82*(3), pp. 328-333.

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2016. Transformation of serum-susceptible *Escherichia coli* O111 with p16Slux plasmid to allow for real-time monitoring of complement-based inactivation of bacterial growth in bovine milk. *Journal of Dairy Science*, *99*(1), pp. 112-119.

MAYE, S., FLYNN, J., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., Bovine intra-mammary challenge with *Streptococcus dysgalactiae* spp. *Dysgalactiae* to explore the effect on the response of Complement activity. Submitted (2016).

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., Determination of breed variation on the Complement response of bovine milk. In proceedings of Agricultural Research Forum, Tullamore, 10th and 11th of March, 2014.

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., Evidence of Innate immune proteins in Milk – development of a bactericidal sequestration technique to detect and monitor Complement Bioavailability. In proceedings at Walsh Fellowships seminar 2013, RDS Dublin, 28th November, 2013.

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., Exploring Interactions between Milk Fat Globule Membrane and the Complement system of milk in relation to bactericidal sequestration. In proceedings at the 41st Annual

Food Research Conference, 26th and 27th April, 2012.

ABBREVIATIONS

BoLA	Bovine lymphocyte antigen
C3	Complement component 3
C5	Complement component 5
C5a	Complement component 5a
Ca	Calcium
CFU	Colony forming units
Cl	Chlorine
CLRS	C-type lectin receptors
CMP	Caseinomacropeptide
E	*Escherichia*
ELISA	Enzyme Linked Immune-Sorbent Assay
EHEC	Enterohaemorragic *E. coli*
EPEC	Enteropathogenic *E. coli*
FGS	Fat globule size
FOS	Fructooligosaccharides
GI	Gastrointestinal
GMP	Glycomacropeptide
GOS	Galactooligosaccharides
HBM	Human breast milk
HMFGM	Human milk fat globule membrane
HIR	High immune response
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon
IL	Interleukin
IMF	Infant milk formula
K	Potassium
KCGP	Kappa-*casein* glycopeptide
KCP	Non-glycosylated *Kappa-Casein peptide*
LB	Luria Bertani
LPS	Lipopolysaccharides
Lyz	Lysozyme
MDPC	Moorepark dairy production centre
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MIC	Minimum inhibitory concentration
Mg	Magnesium
MHC	Major histocompatibility complex
ml	Millilitres
mtDNA	Mitochondrial DNA
MUC1	Mucin 1
MUC15	Mucin 15
Na	Sodium
NAM	N-acetylmuramic
NAG	N-acetylglucosamine

NAGase	N-acetyl-beta-D-glucosaminidase
NLR	NOD-like receptors
NR	Norwegian red
PRRs	Pattern-recognition receptors
P	Phosphorus
PI	Post inoculation
ROI	Region of interest
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SCC	Somatic cell count
SEq	Standard equation
SMM	Skimmed milk membrane
SNP	Single nucleotide polymorphism
St.	*Staphylococcus*
Str	*Streptococcus*
Spp	Species
TGF	Tumour growth factor
TLR	Toll-like receptors
WHO	World health organisation
WT	Wildtype
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
XDH	Xanthine dehydrogenase

Dedicated to Roy and Chloe, for your patience and support

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge the opportunity presented to me by Teagasc to carry out this work in the Food Research Centre, Moorepark. I would like to express my gratitude to the Teagasc Walsh Fellowship scheme and the Department of Agriculture, Food and the Marine for funding my research.

Huge thanks to Dr Phil Kelly, throughout my PhD he has given me invaluable help and encouragement and shared his immense knowledge. His guidance helped me throughout the research and writing of this thesis. I could not have imagined having a better advisor and mentor for my PhD study.

Thanks to Professor Catherine Stanton and Professor Ger Fitzgerald for vital comments, suggestions and support throughout.

I would like to thank all students and staff in the Moorepark Food Research Centre for their support and friendship. Over the years I have made some great office friends who made my time in Moorepark so memorable Louise, Like, Meng, Ronan and Chen thank you for being great friends.

To Sarah Hen and Cian thank you for helping me form Gang 71, we had some great fun in the office and some serious lols were had!

To Anne Marie and Sarah Cooney thanks for being great friends and always listening to me rambling on….. and for continuing to do so!

Owen, Buggy, Sarah Ross, Sinéad Morrin for all the memorable moments spent together over tea, I can definitely say I have had some of my best times of my PhD in the canteen at Moorepark.

To those I started out with but have moved on in their careers; Colette, Ariel, Anil, Lorna, Noelle and Bríd, thanks for bringing me for tea that first day and introducing me to the Moorepark social scene.

Big thanks to the Moorepark crowd, Nuria, Grace, Sandra, Soléne, Lisa, Kate, Deirdre, Sinéad Mackey, Noel, John, Dave, Eoin, Ian, Jonny, Aoife T, Erinn, Alan and Pa for the great tag nights out and social events!

Thanks to Dr Mary Rea and Dr Caitriona Guinane for help and guidance in microbiological studies and lab work.

I would also like to acknowledge the staff in the MDPC for all their help throughout my PhD. I would like also thank the local farmers who allowed me to sample from their herds, as well as this I would like to extend sincere gratitude to the Kerry Cattle association and specifically Remonde Hilliard for sharing her extensive knowledge on the Kerry breed and for allowing me to sample from her cows. Huge thanks to Jim Flynn for his help and advice throughout my studies.

Finally my family, the warmest thanks to my parents, Bernard and Mary Maye and my three sisters Claire, Aisláinn and Tara for their love throughout my PhD.

Chapter I Literature Review

1.1. Introduction

Human breast milk (HBM) is the universally preferred source of nutrition for the neonate, as it contains the correct balance of nutrients and functional components to

both protect and promote early growth during the initial stages of life. These protective elements of HBM support the new-born in these critical stages pending the development of its own immune system. In the event that HBM is replaced with regular first age infant formulae, it is essential that it meets the compositional and nutritional requirements as set out by Codex Alimentarius for infants during the first months of life (Codex Standard for Infant formula, 1981). Over the years, considerable strides have been made to 'humanise', i.e., align the composition of bovine milk to resemble more closely that of HBM. However as of yet, there is no requirement which sets out the levels of protection in the form of defensive components which should be incorporated in infant milk formula. Consequently, formula fed infants are likely to be at a disadvantage by not having the comprehensive physiological protection afforded by components of HBM. Several studies report that the absence of breastfeeding may be associated with an increased incidence of infection, increased possibility of childhood obesity, type 1 and type 2 diabetes, and leukemia, (Lönnerdal et al., 1976, Kostraba et al., 1993, Horta et al., 2007).

It is well known that HBM provides passive protective elements from the mother's immune system such as Immunoglobulin A (IgA), Immunoglobulin G (IgG), Immunoglobulin M (IgM) as well as non-immune components like lactoferrin, lactoperoxidase and lipids. Human milk shares many of these components with bovine milk (Hamosh et al., 1999). In addition, bovine milk is reported to provide other biologically active compounds that can defend neonates, such as antibacterial peptides, antimicrobial proteins, oligosaccharides, and lipids, besides many other components at low concentrations. Complement is another protective component in HBM, which features prominently in the innate immune response and also contributes to the adapted

immune system (Carroll, 2004, Ogundele, 2001). The Complement system is represented by a multi-step pathway or cascade consisting of more than 30 serum proteins and cell surface receptors that act in a number of ways, from cell lysis to the promotion of specific B and T cell responses.

It is postulated that the Complement components bind to the milk fat globule membrane (MFGM) where in the presence of bacteria, the activation of the Complement reaction cascade is triggered (Ogundele, 1999a). It is thought that the MFGM in human milk acts as a preferred site for the initiation and activation of the Complement reaction cascade, localising concentration so as to apply maximal effect on the membrane-bound antigens. Furthermore, this association may increase the sequestration by MFGM of other antigens present. As well as direct antimicrobial effects, Complement can limit the development of harmful immune complexes and may sustain the antigens by coating with covalently attached fragments (Complement component C3) which inhibit the precipitation of Immunoglobulins (Igs) and promote uptake and removal by phagocytes (Korhonen et al., 2000). Another constituent of interest, Complement component C5a, is a pro-inflammatory mediator and is described as one of the most biologically significant peptides (Rainard, 2003). While C5a is reported to be very low in normal milk (Rainard, 1998), it is speculated that it may be produced by the activation of the Complement with zymosan, indicating that a C5-convertase can be assembled in normal milk. Further effects of the Complement system include the formation of a membrane-attacking complex which can be damaging to bacteria (Janeway Jr et al., 2001).

1.2. Nutritional and immune composition of human milk

The neonatal period is one of the most critical and vulnerable periods in mammalian life. Early post-partum milk secretions in the form of colostrum are species specific in terms of composition and physiological effects. Within days, the compositions of both human and bovine milk adjust to the levels typically associated with their respective full lactations (Kulski and Hartmann, 1981). Moreover, its composition varies with lactation stage and in line with the infants evolving nutritional needs ("The Surgeon General's Call to Action to Support Breastfeeding", 2011). A comprehensive understanding of human milk composition provides an important guideline for the management of infant feeding, particularly of fragile, high-risk infants. Such an understanding is also critical for the adaptation of bovine milk and the potential impact of processing induced effects.

Considerable strides have been made to mimic HBM, e.g., reversal of the ratio of casein to whey proteins and realignment of fatty acids, quality and quantity of nonprotein nitrogen, availability of immunoglobulins as well as other immune components (Emmett and Rogers, 1997). However, care is required with the addition of bioactive components derived from bovine milk to infant milk formula (IMF). For example, in the case of osteopontin, the bovine form differs from that found HBM in primary structure, as well as some post-translational modifications. Thus, it is advisable to carry out *in vitro* studies, along with animal models or clinical trials in order to assess the overall effect and potential benefits of including these proteins

(Lönnerdal, 2014).

Human milk is the confluence of sources, i.e., component synthesis in the lactocyte, permeation of constituents of dietary origin, and those released from maternal stores (Ballard and Morrow, 2013). Human maternal lactation is typically divided into three time periods: colostrum during the first 5 days post-partum; transitional milk during the second 5 days post-partum and mature milk thereafter. The general composition of mature human milk is; 3%-5% fat, 0.8%-0.9% protein, 6.9%-7.2% carbohydrate and 0.2% mineral constituents (Jenness, 1979). However, protein and fat contents vary in earlier stage milk forms, i.e., colostrum. The total protein content declines from approx. 14-16 g/l during early lactation to 8-10 g/l at the 3-4 month stage of lactation and finally 7-8 g/l at 6 months and thereafter (Lönnerdal, 2003). The major proteins in human milk are beta-casein, alpha-lactalbumin, lactoferrin, IgA, lysozyme, and serum albumin, while the essential amino acid pattern optimally matches infant nutrition needs. The fat content of HBM is characterized by high contents of palmitic and oleic acids. The main mineral components are Na, K, Ca, Mg, P, and Cl. In HBM, like other mammalian milks, the lipid phase is dispersed as droplets which are surrounded by a MFGM generated by the blebbing process in the mammary gland cells. MFGM is comprised mostly of phospholipids, specific proteins and as well as cholesterol. In the case of HBM, the fat globules range in size from approx. 0.1μm up to 15μm (Gallier et al., 2015).

HBM contains a number of bioactive components that are regarded as either essential or non-essential in terms of their effect on human health (Biesalski et al., 2009). Maternal milk provides passive protection to the young neonate by providing immune constituents and bioactive components which may act synergistically (Korhonen et al.,

2009). More recently, the Complement system has also been implicated in the provision of protection against the continual threat of foreign invaders (Ogundele, 1999b). A previous study suggested that the presence of Complement secretion plays an important role in the initiation of localised immune reactions (Cole et al., 1982). It was originally believed that Complement was present in milk at such low levels that it was unlikely to have any biological effect (Mueller et al., 1982), however, recent evidence proves the opposite effect may be true (Rainard and Riollet, 2006). As discussed later, the Complement system has three major pathways; classical, alternative and lectin. The classical Complement pathway is activated by antibody–antigen complexes on the bacterial surface, whereas the alternative and mannosebinding lectin pathways are activated directly by bacterial cell surface components. The classical pathway of the Complement system appears to be low in bovine milk, while, on the other hand, the identification of components C3b and C3bi, as well as C5-convertase, indicates that the alternative pathway is active (Yingming Zhao, 1997, Rainard, 2003). This pathway is activated in plasma whilst in the fluid phase it is induced by a spontaneous conformational change of C3, which provokes formation of an enzyme complex that cleaves further molecules in the cascade and sets in motion an amplification reaction that activates the Complement (Zipfel and Skerka, 2008). The benefits of such innate immunological components in breast milk has been credited with improved developmental in premature infants, and reduced incidence of GI infections, obesity and eczema (Von Kries et al., 1999, Howie et al., 1990).

1.3. Milk fat globule membrane – MFGM

The MFGM is a biological membrane that envelopes the fat droplets dispersed in Milk. MFGM is understood to be a phospholipid tri-layer that carries proteins and lipids from the endoplasmic reticulum membrane and the mammary epithelium cells' membrane (Mather and Keenan, 1975). As well as being a membrane enveloping the milk fat globules in milk, these colloidal assemblies have been identified as the carriers of fat-soluble nutrients as well as biologically active molecules, which include phospholipids, sphingolipids, cholesterol and other MFGM proteins (Lopez, 2011). Milk fat globules and their surrounding MFGM originate from the plasma membrane and more specifically the endoplasmic reticulum during milk secretion (Zaczek and Keenan, 1990).

It has been found that the MFGM is comprised of a number of proteins and lipids which represent between 1–2% of the protein in milk (Le et al., 2013). Although its composition is highly variable, it is mostly comprised of glycoproteins, phospholipids and sphingolipids (Table 1.1), which are of considerable interest because of their potential bioactivity. The phospholipid fractions include phosphatidylethanolamine and phosphatidylcholine and in lesser quantities phosphatidylserine and phosphatidylinositol. The sphingolipids include sphingomyelin as well as ceramides and gangliosides (Park, 2009). The bioactive proteins associated with the MFGM are known to have anti-carcinogenic properties as well as moderating the progression of some age-related diseases, stress responses, apoptosis and development of Alzheimer's disease (Spitsberg, 2005). Furthermore, butyric acid and butyrate are capable of inhibiting development of colon and mammary tumours (Parodi, 1998). Mostly, MFGM protects against bacteria and viruses by binding to their outer layers. Mucin components associated with the MFGM (MUC-1 and MUC-15) have been

identified as having a protective role by inhibiting epithelial cell apoptosis. Moreover, they play a significant role regulating chemokine secretion in gastrointestinal tissue (Chatterton et al., 2013). CD14 is a minor constituent of MFGM that is recognised as an important receptor that plays a key role in the innate recognition of bacteria. This component is believed to trigger a potent immune response and may also have a significant role in education of the neonatal immune system (Bianchi et al., 2009). Milk also contains hormones, growth factors and cytokines that are biologically active at low concentrations (Friel and Qasem, 2016).

Component	**mg/100g fat globules**	**mg/100g MFGM dry matter**
Protein	1800	70
Phospholipids	650	25
Cerobrosides	80	3
Cholesterol	40	2
Carotenoids + Vit. A	0.04	0.0
Iron (Fe)	0.3	0.0
Copper (Cu)	0.01	0.0
Total	>2570	100

Table 1.1 Average composition of MFGM (adapted from Dewettinck et al., 2008).

Human milk fat globule membrane (HMFGM) is known to prevent bacterial adherence that causes agglutination (Hamosh 2001). Moreover, it is hypothesised that MFGM provides the major site where Complement is activated and comes into contact with particulate antigens (Ogundele, 1999a).

The clustering of milk fat globules during cream separation has been likened to the agglutination of bacteria or red blood cells due to the action of IgM (Walstra, 1995). It is hypothesised that cold agglutination involves three components; MFGs, IgM and the skim milk membrane (SMM). The indigenous immunoglobulins precipitate onto fat globules at low temperatures, and thus, are termed cryoglobulins These components interact using carbohydrate moieties and it is reported the fat globules cluster to a limited degree by IgM alone; however, clustering is improved in the presence of SMM, which may act as a cross-linking agent (Euber and Brunner, 1984). It is apparent that this process is accelerated by cryoglobulins, as seen from the fact that ovine, caprine or buffalo milk, which lack cryoglobulins, cream much more slowly than bovine milk (Huppertz and Kelly, 2006).

1.4. Antimicrobial agents in Bovine Milk

Transfer of antimicrobial components during milk secretion is a key biological function which provides protection to the suckling neonate. It is reasonable to expect that maximum protection is transferred during the early stages of life *via* colostrum and transition to early lactation milk. Within milk, a range of proteins, peptides, enzymes and complex carbohydrates are known to possess antimicrobial activities, by which they restrict the growth of and / or clear pathogenic bacteria, viruses and fungi. A brief overview of these components is now outlined.

Immunoglobulins

Immunoglobulins (Igs), generically referred to as antibodies, constitute 1-2% of total protein in normal bovine milk and are sub-divided into different classes based on their

structure and functions. The major immunoglobulins identified in both human and bovine milk are IgA, IgG and IgM (Korhonen and Marnila, 2009). Ig preparations derived from human serum, as well as bovine colostrum and serum, have proven effective in human clinical trials when treating a variety of enteric microbial infections and other conditions which cause diarrhoea (Jasion and Burnett, 2005). The heat-labile nature of Igs raises concerns as to the effect of processing on their biological efficacy. In the case of IgG, a high percentage of the bioactivity is retained following processing e.g. 59 – 76% after high temperature, short time (HTST) treatment (Chan et al., 1995). However, some caution is required at the point of digestion, as IgG binds only to antigens within the gastrointestinal (GI) tract if the Fab (fragment antigen-binding) structure is intact and not extensively denatured through exposure to acidic pH or the action of digestive proteolytic enzymes (Jasion and Burnett, 2005). Immunoglobulins also act synergistically along with other defence factors in milk such as lactoferrin, and the Complement system (Ellison, 1994); in fact, possibly one of the Igs' most important functions is the activation of Complement-mediated bacteriolytic reactions (Korhonen et al., 2000).

Lactoferrin

Lactoferrin is an iron-binding glycoprotein that is considered to be part of the innate immune system, but has a capacity to take part in specific immune reactions (Legrand et al., 2005). While occurring at low concentration, lactoferrin levels are considerably higher in human compared to bovine milk. Moreover, levels are elevated (~7g/L) in both colostrum and first milkings (Farnaud and Evans, 2003) which emphasises its importance in early life nutritional and biological support. A number of mechanisms

have been postulated by which lactoferrin is effective as an antimicrobial agent, e.g.(i) withholding iron from iron-requiring pathogens (Ellison, 1994), (ii) binding of the microbial membrane by interacting with lipopolysaccharide (LPS) on the surface (Appelmelk, et al., 1994), and (iii) prevention of microbial attachment to epithelial cells or enterocytes (González-Chávez et al., 2009). The amino terminal domain of lactoferrin, responsible for its outer membrane effects, has been identified as the lactoferricin peptide, which is released on digestion by pepsin. Lactoferricin operates by degrading the protein structures which are essential for bacterial attachment and invasion (Gifford et al., 2005). The broad bactericidal activity of lactoferricin and its effect on bacterial morphology are quite distinct from those of whole lactoferrin (Ellison, 1994).

Lactoperoxidase

The 'Lactoperoxidase System of Raw Milk Preservation' is currently the only approved method of raw milk preservation, apart from refrigeration, adopted by Codex Alimentarius as a guideline in 1991 (CAC GL 13/91). The Lactoperoxidase System (LP-system) operates by reactivation of the enzyme lactoperoxidase naturally present in raw milk by the addition of thiocyanate and a source of peroxide. This results in a blocking of bacterial metabolism thereby preventing the multiplication of bacteria present in the milk. The effect is classified as bacteriostatic and of limited duration due to its temperature dependency. Heme-containing peroxidase acts in combination with hydrogen peroxide to form hypothiocyanate that kills both gram-positive and gram-negative bacteria. The lactoperoxidase reaction with nitrite to produce nitric dioxide induces interactions with xanthine oxidase (XO) to regulate local inflammatory

responses and control infectious events (Silanikove et al., 2005). This native antimicrobial LP-system present in milk is actively encouraged in developing dairy countries, particularly in areas where farmers experience problems with inadequate milk storage and transport logistics. The LP-system provides badly needed shelf-life of milk samples collected under tropical conditions, and supports farm sales outside of its immediate catchment (Asaah et al, 2007, Bennett, 2005).

Lysozyme

Lysozyme is a bacterial hydrolase that catalyses the breakdown of peptidoglycan polymers of the bacterial cell wall at the β1-4 bonds between N-acetylmuramic (NAM) acid and N-acetylglucosamine (NAG) residues, thereby lysing sensitive bacteria.

Apart from its bacterial effects, lysozyme's ability to inhibit viruses and eukaryotic microorganisms that are devoid of a typical peptidoglycan layer suggests the involvement of mechanisms other than hydrolytic activity, most probably an interaction with the lipid layer of the inner membrane. However, microorganisms with a natural resistance to lysozyme are common, and many mechanisms of resistance have been discussed (Benkerroum, 2008). As an example of commercial application, egg-white sourced lysozyme is employed as an antimicrobial to lyse *Clostridium tyrobutyricum*, a spore-forming spoilage bacterium that is particularly associated with 'gas blowing' in brined-cheeses such as Gouda and Grana Padano (Fox, 2003).

Furthermore, it has been shown that a lysozyme-supplemented milk has a positive influence on the development of premature infants and can help to combat infection (Zimecki and Artym, 2005). Notably, indirect and direct interactions have been identified between the Complement system and lysozyme, whereby, the response of

polymorphonuclear leukocytes (PMN) chemotaxins to the Complement-derived is inhibited. Lysozyme's affinity for calcium is thought to inhibit Complement by chelating the divalent ions required for Complement activation (Ogundele, 1998).

Antimicrobial proteins of milk fat globule membrane (MFGM)

MFGM proteins generally that are recognised to possess antimicrobial activity are:

- Mucin 1 (MUC1) – exhibits antiviral effects and is anti-rotavirus especially in neonates;
- Mucin 15 (MUC15 or PAS III), which possesses strong antiviral properties;
- Xanthine oxidase (XO), is a bactericidal agent that works in the gut by reducing oxygen;
- Cluster of differentiation (CD36 or PAS IV), these are glycoproteins that act as receptors due to high sugar content.

Glycosylyated (glyco) proteins in MFGM are believed to prevent bacterial adhesion to the gastrointestinal wall. MFGM fractions enriched with various (glyco) proteins, particularly MUC1, stimulate the production of short chain fatty acids (butyrate) and ammonia, as well as altering the bacterial community structure following incubation with colonic microbiota for 48h (Struijs et al., 2013). Mucins play an important role in many biological processes, including the protection of epithelial surfaces in the course of immune response adhesion, inflammation, and tumour genesis. MUC15 is a high molecular weight glycoprotein which may function in an antiviral capacity (Fong and Norris, 2009).

Nearly half of XO activity in cow's milk is associated with MFGM (Charalambous, 2012). XO, also recognised as xanthine oxidoreductase (XOR) and xanthine dehydrogenase (XDH), is a molybdoflavoenzyme found at high levels in bovine mammary epithelial cells. Purified XO has long been known to inhibit the growth of bacteria such as *Staphylococcus aureus, Escherichia coli* and *Salmonella enterides* (Green and Pauli, 1943, Stevens et al., 2000). It is well documented that this antibacterial activity is attributable to the presence of a reducing substrate such as hydrogen peroxide (Hancock et al., 2002). The antibacterial activity of several MFGM hydrolysates against test food pathogens on agar plates prepared with M9 minimal media was independent of xanthine oxidase enzymatic activity (Clare et al., 2008). However, Clare et al. (2008) also observed antimicrobial effects by MFGM hydrolysates due to the generation of H_2O_2 by xanthine oxidase when brain heart infusion (BHI)-based media were used Additional MFGM constituents like CD36 (cluster of differentiation), an integral membrane protein, occurs in a wide range of cells and tissues and is recognised to play major roles in adhesion, signal transduction, and hematopathology (Greenwalt et al., 1992). It has been demonstrated that CD36 facilitates adhesion and sequestration of infected erythrocytes as well as phagocytosis of apoptotic neutrophils and in this way may afford protection (Ramussen et al., 1998).

Milk protein hydrolysates and peptides

Bioinformatics tools are increasingly being used to guide the mining of potential antimicrobial peptides encrypted in milk protein sequences. As an example, Dziuba

and Dziuba (2014) identified 11 new peptides with potential antimicrobial activity from all peptides released during *in silico* proteolysis of milk proteins using 28 enzymes. In 2006, it was reported that protein hydrolysates generated from individual milk protein fractions (casein, α-lactalbumin, β-lactoglobulin, and serum albumin) prepared by four gastrointestinal proteinases (trypsin, α-chymotrypsin, pepsin and pancreatin) exhibited varying degrees of antimicrobial activity (all hydrolysates of: casein > α-lactalbumin > β-lactoglobulin > serum albumin > ovalbumin h) towards 24 microbial strains tested (Biziulevičius et al., 2006). The influence of the proteolytic enzyme used is also influential: tryptic hydrolysates > peptic hydrolysates > chymotryptic hydrolysates > pancreatic hydrolysates (Biziulevičius et al., 2006). The antimicrobial effects of such protein hydrolysates adds to earlier studies and are consistent with the overview that mammalian body fluids containing cationic peptides/proteins (such as lysozyme, neutrophil-derived permeability-increasing peptides, defensins, etc.) are capable of killing bacteria by activating their autolytic enzymes, thus causing bacteriolysis (Biziulevičius et al., 2006).

While κ-casein is a significant, highly functional fraction of milk proteins, the antimicrobial interest focuses on its caseinomacropeptide (CMP). CMP is a heterogeneous C-terminal fragment released into whey during cheese-making as a result of chymosin-induced cleavage of the phenylalanine-methionine peptide bond at amino acid residues 105 and 106 of κ-casein. Malkoski et al. (2001) provided an early indication of the capacity of CMP to inhibit the growth of both *Streptococcus mutans*, *Porphyromonas gingivalis* and *Esherichia coli*. The sialic acid content of CMP has been shown to be responsible for the peptide's binding to *Salmonella enteritidis* and Enterohaemorrhagic *Esherichia coli* (EHEC) O157 (Nakajima et al. 2005). Using a Caco 2 cell line, Nakajima et al. (2005) also demonstrated that CMP

(*Glycomacropeptide* (GMP), as referred to by the authors) inhibited the adhesion of EHEC O157 in a dose-dependent manner, but the adhesion was not as potent when challenged with *Salmonella* infection. Both glycosylated κ-*casein* glycopeptide (KCGP) and non-glycosylated κ-*casein peptide* (KCP) forms of CMP inhibited growth of *Enterococcus faecalis* in planktonic culture equally well. However, KCGP was the significantly better of the two at inhibiting the growth of *Enterococcus faecalis* (Liu et al. 2012).

Milk oligosaccharides

Complex carbohydrates are increasingly used in the formulation of infant milks. Initial interest was driven by the prebiotic potential of fructo-oligosaccharides (FOS). Synergistic combinations of FOS and galacto-oligosaccharides (GOS) soon followed which possessed antimicrobial efficacy in addition to promoting gut microbiota (Bruzzese, et al., 2009). Oligosaccharides are polymers of monosaccharides, the prebiotic effects of which are known to promote survival of specific probiotic strains e.g. *Bifidobacterium infantis.* Acidic, also known as sialyl-, oligosaccharides featuring sialic acid attachments confer additional functionality in the form of antiadhesion due to the stereo-specificity of their sialyl containing structures (Mehra and Kelly, 2006). These decoy receptors mimic epithelial cell surface glycans in order to restrict pathogen attachment to infant mucosal surfaces (Bode, 2012). Bovine milk has a much lower concentration of sialyl oligosaccharides when compared to human milk. Given the prophylactic benefits of these milk-based carbohydrate structures, further research is needed to fully utilise these components from bovine milk (Zivkovic and Barile, 2011). Studies have focused on chemically synthesising these structures or

reproducing them through bio-fermentation using genetically modified bacteria (Espinosa, et al., 2007).

Clearly, milk contains a wide array of naturally occurring antimicrobial proteins. If these are to be fully exploited to promote human and animal health, attention must be devoted to fully understanding the possible inactivating effects which may occur during milk processing and transformation into nutritional ingredients. Such beneficial functionalities could be applied in a variety of applications which include, but may not be exclusively limited to, the reduction of mastitis in cows and improving the health of infants.

1.5. Innate immune system

Immunity refers to the ability of a host system to protect itself; the first response is the non-specific innate immune response, as this system fights microbes and plays a key role in the initialisation of the specific/adaptive immune system. As well as functioning separately, some components of the innate and adaptive immune system components play a role in both systems (Figure 1.1). Major constituents of the innate immune system include phagocytes, complement, toll-like receptors, natural killer cells and macrophages. Furthermore, there are physical barriers such as skin, mucosal surfaces of the digestive and respiratory systems, and low pH levels in the stomach.

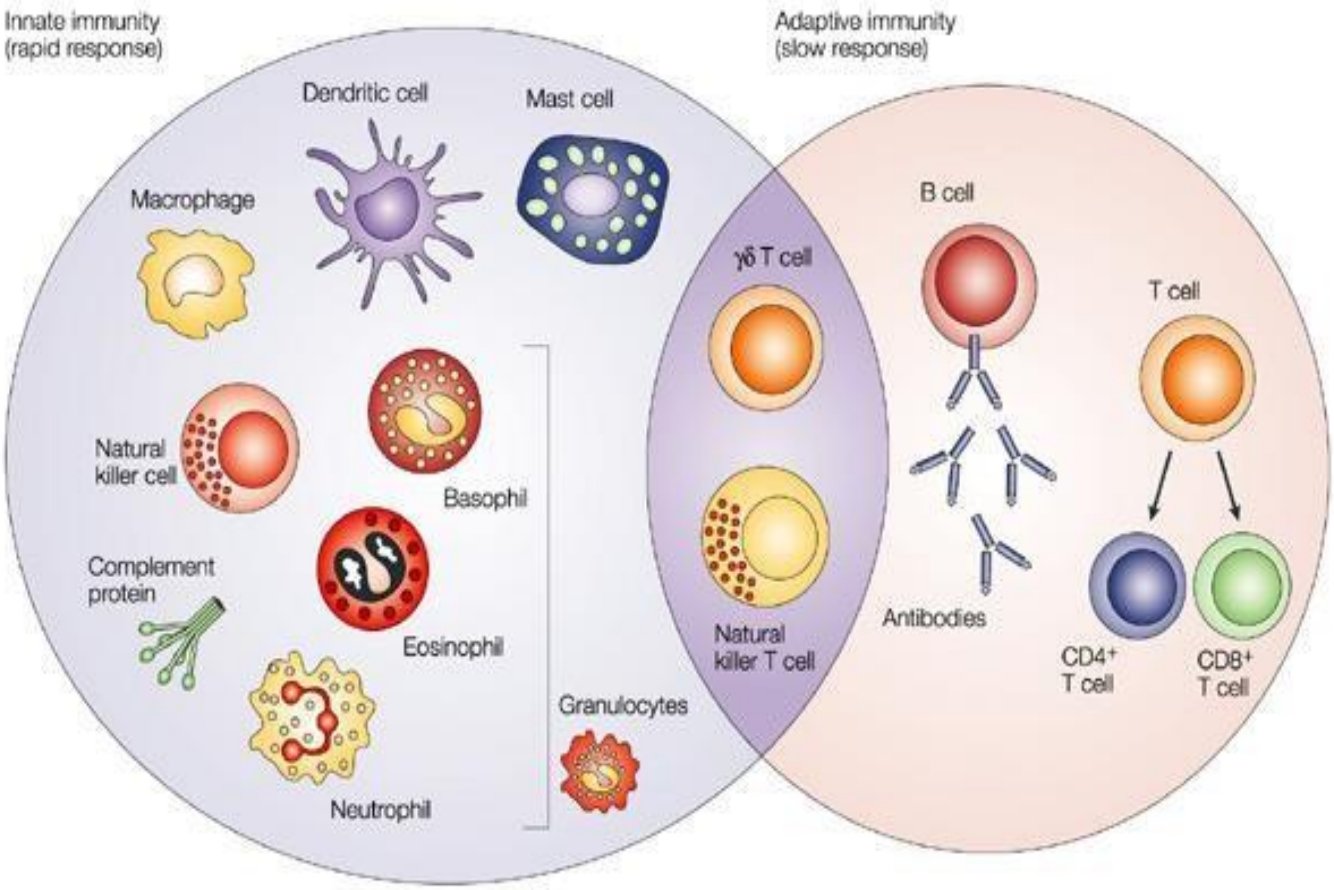


Figure 1.1 Summary of the cells of the innate and adaptive immune system (Dranoff, 2004)

During early stage infection, the innate immune system is initiated and triggers an inflammatory reaction. This inflammatory response at the site of infection, recruits macrophages, polymorphonuclear leukocytes, and mast cells through their innate immune receptors (Song et al., 2000). Phagocytes engulf the microorganisms, which in turn may release bactericidal proteins, peptides, and enzymes, which act against invading microorganisms. Complement plays a multifaceted role in the inflammatory response which includes coordinating different components and events. Activation of the Complement system leads to the formation of anaphylatoxins C3a, C4a, and C5a which are reported as potent inflammatory mediators (Markiewski, 2007). Over time,

the capacity of the innate immune system has evolved improving its capability to recognise microbial pathogens using specific identifiers which recognise invading organisms (Beutler, 2004). Pattern-recognition receptors (PRRs) have a central role in identifying components of invading microorganisms and subsequently launch a response aimed at their elimination. Inappropriate activation of PRRs can lead to prolonged inflammation which may be a root cause of some autoimmune and inflammatory diseases. Well-reported PRRs include toll-like receptors (TLRs) and Ctype Lectin Receptors (CLRs), as well as cytoplasmic NOD-like receptors (NLRs). Thus, these components are regulated by the degradation or translocation of the innate receptors themselves and through the involvement of intracellular regulators or amplifiers (Cao, 2016).

Milk produced by a healthy bovine udder should contain relatively low numbers of somatic cells (monitored by somatic cell counts (SCC)) and, thus, reflect low levels of inflammation. Milk somatic cells consist of a number of different cell types; neutrophils, macrophages, lymphocytes, and epithelial cells. In self-limiting mastitis, bacteria are eliminated after the initial inflammatory phase, neutrophil recruitment ceases and SCC returns to healthy (low) levels. Prolonged infection invokes the production of more specific immune components and also has implications for milk yield and the quality (Ogola et al., 2007, Riollet et al., 2002)

1.6.Complement system

Complement, as a fundamental part of the innate immune system, was originally identified by Jules Bordet in 1899 as a component of the plasma that enhances the

opsonisation and bactericidal effects of antibodies. The Complement system delivers a rapid and proficient means to protect the host from invading microbes. Due to its diverse biological activities, Complement is a central component in inflammation, a natural response of the host tissue to any injury. There is also increasing evidence that Complement significantly contributes to the regulation of the adaptive immune response (Kirschfink, 1997). Complement consists of a group of more than 30 plasma proteins, the main function of which are the recognition and elimination of invading microbes. Complement can be triggered either by a target-bound antibody (the classical pathway), recognition of microbial polysaccharide structures (the lectin pathway) or recognition of other foreign surface structures as yet uncharacterized (the alternative or properdin pathway) (Kuby, 1997). Once activated, it progresses in a cascading movement, whereby the activation and production of one protein induces the next, which in turn produces the next required protein and so on (Rainard and Riollet, 2003). By acting in a sequential cascading action, the Complement system stimulates a plethora of physiological responses. All three activation pathways share the common step of activating the component C3, but differ in their mechanisms of recognition. Complement plays a vital role in phagocytosis as part of the innate immune response and is also important in initiation and control of the inflammatory response (Barrio et al., 2003). Three distinct aforementioned physiological pathways, classical, alternative and the mannan binding or lectin pathway (Fig 1.2). While initiated in different ways, all converge at the C3 convertase activation stage of the cascade. The classical pathway is initiated by the binding of antigen-antibody complexes as well as due to presence of particular microbes. The mannan binding lectin (MBL) pathway may be activated by the recognition and binding of PAMPs by lectin proteins (Holmskov et al., 1996). Thus far, three members of this pathway have

been identified: MBL15, ficolin H and ficolin L16 (Carroll, 2004). The lectin pathway is a member of the collectin family of proteins.

Furthermore, the Complement system may be stimulated in the absence of antibodies as a fundamental component of the innate immune system (Kuby, 1997). The alternative pathway may be activated in circumstances where antibodies are not required, in which case a spontaneous conformational change of C3 provokes formation of an enzyme complex that cleaves further molecules in the cascade and sets in motion an amplification reaction that activates the complement (Zipfel and Skerka, 2008). The alternative pathway may be stimulated by specific bacterial cell membrane components, i.e., lipopolysaccharides. This pathway differs from the other two in that it is always 'turned on' because of the spontaneous activation of C3 and its ability to bind to a range of acceptor sites. During studies of the alternative immune system, it has been found that classical pathway is less active due to a shortage of component C1q (Rainard, 2003). The degradation of Complement component C3 into C3b and C3bi due to the ability of Factor I and Factor H confirms that the alternative pathway is active (Pangburn and Müller-Eberhard, 1984). This pathway is triggered by a spontaneous conformational change of C3, which provokes formation of an enzyme complex that cleaves further molecules in the cascade and sets in motion an amplification reaction (Zipfel and Skerka, 2008). The manner by which Complement proteins migrate into milk is still a matter of speculation.

The importance of the presence of the Complement system in HBM and mastitic bovine milk has been previously reported (Rainard and Riollet, 2006, Ogundele, 2000). Typically, it was thought that the levels of Complement in healthy bovine milk was not sufficient for chemotaxis or opsonisation; thus, the consensus has been that

the biological significance of the Complement was low (Rainard and Riollet, 2006, Rainard, 2003). It is also speculated that there may be some synergism when the antimicrobial effects of MFGM and Complement in human milk are combined (Ogundele, 1999a).

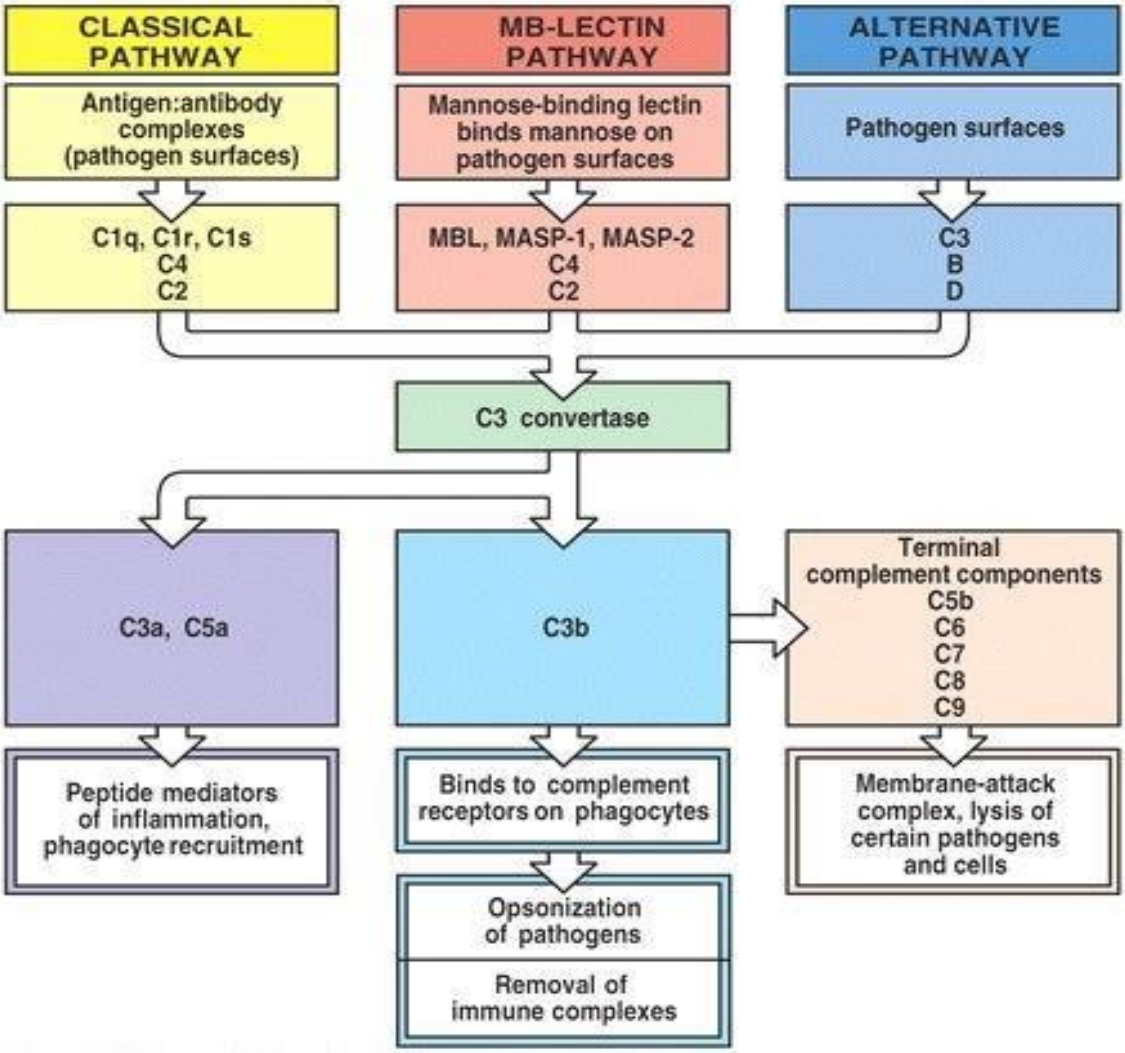


Figure 1.2 Illustration of the three Complement pathways – Classic, Alternative and MBL pathways (Janeway et al, 2005).

1.7.Mastitis

Currently the dairy food sector is one of Ireland's most important indigenous manufacturing divisions. With 85% of all dairy output exported, Ireland is ranked the

10th largest dairy export nation in the world. In 2013, export values for Irish dairy product and ingredients exceeded €3 billion. The strongest performing product categories were butter, cheese, infant formula, skim milk powder, whole milk powder and whey protein ingredients. Mastitis in dairy herds is a common occurrence which impacts on production cost and out-turns on dairy farms. Further losses may be due to the effect on milk quality, treatment costs as well as animal replacement (Whist and Østerås, 2007). Milk yield losses of approx. 100-150 kg milk/cow/lactation can also result (Erb et al., 1985, Seegers et al., 2003). In 2013, mastitis was estimated to have caused Irish farmers a net farm profit decrease of €19,504 for a 40 hectare farm as SCC increased from <100,000 cells/ml to >400,000 cells/ml (ICBF). Several studies have found relatively large associations between mastitis and fertility, thus, it can have an even greater cost for dairy farmers (Loeffler et al., 1999). Mastitis can be a persistent inflammatory response and in extreme cases can be potentially fatal. SCC is a useful secondary indicator of mastitis, it is used in quality control programmes to assist with reduction in the incidence of mastitis (Mrode and Swanson, 2003).

Bacterial mastitis pathogens have been loosely classified as either environmental or contagious pathogens. Contagious pathogens are a sub-group of bacteria which have adapted to survive within the host, and can be indicators of the presence of intramammary infections in the herd (Riekerink et al., 2006). Mastitis is mainly caused by bacterial infection - the more well-known infective strains include *Streptococcus agalactiae (Str. agalactiae), Staphylococcus aureus (S. aureus), Mycoplasma, Strep. Dysgalactiae (Str. dysgalactiae), Streptococcus uberis (Str. uberis), Pseudomonas spp, Prototheca* and yeasts. Inflammation triggered by a gram-positive bacterium *Str. agalactiae* blocks mammary ducts, leading to decreased milk production, increased

SCC and eventual involution. *S. aureus* has been identified in a number of cases of mastitis where it causes per-acute, acute, chronic, and subclinical mastitis. A further issue with this contagious form of mastitis is that some strains are antibiotic-resistant (Tenhagen et al., 2006). Another causative species of bacteria is *Prototheca*, which survive in muddy soil conditions and can induce chronic cases of mastitis; *Prototheca* infections are typically associated with cases of environmental mastitis (Costa et al., 1998). Finally, coliform-induced mastitis is usually caused by gram negative *Esherichia coli (E. coli), Klebsiella spp., Enterobacter spp*., and *Citrobacter spp* (Smith et al., 1985).

Consequently, stringent hygiene practices in milking parlours, including clean bedding, clean housing and sanitisation of equipment and teat-dipping, are advised. Many countries including UK, Germany and Finland, which have succeeded in reducing the incidence of subclinical mastitis through good hygienic practices have also reduced the prevalence of the main mastitis causing pathogen (Pitkälä et al., 2004).

1.8.Assaying the bacteriostatic/bactericidal effects of milk

Milk has long been recognised to resist infection; originally the bacteriostatic qualities of milk were reported by Hanssen in (1924) who speculated that the presence of oxidising enzymes had bactericidal properties. Moreover, lactoferrin and IgA in milk

have been identified to contribute to protection. However, it has been determined that serum proteins, specifically Complement, are lethal to some specific gram negative bacteria (Taylor, 1983). Exploiting this knowledge, a more specific assay was customised to test milk samples; a bactericidal sequestration assay was developed in 1975 to help with the identification of the bactericidal and haemolytic activity, which more recently has been used to assess the Complement activity of human milk samples (Brock et al., 1974; Ogundele, 2001). Brock et al reported further evidence of the bactericidal effects of milk and colostrum but did not differentiate whether it was due to Complement-mediated response or the activity of immunoglobulins (IgG, IgM) in milk, or both. The background of this assay is based on a reported conformation change in serum-sensitive bacteria, whereby increased sensitivity to bactericidal action is noted. This structural change involves the loss of lipopolysaccharide as well as other characteristics, and thus, may provide some explanations as to why the group of bacteria with the O- antigen are more susceptible to this (Rowley, 1968). The bactericidal sequestration assay established in this study utilises the knowledge that gram-negative bacteria (e.g. *E. coli*) are sensitive to complement lytic action.

1.9. *Escherichia coli* O111

Escherichia coli (*E. coli*) is a well-recognised gram negative bacterium which is commonly found in intestinal flora of warm blooded organisms. The rod shaped bacterium is characterised as a facultative anaerobe (Olsvik et al., 1991). *E. coli* is one of the most extensively studied prokaryotic model organisms that plays a major role

in biotechnology and microbiology. Furthermore, it is a major species among the facultative anaerobic normal flora of the intestine and plays an important role in maintaining intestinal physiology. *E. coli* O111 was originally discovered in 1945 and was isolated from a faecal sample of gastroenteritis; it has also been associated with neonatal diarrhoea in infants (Reiter and Brock, 1975, Coleman et al., 1977). It has since been found in other sources, i.e., bovine mastitis, chicken faeces, piglet enteritis, as well as cats, dogs and kittens (Taylor 1961). It was one of the first strains implicated in cases of gastroenteritis in the 1940s, and has been classified as a classic serogroups of enteropathogenic *E. coli* (EPEC). EPEC's are often responsible for infantile GI infections, thus, antibodies against these bacteria play a significant part of the protection in human milk (Marcy, 1976, Mietens, et al, 1975). *E. coli* O111 has been implicated in numerous outbreaks of enteric disease, including relapsing diarrheal illness and in a community outbreak of diarrhoea in Finland (Campos et al., 1994).

The *E. coli* O111 strain has been identified to be susceptible to Complement activity, it has been considered that the co-evolution of bacteria with complement systems for millions of years, allowed some specific microorganisms to develop the ability to inhibit the activation of complement pathways.

1.10. Effect of cow breed on milk composition

Domesticated cattle play an important role in modern farming and contribute to global populations nutritional requirements through milk, meat ingredients and by-products. Cattle have long been farmed, and different breeds have evolved due to the influence of man and the evolutionary effects of climate, disease and geographic separation

(Blott et al., 1998). Cattle genetics have been monitored since the eighteenth century by considering physical appearances such as horn morphology and coat colouring in the course of adapting breeds for domestication and commercial purposes. There are two taxa of cattle; zebu (*Bos indicus*) and taurine (*Bos taurus*) (MacHugh et al., 1997); in total, there 790 breeds of cattle have been identified and, of this, approximately 270 breeds are native to Europe (Blott et al., 1998). The major distinctions which separate the two taxa of domestic cattle are the presence of a hump, large dewlap, skin anatomy, and physiological characteristics that involve adaptation to arid conditions (Bradley et al., 1998). Recent studies have used mitochondrial DNA to investigate the sequence diversity of cattle populations; analysis of mtDNA displacement loop sequences have indicated that the two main domestic cattle species have distinct haplotype profiles (Hristov et al., 2015). There have been reports that inherited traits may play a significant role in host immunity, bovine lymphocyte antigen (BoLA) alleles are associated with disease incidence, i.e., class I and class II BoLA genes have been associated with the incidence of mastitis, bovine leukaemia virus infection, chronic posterior spinal paresis, and total parasitic load (Dietz et al., 1997).

Cattle genetics and breeding may be utilised in the course of selecting for increased resistance to clinical mastitis, while until now indirect selection has been practiced widely by monitoring SCCs. It has been suggested that the bacteriolytic enzyme lysozyme may be used as an index of macrophage functional status; in this way, the Lyz gene is suggested as a candidate marker for improvement of mastitis resistance (Chen et al., 2013).

1.11. Concluding remarks

The review of literature in this Chapter has focussed largely on profiling the protective effects of components in bovine milk. In contrast to HBM, there is a paucity of information relating to the transfer of immune components to the milk and, indeed, the comparability between the biological efficacy of such components contributed by the human and bovine. Formula-fed infants would appear to be at a disadvantage compared to their breast-fed counterparts as continuing efforts at the 'humanisation' of bovine milk to date have stopped short of addressing such a gap. Thus, it is becoming an area of great interest for the dairy sector, and there is a need to improve the health benefits and functionality of infant formulas. To begin with, it is important to understand more about the extent to which Complement as a major innate immune component occurs in bovine milk and how well it compares with that of HBM. It is also of interest to understand whether Complement in bovine milk associates with MFGM. Thus, in the following Chapter, an initial assessment of Complement activity in freshly drawn bovine milk is explored.

1.12. Thesis Objectives

1. In the absence of established data about the presence and activity of Complement in bovine milk, it was firstly necessary to set up the appropriate analytical methodology and a milk sample collection plan to monitor

Complement activity in milk.

2. To study the effects of heat treatment and cream separation, by simulating the effects of industrial dairy manufacturing processes.

3. To explore the possible association between Complement and milk fat globule membrane.

4. To evaluate an improvement of the bacterial sequestration assay for monitoring of Complement using a biotechnological adaptation to confer fluorescence functionality to the Complement-sensitive target organism.

5. Evaluate differences in Complement activity between the infected and healthy quarters of lactating dairy cows during induced mastitis in udder quarters.

6. To monitor the contribution of dairy breed to Complement activity in milk by milk sampling from purebred and cross-bred herds of commercially important milking breeds

1.13. References

ADLEROVA, L., BARTOSKOVA, A. AND FALDYNA, M., 2008. Lactoferrin: a review. *Veterinarni Medicina*, *53*(9), pp. 457-468.

ASAAH, N.O., FONTEH, F., KAMGA, P., MENDI, S. AND IMELE, H., 2007. Activation of the lactoperoxidase system as a method of preserving raw milk in areas without cooling facilities. *African Journal of Food, Agriculture, Nutrition and Development, 7*(2), pp. 1-15.

AMERICAN ACADEMY OF PEDIATRICS, TASK FORCE ON SUDDEN INFANT DEATH SYNDROME. 2005. The changing concept of sudden infant death syndrome: diagnostic coding shifts, controversies regarding the sleeping environment, and new variables to consider in reducing risks. *Paediatrics, 116*(5), pp. 1245–1255.

APPELMELK, B.J., AN, Y.Q., GEERTS, M., THIJS, B.G., DE BOER, H.A., MACLAREN, D.M., DE GRAAFF, J. AND NUIJENS, J.H., 1994. Lactoferrin is a lipid A-binding protein. *Infection and Immunity*, *62*(6), pp. 2628-2632.

BALLARD, O. AND MORROW, A. L. 2013. Human milk composition: nutrients and bioactive factors. *Pediatric Clinics of North America, 60*(1)**,** pp. 49-74.

BARRIO, M. B., RAINARD, P. AND POUTREL, B. 2003. Milk complement and the opsonophagocytosis and killing of *Staphylococcus aureus* mastitis isolates by bovine neutrophils. *Microbial Pathogenesis, 34*(1)**,** pp. 1-9.

BENKERROUM, N., 2008. Antimicrobial activity of lysozyme with special relevance to milk. *African Journal of Biotechnology*, *72*(25), pp. 4856-4867.

BENNETT A. 2005. Benefits and potential risks of the lactoperoxidase system of raw milk preservation: report of an FAO/WHO technical meeting. Rome: FAO headquarters, November 28 – December 2; [report coordinated by Anthony Bennett . . .[et al.]; edited by James Edge] [M]. Food & Agriculture Org., 2006.

BEUTLER, B., 2004. Innate immunity: an overview. *Molecular Immunology*, *40*(12), pp. 845-859.

BIANCHI, L., PUGLIA, M., LANDI, C., MATTEONI, S., PERINI, D., ARMINI, A., VERANI, M., TROMBETTA, C., SOLDANI, P., RONCADA, P., GREPPI, G., PALLINI, V. AND BINI, L. 2009. Solubilization methods and reference 2-DE map of cow milk fat globules. *Journal of Proteomics, 72*(5), pp. 853-864.

BIESALSKI, H.-K., DRAGSTED, L. O., ELMADFA, I., GROSSKLAUS, R., MÜLLER, M., SCHRENK, D., WALTER, P. AND WEBER, P. 2009. Bioactive compounds: Definition and assessment of activity. *Nutrition, 25*(11)**,** pp. 1202-1205.

BIZIULEVIČIUS, G.A., KISLUKHINA, O.V., KAZLAUSKAITĖ, J. AND ŽUKAITĖ, V., 2006. Food-protein enzymatic hydrolysates possess both antimicrobial and immune-stimulatory activities: a ‘cause and effect’ theory of bi-functionality. *FEMS Immunology and Medical Microbiology*, *46*(1), pp. 131-138.

BLOTT, S. C., WILLIAMS, J. L. AND HALEY, C. S. 1998. Genetic relationships among European cattle breeds. *Animal Genetics*, *29*(4), pp. 273-282.

BODE, L., 2012. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*, *22*(9), pp. 1147-1162.

BRADLEY, D. G., LOFTUS, R. T., CUNNINGHAM, P. AND MACHUGH, D. E. 1998. Genetics and domestic cattle origins. *Evolutionary Anthropology: Issues, News, and Reviews, 6*(3), pp. 79-86.

BROCK, J.H., ORTEGA, F. AND PINEIRO, A. 1974. Bactericidal and haemolytic activity of complement in bovine colostrum and serum: effect of proteolytic enzymes and ethylene glycol tetraacetic acid (EGTA). *Annales D'Immunologie*, *126*(4), pp. 439-451.

BRUZZESE, E., VOLPICELLI, M., SQUEGLIA, V., BRUZZESE, D., SALVINI, F., BISCEGLIA, M., LIONETTI, P., CINQUETTI, M., IACONO, G., AMARRI, S. AND GUARINO, A., 2009. A formula containing galacto-and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: an observational study. *Clinical Nutrition*, *28*(2), pp. 156-161.

CAMPOS, L. C., WHITTAM, T. S., GOMES, T., ANDRADE, J. AND TRABULSI, L. R. 1994. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infection and Immunity, 62*(8), pp. 3282-3288.

CAO, X. 2016. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nature Reviews Immunology, 16*(1), pp. 35-50.

CARROLL, M.C., 2004. The complement system in regulation of adaptive immunity. *Nature Immunology*, *5*(10), pp. 981-986.

CHARALAMBOUS, G., 2012. Bovine milk xanthine oxidase. *Instrumental Analysis of Food V2: Recent Progress* (2). Elsevier.

CHATTERTON, D. E., NGUYEN, D. N., BERING, S. B. AND SANGILD, P. T. 2013. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The International Journal of Biochemistry and Cell Biology, 45*(8), pp. 1730-1747.

CHEN, R., WANG, Z., YANG, Z., MAO, Y., JI, D., ZHU, X. AND HAMZA, A. E. 2013. A novel SNP of lysozyme gene and its association with mastitis trait in Chinese Holstein. *Arch Tierz, 56*, pp. 58.

CLARE, D.A., ZHENG, Z., HASSAN, H.M., SWAISGOOD, H.E. AND CATIGNANI, G.L., 2008. Antimicrobial properties of milkfat globule membrane fractions. *Journal of Food Protection*, *71*(1), pp. 126-133.

CODEX ALIMENTARIUS., 1991 Guidelines for the preservation of raw milk by use of the Lactoperoxidase system. CAC/GL pp. 13-1991.

CODEX STANDARD FOR INFANT FORMULA CODEX STAN 72-1981 (amended 1983, 1985, 1987, 1997). *Food and Agriculture Organization of the United Nations and World Health Organization, Rome, Italy.* Codex Alimentarius Commission.

COLE, F., SCHNEEBERGER, E., LICHTENBERG, N. AND COLTEN, H. 1982. Complement biosynthesis in human breast-milk macrophages and blood monocytes. *Immunology, 46*(2), pp. 429.

COLEMAN, W., GOEBEL, P. J. AND LEIVE, L. 1977. Genetic analysis of *Escherichia coli* O111: B4, a strain of medical and biochemical interest. *Journal of Bacteriology, 130*(2), pp. 656-660.

COSTA, E.O., RIBEIRO, A.R., WATANABE, E.T. AND MELVILLE, P.A., 1998. Infectious bovine mastitis caused by environmental organisms. *Journal of Veterinary Medicine, Series B, 45*(1-10), pp. 65-71.

DEWETTINCK, K., ROMBAUT, R., THIENPONT, N., LE, T. T., MESSENS, K. AND VAN CAMP, J. 2008. Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal, 18*(5), pp. 436-457.

DIETZ, A. B., DETILLEUX, J., FREEMAN, A., KELLEY, D., STABEL, J. AND KEHRLI, M. 1997. Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle. *Journal of Dairy Science, 80*(2), pp. 400-405.

DRANOFF, G. 2004. Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer, 4*(1)**,** pp. 11-22.

DZIUBA, B. AND DZIUBA, M., 2014. New milk protein-derived peptides with potential antimicrobial activity: An approach based on bioinformatic studies. *International Journal of Molecular Sciences, 15*(8), pp. 14531-14545.

ELLISON III, R.T., 1994. The effects of lactoferrin on gram-negative bacteria. *Lactoferrin*, pp. 71-90. Springer US.

EMMETT, P. M. AND ROGERS, I. S. 1997. Properties of human milk and their relationship with maternal nutrition. *Early Human Development, 49,* pp. S7-S28.

ERB, H., SMITH, R., OLTENACU, P., GUARD, C., HILLMAN, R., POWERS, P., SMITH, M. AND WHITE, M. 1985. Path model of reproductive disorders and performance, milk fever, mastitis, milk yield, and culling in Holstein cows. *Journal of Dairy Science, 68*(12), pp. 3337-3349.

ESPINOSA, R.M., TAMÉZ, M. AND PRIETO, P., 2007. Efforts to emulate human milk oligosaccharides. *British Journal of Nutrition*, *98*(1), pp. S74-S79.

EUBER, J. AND BRUNNER, J. 1984. Re-examination of fat globule clustering and creaming in cow milk. *Journal of Dairy Science*, *67*(12), pp. 2821-2832.

FARNAUD, S. AND EVANS, R.W., 2003. Lactoferrin—a multifunctional protein with antimicrobial properties. *Molecular Immunology*, *40*(7), pp. 395-405.

FONG, B.Y. AND NORRIS, C.S., 2009. Quantification of milk fat globule membrane proteins using selected reaction monitoring mass spectrometry. *Journal of Agricultural and Food Chemistry*, *57*(14), pp. 6021-6028.

FOX, P.F., 2003. Significance of indigenous enzymes in milk and dairy products. Handbook of Food Enzymology - Food Science and Technology, New York – Marcel Dekker, pp. 255-278.

FRIEL, J.K. AND QASEM, W.A., 2016. Human Milk and Infant Formula: Nutritional Content and Health Benefits. In *Beverage Impacts on Health and Nutrition* (pp. 163-177). Springer International Publishing.

GALLIER, S., VOCKING, K., POST, J. A., VAN DE HEIJNING, B., ACTON, D., VAN DER BEEK, E. M. AND VAN BAALEN, T. 2015. A novel infant milk formula concept: Mimicking the human milk fat globule structure. *Colloids and Surfaces B: Biointerfaces,* 136, pp. 329-339.

GENERAL, U.S., 2011. The Surgeon General's call to action to support breastfeeding. *Rockville (MD)*.

GIFFORD, J.L., HUNTER, H.N. AND VOGEL, H.J., 2005. Lactoferricin. *Cellular and Molecular Life Sciences*, *62*(22), pp. 2588-2598.

GONZÁLEZ-CHÁVEZ, S.A., ARÉVALO-GALLEGOS, S. AND RASCÓN-CRUZ, Q., 2009. Lactoferrin: structure, function and applications. *International Journal of Antimicrobial Agents*, *33*(4), pp. 301-e1.

GREEN, D. E., AND PAULI, R., 1943. The antibacterial action of the xanthine oxidase system. Proceedings of the *Society for Experimental Biology and Medicine*, *54*(1), pp. 148–150.

GREENWALT, D.E., LIPSKY, R.H., OCKENHOUSE, C.F., IKEDA, H., TANDON, N.N. AND JAMIESON, G.A., 1992. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood*, *80*(5), pp. 1105-1115.

HAMOSH, M., PETERSON, J. A., HENDERSON, T. R., SCALLAN, C. D., KIWAN, R., CERIANI, R. L., ARMAND, M., MEHTA, N. R. AND HAMOSH, P. 1999. Protective function of human milk: the milk fat globule. *Seminars in Perinatology*, *23*(3), pp. 242-249.

HAMOSH, M., 2001. Bioactive factors in human milk. *Pediatric Clinics of North America*, *48*(1), pp. 69-86.

HANCOCK, J.T., SALISBURY, V., OVEJERO-BOGLIONE, M.C., CHERRY, R., HOARE, C., EISENTHAL, R. AND HARRISON. R., 2002. Antimicrobial properties of milk: dependence on presence of xanthine oxidase and nitrite. *Antimicrobial Agents and Chemotherapy*, *46*(10), pp. 3308-3310.

HOLMSKOV, U., FISCHER, P. B., ROTHMANN, A. AND HØJRUP, P. 1996. Affinity and kinetic analysis of the bovine plasma C-type lectin collectin-43 (CL-43) interacting with mannan. *FEBS Letters, 393*(2-3)**,** pp. 314-316.

HORTA B, BAHL R, MARTINES J, VICTORA CG. 2006. Evidence on the long-term effects of breastfeeding: systematic reviews and meta-analyses. Study commissioned by WHO/CAH.

HOWIE, P. W., FORSYTH, J. S., OGSTON, S. A., CLARK, A. AND FLOREY, C. 1990. Protective effect of breast feeding against infection. *BMJ, 300* (6716)**,** pp. 11-16.

HRISTOV, P., TEOFANOVA, D., NEOV, B., SHIVACHEV, B. AND RADOSLAVOV, G. 2015. Mitochondrial diversity in autochthonous cattle breeds from the Balkan Peninsula. *Czech Journal of Animal Sciences 60***,** pp. 311-318.

HUPPERTZ, T. AND KELLY, A.L., 2006. Physical chemistry of milk fat globules. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 173-212). Springer US.

ICBF. *Mastitis* [Online]. Available: http://www.icbf.com/?page_id=2274.

JANEWAY CJ, TRAVERS P, WALPORT M, SHLOMCHIK M. 2005, The distribution and functions of immunoglobulin isotypes. In *Immunobiology: The Immune System in Health and Disease*. 5th edition. Garland Science.

JANEWAY JR, C. A., TRAVERS, P., WALPORT, M. AND SHLOMCHIK, M. J. *The distribution and functions of 1370 immunoglobulin isotypes*. Garland Science; 2001.

JASION, V.S. AND BURNETT, B.P., 2015. Survival and digestibility of orallyadministered immunoglobulin preparations containing IgG through the gastrointestinal tract in humans. *Nutrition Journal*, *14*(1).

JENNESS, R., 1979. The composition of human milk. In *Seminars in Perinatology*, *3*(3), pp. 225-239.

KAZLAUSKAITĖ, J., BIZIULEVIČIUS, G.A., ŽUKAITĖ, V., BIZIULEVIČIENĖ, G., MILIUKIENĖ, V. AND ŠIAURYS, A., 2005. Oral tryptic casein hydrolysate enhances phagocytosis by mouse peritoneal and blood phagocytic cells but fails to prevent induced inflammation. *International Immunopharmacology*, *5*(13), pp. 1936-1944.

KIRSCHFINK, M., 1997. Controlling the complement system in inflammation. *Immunopharmacology*, *38*(1), pp. 51-62.

KORHONEN, H. AND MARNILA, P., 2009. *Bovine milk immunoglobulins against microbial human diseases*, pp. 269-289. Oxford, UK: Woodhead.

KORHONEN, H. J., MARNILA, P. AND CORREDIG, M. 2009. Bovine milk immunoglobulins against microbial human diseases. *Dairy-Derived Ingredients: Food and Nutraceutical uses***,** Elsevier, pp. 269-289.

KORHONEN, H., MARNILA, P. AND GILL, H. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition, 84***,** pp. 75-80.

KORHONEN, H., MARNILA, P. AND GILL, H.S., 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition*, *84*, pp. 75-80.

KOSTRABA, J. N., CRUICKSHANKS, K. J., LAWLER-HEAVNER, J., JOBIM, L. F., REWERS, M. J., GAY, E. C., CHASE, H. P., KLINGENSMITH, G. AND HAMMAN, **R. F.** 1993. Early exposure to cow's milk and solid foods in infancy, genetic predisposition, and risk of IDDM. *Diabetes, 42*(2)**,** pp. 288-295.

KUBY, J. 1997. Immunology. W. H. Freeman and Company, New York, NY, USA.

KULSKI, J.K. AND HARTMANN, P.E., 1981. Changes in human milk composition during the initiation of lactation. *The Australian journal of experimental biology and medical science, 59*(1), pp. 101-114.

LE, T.T., DEBYSER, G., GILBERT, W., STRUIJS, K., VAN CAMP, J., VAN DE WIELE, T., DEVREESE, B. AND DEWETTINCK, K., 2013. Distribution and isolation of milk fat globule membrane proteins during dairy processing as revealed by proteomic analysis. *International Dairy Journal*, *32*(2), pp. 110-120.

LEGRAND D., ELASS E., CARPENTIER M., MAZURIER J., 2005. Lactoferrin: a modulator of immune and inflammatory responses. *Cellular and Molecular Life Sciences*, *62*(22), pp. 2549–2559.

LI-CHAN, E., KUMMER, A., LOSSO, J.N., KITTS, D.D. AND NAKAI, S., 1995. Stability of bovine immunoglobulins to thermal treatment and processing. *Food Research International*, *28*(1), pp. 9-16.

LIU, V., DASHPER, S., PARASHOS, P., LIU, S.W., STANTON, D., SHEN, P., CHIVATXARANUKUL, P. AND REYNOLDS, E.C., 2012. Antibacterial efficacy of casein-derived peptides against *Enterococcus faecalis*. *Australian Dental Journal*, *57*(3), pp. 339-343.

LOEFFLER, S. H., DE VRIES, M. J. AND SCHUKKEN, Y. H. 1999. The

effects of time of disease occurrence, milk yield, and body condition on fertility of dairy cows. *Journal of Dairy Science*, *82*(12), pp. 2589-2604.

LÖNNERDAL, B., FORSUM, E., GEBRE-MEDHIN, M. AND HAMBRAEUS, L. 1976. Breast milk composition in Ethiopian and Swedish mothers. II. Lactose, nitrogen, and protein contents. *The American Journal of Clinical Nutrition, 29*(10), pp. 1134-1141.

LÖNNERDAL, B., 2003. Nutritional and physiologic significance of human milk proteins. *The American Journal of Clinical Nutrition*, *77*(6), pp. 1537S-1543S.

LÖNNERDAL, B., 2014. Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. *The American Journal of Clinical Nutrition*, *99*(3), pp. 712S-717S.

LOPEZ, C. 2011. Milk fat globules enveloped by their biological membrane: unique colloidal assemblies with a specific composition and structure. *Current Opinion in Colloid and Interface Science, 16*(5), pp. 391-404.

MACHUGH, D. E., SHRIVER, M. D., LOFTUS, R. T., CUNNINGHAM, P. AND BRADLEY, **D. G.** 1997. Microsatellite DNA variation and the evolution, domestication and phylogeography of taurine and zebu cattle (Bos taurus and Bos indicus). *Genetics, 146*(3), pp. 1071-1086.

MALKOSKI, M., DASHPER, S.G., O'BRIEN-SIMPSON, N.M., TALBO, G.H., MACRIS, M., CROSS, K.J. AND REYNOLDS, E.C., 2001. Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrobial Agents and Chemotherapy*, *45*(8), pp. 2309-2315.

MARCY, M. S. 1976. Microorganisms responsible for neonatal diarrea. In: *Infections Diseases of the Foetus and Newborn Infant*, edited by Remington and Kleim Saunders Co. 1976.

MARKIEWSKI, M.M. AND LAMBRIS, J.D., 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *The American Journal of Pathology*, *171*(3), pp. 715-727.

MATHER, I. H. AND KEENAN, T. 1975. Studies on the structure of milk fat globule membrane. *Journal of Membrane Biology*, *21*(1)**,** pp. 65-85.

MEHRA, R. AND KELLY, P., 2006 Milk oligosaccharides: structural and technological aspects. *International Dairy Journal 16*(11), pp. 1334-1340.

MIETENS, C., KEINHORST, H., HILPERT, H., GERBER, H., AMSTER, H. AND PAHUD, J.J., 1979. Treatment of infantile *E. coli* gastroenteritis with specific bovine anti- *E. coli* milk immunoglobulins. *European Journal of Pediatrics*, *132*(4), pp. 239-252.

MRODE, R.A. AND SWANSON, G.J.T., 2003. Estimation of genetic parameters

for somatic cell count in the first three lactations using random regression. *Livestock Production Science*, *79*(2), pp. 239-247.

MUELLER, R., CARROLL, E.J. AND PANICO, L., 1982. Complement C 3 levels and haemolytic activity in normal and mastitic whey. *Zentralblatt für Veterinärmedizin Reihe B*, *29*(2), pp. 99-106.

NAKAJIMA, K., TAMURA, N., KOBAYASHI-HATTORI, K., YOSHIDA, T., HARA-KUDO, Y., IKEDO, M., SUGITA-KONISHI, Y. AND HATTORI, M., 2005. Prevention of intestinal infection by glycomacropeptide. *Bioscience, Biotechnology, and Biochemistry*, *69*(12), pp. 2294-2301.

OGOLA, H., SHITANDI, A. AND NANUA, J., 2007. Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science*, *8*(3), pp. 237-242.

OGUNDELE, M.O., 1998. A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation. *Mediators of Inflammation*, *7*(5), pp. 363-365.

OGUNDELE, M.O., 1999a. Anti-complement activities of human breast-milk. *Inflammation Research*, *48*(8), pp. 437-445.

OGUNDELE, M.O., 1999b. Inhibitors of complement activity in human breastmilk: a proposed hypothesis of their physiological significance. *Mediators of Inflammation*, *8*(2), pp. 69-75.

OGUNDELE, M. O. 2000. Activation and deposition of human breast-milk complement C3 opsonins on serum sensitive *Escherichia coli* O111. *Journal of Reproductive Immunology*, *48*(2), pp. 99-105.

OGUNDELE, M.O., 2001. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. *Immunology and Cell Biology*, *79*(1), pp. 1-10.

OLSVIK, Ø., WASTESON, Y., LUND, A. AND HORNES, E. 1991. Pathogenic *Escherichia coli* found in food. *International Journal of Food Microbiology*, *12*(1), pp. 103-113.

PANGBURN, M. K. AND MÜLLER-EBERHARD, H. J. 1984. *The alternative pathway of complement*. Springer Seminars in Immunopathology. Springer, (Vol 2-3) pp. 163-192.

PARK, Y. W. 2009. *Bioactive components in milk and dairy products*, John Wiley Blackwell.

PARODI, P. W. 1998. A role for milk proteins in cancer prevention. *Australian Journal of Dairy Technology*, *53*(1), pp. 37.

PITKÄLÄ, A., HAVERI, M., PYÖRÄLÄ, S., MYLLYS, V. AND HONKANEN-BUZALSKI, T., 2004. Bovine mastitis in Finland 2001—Prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science*, *87*(8), pp.

2433-2441.

RAINARD, P. AND POUTREL, B. 1995. Deposition of complement components on Streptococcus agalactiae in bovine milk in the absence of inflammation. *Infection and Immunity*, *63*(9), pp. 3422-3427.

RAINARD, P., SARRADIN, P., PAAPE, M.J. AND POUTREL, B., 1998. Quantification of C5a/C5adesArg in bovine plasma, serum and milk. *Veterinary Research*, *29*(1), pp. 73-88.

RAINARD, P. AND POUTREL, B. 2000. Generation of complement fragment C5a in milk is variable among cows. *Journal of Dairy Science*, *83*(5), pp. 945-951.

RAINARD, P. AND RIOLLET, C. 2006. Innate immunity of the bovine mammary gland. *Veterinary Research*, *37*(3), pp. 369-400.

RAINARD, P. 2003. The complement in milk and defense of the bovine mammary gland against infections. *Veterinary Research*, *34*(5), pp. 647-670.

RAINARD, P. AND RIOLLET, C., 2003. Mobilization of neutrophils and defense of the bovine mammary gland. *Reproduction Nutrition Development*, *43*(5), pp. 439-457.

RASMUSSEN, J.T., BERGLUND, L., RASMUSSEN, M.S. AND PETERSEN, T.E., 1998. Assignment of di-sulfide bridges in bovine CD36. *European*

Journal of Biochemistry, *257*(2), pp. 488-494.

REITER, B. AND BROCK, J. 1975. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype O111. *Immunology*, *28*(1), pp. 71.

RIEKERINK, R., BARKEMA, H. W., VEENSTRA, S., POOLE, D. E., DINGWELL, R. T. AND KEEFE, G. P. 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. *Canadian Veterinary Journal*, *47*(6), pp. 567.

RIOLLET, C., RAINARD, P. AND POUTREL, B., 2002. Cells and cytokines in inflammatory secretions of bovine mammary gland. In *Biology of the Mammary Gland*, pp. 247-258. Springer US.

ROWLEY, D. 1968. Sensitivity of rough gram-negative bacteria to the bactericidal action of serum. *Journal of Bacteriology*, *95*(5), pp. 1647-1650.

SEEGERS, H., FOURICHON, C. AND BEAUDEAU, F. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research*, *34*(5), pp. 475-491.

SILANIKOVE, N., SHAPIRO, F., SHAMAY, A. AND LEITNER, G., 2005. Role of xanthine oxidase, lactoperoxidase, and NO in the innate immune system of

mammary secretion during active involution in dairy cows: manipulation with casein hydrolyzates. *Free Radical Biology and Medicine*, *38*(9), pp. 1139-1151.

SMITH, K.L., TODHUNTER, D.A. AND SCHOENBERGER, P.S., 1985. Environmental Mastitis: Cause, Prevalence, Prevention 1, 2. *Journal of Dairy Science*, *68*(6), pp. 1531-1553.

SONG, W.-C., ROSA SARRIAS, M. AND LAMBRIS, J. D. 2000. Complement and innate immunity. *Immunopharmacology*, *49*(1), pp. 187-198.

SPITSBERG, V. L. 2005. Invited Review: Bovine Milk Fat Globule Membrane as a Potential Nutraceutical. *Journal of Dairy Science*, *88*(7), pp. 2289-2294.

STEVENS, C.R., MILLAR, T.M., CLINCH, J.G., KANCZLER, J.M., BODAMYALI, T. AND BLAKE, D.R., 2000. Antibacterial properties of xanthine oxidase in human milk. *The Lancet*, *356*(9232), pp. 829-830.

STRUIJS, K., VAN DE WIELE, T., LE, T.T., DEBYSER, G., DEWETTINCK, K., DEVREESE, B. AND VAN CAMP, J., 2013. Milk fat globule membrane glycoproteins prevent adhesion of the colonic microbiota and result in increased bacterial butyrate production. *International Dairy Journal*, *32*(2), pp. 99-109.

TAYLOR , J. 1961. Host specificity and enteropathogenicity of *Escherichia coli*. *Journal of Applied Microbiology*, *24*(3), pp. 316-325.

TAYLOR, P.W., 1983. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiological Reviews*, *47*(1), pp. 46.

TENHAGEN, B.A., KÖSTER, G., WALLMANN, J. AND HEUWIESER, W., 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *Journal of Dairy Science*, *89*(7), pp. 2542-2551.

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, U.S. PUBLIC HEALTH SERVICE, OFFICE OF THE SURGEON GENERAL; U.S. PUBLIC HEALTH SERVICE, OFFICE ON WOMEN'S HEALTH. The Surgeon General's Call to Action to Support Breastfeeding. Washington: U.S. Department of Health and Human Services, U.S. Public Health Service, Office of the Surgeon General; 2011.

WHIST, A.C. AND ØSTERÅS, O., 2007. Associations between somatic cell counts at calving or prior to drying-off and clinical mastitis in the remaining or subsequent lactation. *Journal of Dairy Research*, *74*(01), pp. 66-73.

VAN HOOIJDONK, A.C., KUSSENDRAGER, K.D. AND STEIJNS, J.M., 2000. In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *British Journal of Nutrition*, *84*(S1), pp. 127-134.

VON KRIES, R., KOLETZKO, B., SAUERWALD, T., VON MUTIUS, E., BARNERT, D., GRUNERT, V. AND VON VOSS, H. 1999. Breast feeding and obesity: cross sectional study. *British Medical Journal, 319*(7203)**,** pp. 147-150.

WALSTRA, P., 1995. Physical chemistry of milk fat globules. *Advanced Dairy Chemistry – 2*, Chapman and Hall, London.

ZACZEK, M. AND KEENAN, T. 1990. Morphological evidence for an endoplasmic reticulum origin of milk lipid globules obtained using lipidselective staining procedures. *Protoplasma, 159*(2-3)**,** pp. 179-183.

ZHAO, Y., KENT, S.B. AND CHAIT, B.T., 1997. Rapid, sensitive structure analysis of oligosaccharides. *Proceedings of the National Academy of Sciences*, *94*(5), pp. 1629-1633.

ZIMECKI, M., ARTYM, J., 2005. Therapeutic properties of proteins and peptides from colostrum and milk. *Postepy Hig. Med. Dosw*., *59*, pp. 309-323

ZIPFEL, P. F. AND SKERKA, C. 2008. Complement: The Alternative Pathway. *Encyclopedia of Life Science*.

ZIVKOVIC, A.M. AND BARILE, D., 2011. Bovine milk as a source of functional oligosaccharides for improving human health. *Advances in Nutrition: An International Review Journal*, *2*(3), pp. 284-289.

Chapter II Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research*, *82*(3), pp. 328-333.

2.1. Abstract

While the Complement protein system in human milk is well characterised, there is little information on its presence and activity in bovine milk. Complement forms part of the innate immune system, hence the importance of its contribution during milk ingestion to the overall defences of the neonate. A bactericidal sequestration assay, featuring a Complement sensitive strain, *E. coli* O111, originally used to characterise Complement activity in human milk, was successfully applied to freshly drawn bovine milk samples. Consequently, Complement activities in both human and bovine milks were compared. Although not identical in response, the levels of Complement activity in bovine milk were found to be closely comparable with that of human milk. Differential counts of *E. coli* O111 derived from the initial and final CFU after the 2h

incubation were 6.20 and 6.04 log CFU/ml for raw bovine and human milks, respectively, the lower value representing a stronger Complement response.

Exposing bovine milk to a range of thermal treatments, e.g., 42°C, 45°C, 65°C, 72°C, 85°C or ≥ 95°C for 10 min, progressively inhibited Complement activity with increasing temperature, thus confirming the heat-labile nature of this immune protein system. Low level Complement activity was found, however, in samples heat-treated in 65°C or 72°C and in retailed pasteurised milk, which highlights the limit to which high temperature, short time (HTST) industrial thermal processes should be applied if retention of activity is a priority. Concentration of Complement in the fat phase was evident following cream separation, and this was also reflected in the further loss of activity recorded in low fat variants of retailed pasteurised milk. Laboratory-based churning of the cream during simulated butter making generated an aqueous (buttermilk) phase with higher levels of Complement activity than the fat phase, thus pointing to a likely association with the milk fat globule membrane (MFGM) layer.

2.2. Introduction

As well as being a source of essential nutrients for optimum growth and development, milk provides the neonate with protective elements to support survival of the newborn (Van Hooijdonk et al., 2000, Hanssen, 1924). While the bacteriostatic qualities of milk were recognised as far back as 1924 (Hanssen, 1924), research over the years has focussed on the characterisation of its antimicrobial components, such as lactoferrin, lactoperoxidase, lysozyme, peptides derived *in vivo* during digestion, and sialyl-containing oligosaccharides. However, less consideration appears to have been given

to the role of another line of defence, i.e., the extent to which the innate immune system of the lactating mammal is manifested in its milk secretions. Innate immunity with its physical barriers and cellular components provides non-specific protection as a first line of defence (Ricklin and Lambris, 2007), which is distinguishable from that of the adaptive immune system that typically responds to specific pathogenic challenge (Galyean et al., 1999). Within innate immunity, Complement consists of serum and membrane proteins which circulate as inactive precursors in the blood system. The triggering of these precursors initiates a cascade of protein cleavages which culminates in the amplification of a cell-killing membrane attack complex (Oviedo-Boyso et al., 2007). Though initiated by different mechanisms, all three established Complement pathways, i.e., the classical, alternative and mannan-binding lectin (MBL) converge at the point when complement component C3 becomes active
(Abbas et al., 1994). The classical pathway commences with immune complexation by IgG or IgM to pathogens or foreign antigens, while the alternative pathway is triggered by either carbohydrates, lipids or proteins found on the surface of foreign microbes or cells in the body, and the MBL pathway is activated when either MBL or Ficolin binds to the surface of pathogens due to carbohydrate moieties (Sarma and Ward, 2011).

Efforts to date at addressing the presence and role of Complement in human milk (Ogundele, 2001) also lend support to the view that such innate immune components migrate from the circulatory system in the mammary gland into the secreted milk. However, little is known about its activity in bovine milk and its presence is not even recognised in the recent revision (6^{th}) of the nomenclature of the proteins in cows' (Farrell *et al.,* 2004). A lack of suitable assays may have been a contributing factor to

this paucity of information. Gradually, the bactericidal sequestration assay-based analytical approach was adapted initially to help identify bactericidal and haemolytic activity of bovine colostrum (Reiter and Brock, 1975), bovine mastitic milk (Rainard P, 1984) and human milk (Ogundele, 2001). The uniqueness of the bactericidal sequestration assay in this case is that growth of a specific pathogenic bacterial strain, i.e., *E. coli* O111 is sensitive to the presence of Complement, thus allowing the extent of growth inactivation to be used as an indirect measure of Complement activity present.

Thus, an objective of this study was to adapt and apply the bactericidal sequestration assay used by Ogundele (2002) to establish the level of Complement activity in fresh bovine milk and its comparability with that of human breast milk. A further objective was to evaluate the extent to which such activity may be affected by typical milk processes such as heating and cream separation. This interest was taking place against a backdrop of where advances in the 'humanisation' of bovine milk for use as infant feed formula requires a deeper understanding of the biological comparability between both bovine and human breast milk Complement, so that appropriate strategies may be designed for its protection in freshly secreted bovine milk during the course of dairy manufacturing processes.

2.3.Material and Methods

2.3.1 Sample preparation - Milk samples

Fresh whole bulk bovine milk was collected from the Moorepark Dairy Production Centre (MDPC) farm on three consecutive days. The SCC of the freshly collected milk was determined using Bentley Somacount 300® (Bentley Instruments Inc., Chaska MN, USA), SCC's of <200,000 cells per ml was required for use of the milk. On three consecutive mornings fresh commercial products including cream, full fat milk and low fat milk were purchased from local supermarkets and tested directly.

Human milk was collected from the Western Health and Social Care Trust (Glenshane Road, Londonderry BT47 6SB, United Kingdom) and was stored in aliquots at -80°C until required. The samples were slowly thawed at 4°C overnight before analysis.

2.3.2 Control preparation

Freshly collected raw milk was heat treated at 56°C for 30 min to ensure complete inactivation of the complement protein (Korhonen et al., 2000). All samples were screened using the ISO methods (ISO, 2013, ISO, 2004) and also the milks were streaked on Nutrient and Luria Bertani agar.

2.3.3 Effect of heat treatment

Individual milk samples were exposed to a range of temperatures: 42°C, 45°C, 65°C, 72°C, 85°C and 95°C with 10 min holding time before bactericidal sequestration assay in order to determine the effect of heating on Complement activity. (Korhonen et al., 2000). Milk samples were heat-treated using Hettich Elbanton special products oil

bath (Hettich Benelux B.V., De Aaldor 9 – 4191 PC Geldermalsen, Netherlands) with rocking arm to ensure uniform heat treatment throughout the samples. A control sample was produced by heating milk at 56□C for 30 min and was a reference for complete Complement inactivation.

2.3.4 Milk samples separation and washing

An Armfield disc bowl centrifuge (Armfield, Ringwood, UK) was used to generate cream samples (approximately 40% fat) and skimmed milk (approximately 1% fat) from the fresh raw milk. The cream was then stored at 4°C overnight and the following morning a Kenwood Chef food mixer (Model KM220, Harvant, Hants, UK) was used to disrupt the cream emulsion resulting in the production of butter. From this, buttermilk was released and was filtered through glass wool to remove butter grains. Human milk was thawed overnight at 4°C; this was then separated by centrifugation at 5000 rpm for 15 min at 4°C (Patton and Huston, 1986) in order to separate the fat fraction.

Washed buttermilk was prepared using the Armfield disc bowl centrifuge; however, an additional step was incorporated whereby the cream was suspended in sterile distilled water and held at 37°C for 1h to wash the buttermilk. The separation procedure was repeated (Armfield disc bowl centrifuge) and cream collected. This step was repeated twice more.

2.3.5 Bacterial Strain

E. coli O111 (*E. coli* NCTC 8007, serotype O111 K58(B4)) a pathogenic Complement sensitive strain was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections Porton Down Salisbury Wiltshire, SP4 0JG UK). This strain was routinely grown in Luria-Bertani (LB) medium at 37°C with shaking. Standard LB broth and agar was prepared as described by Sambrook et al. (2001).

2.3.6 Bactericidal sequestration assay

E. coli O111 was prepared for overnight growth at 37°C on LB agar. An isolated colony from replicate plates was inoculated into 3 tubes of LB broth (Merck KGaA, Darmstadt, Germany) and grown over night at 37°C. The overnight cultures were centrifuged at 5000 rpm, a pellet was formed, the supernatant was removed and the pellet was re-suspended in phosphate buffer saline solution (1 x PBS solution); this step was repeated twice. At the final step, the pellet was suspended in LB broth and adjusted to 3 x 10^8 colony forming units (CFU) per ml using the McFarland Method (Goldman et al., 1986). Using 96-well plates, 3 x 20 µl of each of the three replicate cultures was added to each round bottomed well (SARSTEDT Ltd, Wexford, Ireland) and 80 µl of the sample to be tested was then added. The plate was incubated at 37°C for 2h shaking at 200 rpm (Model Mini 4450 SHKA4450-1CE, Fischer Scientific,

Ballycoolin, Dublin). A 20 µl sample was taken from each well after the incubation time, and total viable counts were enumerated on LB agar using the pour plate method.

2.3.7 Serum susceptibility assay

The blood serum susceptibility assay developed by Hogan et al., (1989) was adapted by substituting blood with milk. Approximately 125 µl of freshly collected bovine milk and heat treated milk (control) were added to individual wells of a 96-well plate, also 100 µl Tris-NaCl buffer was added (1x Sigma Chemical Company, St. Louis, MO). The next step was the addition of 25 µl of the *E. coli* O111 inoculum, which had been grown to a level of 10^6 CFU per ml. The 96-well plate was incubated at 37°C and viability of the bacteria evaluated at 0 and 4h. The viable bacteria were plated on LB agar (Merck KGaA, Darmstadt, Germany) for 15h at 37°C, results were collected and the differential growth of the strain from the serum susceptibility assay was presented by subtracting the initial amount of colonies (0h) from the final number of viable colonies (4h).

2.4. Results

2.4.1. Comparison of Complement-associated bactericidal sequestration activity of bovine and human milks

The sequestration assay used relies on the specific sensitivity of *E. coli* O111 to the presence of Complement; thus, an active Complement system in milk would be

expected to restrict growth of the strain. Furthermore, since Complement is known to be inactivated at temperatures >56°C for 30 min (Korhonen et al., 2000) it is thus possible to compare growth of *E. coli* O111 in heated and raw milk, the difference in growth rates between the heated (control) and raw milks being attributable to Complement activity. This was evident when comparing the growth of *E. coli* in heattreated (56°C, 30 min) milk, freshly collected raw bovine milk and human milk (Fig. 2.1) where differential counts after 2h incubation at 37°C were 6.32, 6.20 and 6.06 viable colonies of *E. coli* O111 (log CFU/ml), respectively, the lower value indicating a more effective Complement response.

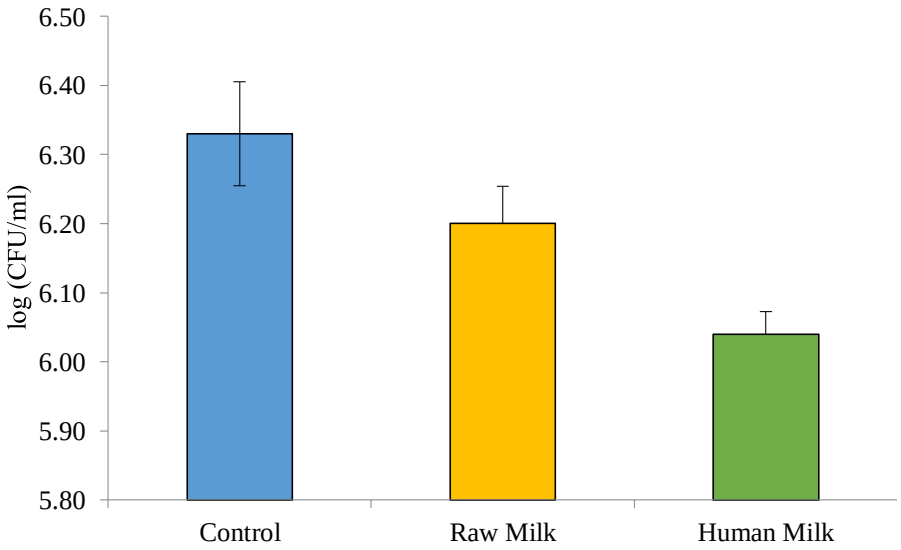


Figure 2.1 Differential reduction of *E. coli* O111 (log10 CFU/ml) during bactericidal sequestration assay of heated bovine milk (control), raw bovine milk and human milk, following inoculation and incubation for 2h incubation at 37°C.

This corresponded to cell reduction factors of 0.12 and 0.26 following incubation with raw bovine milk and human milk respectively. Following analysis for normality, it was determined that a Kruskal-Wallis test should be applied to the differential data, the results of which confirmed that there was a statistical difference ($p < 0.01$) between all

three samples, which was supported further by a Mann-Whitney test to show that both raw bovine milk and human milk were significantly different ($p < 0.01$) from the control (heat-treated milk).

2.4.2. Growth characteristics of *E. coli* O111

The growth characteristics of *E. coli* O111 was compared by incubating the strain separately in LB broth or bovine milk overnight at 37°C in order to have an improved understanding of its vitality during deployment in the bactericidal sequestration assay. The *E. coli* O111 strain grew steadily on LB agar starting from an initial viable colony count of 7.18 to reach ~8.98 log CFU/ml after 24h incubation (Fig. 2.2). Growth of the strain in bovine milk peaked at 8.51 log CFU/ml at 13h during the same period, after which it declined slightly.

Complement activity was also evident when *E. coli* was inhibited in untreated wells during a serum susceptibility assay of forewarmed (56°C, 30 min) and non-heated bovine milk. The plated viable colonies reached peak growth of 8.0 and 8.95 log cells/ml after incubation with the raw bovine and heated bovine milks (control), respectively ($p < 0.05$).

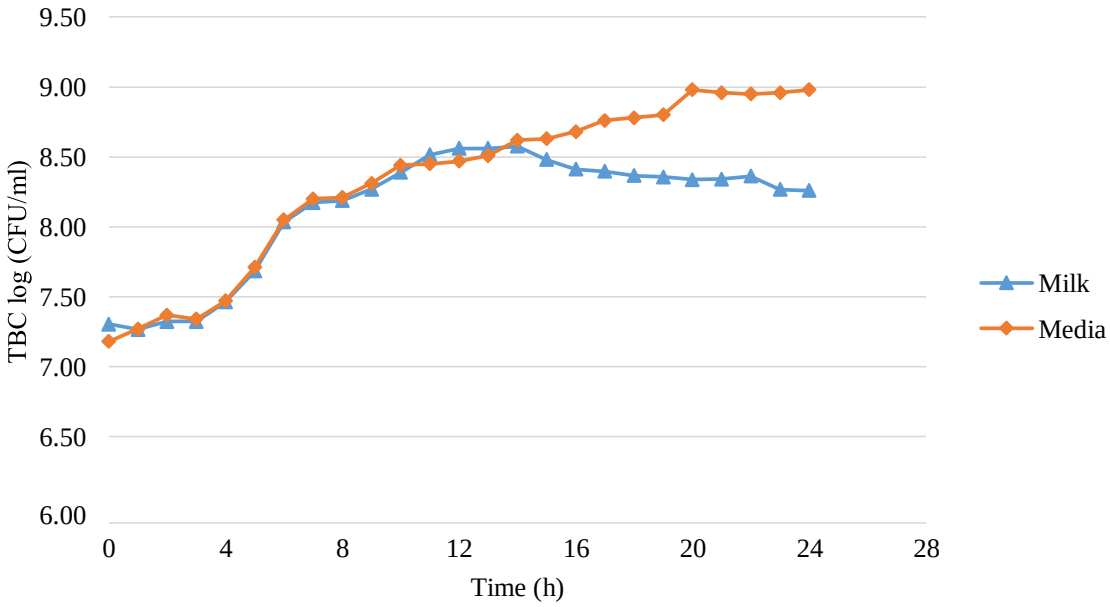


Figure 2.2. Total viable counts of *E. coli* O111 (log10 CFU/ml) during incubation at 37°C for 24h in LB broth and bovine milk.

2.4.3. Investigation of Complement activity in low fat bovine milk

Preliminary laboratory work carried out on the variation between freshly collected bovine milk and in-store purchased full-fat milk was extended to include low-fat (1% fat) retail milks and skim milk prepared by cream separation in the laboratory from freshly collected raw bovine milk (Fig. 2.3). Subsequent to assessment for normality, a Kruskal-Wallis test revealed that there was a statistically significant difference ($p < 0.001$) in colony forming units across the five groups [control (heat inactivated milk), raw milk, retail full-fat milk, skim milk, retail low-fat milk]. The raw untreated bovine milk was more effective in inhibiting growth of *E. coli* O111 than the pasteurised sample. The lower-fat milk variants (in-store purchased and lab-prepared) were also less effective in reducing growth of the Complement-sensitive strain. Further

statistical analysis, which examined individual groups (Mann-Whitney test), confirmed that full fat raw milk was significantly different from all other samples ($p < 0.01$).

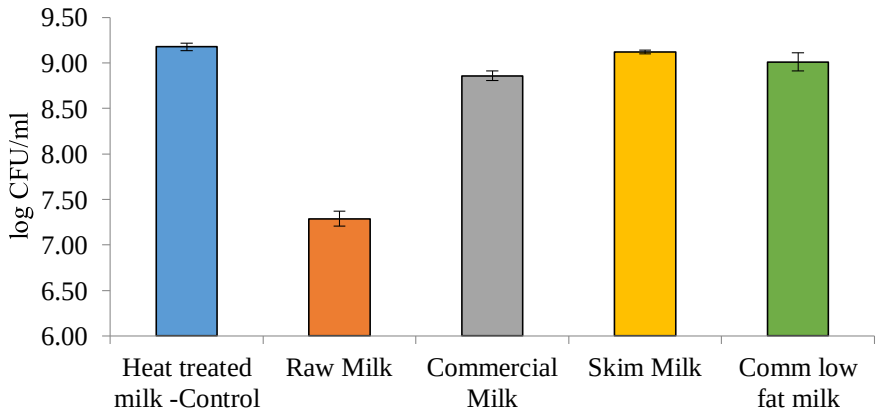


Figure 2.3 Total viable counts of *E. coli* O111 (log10 CFU/ml) after 2h following incubation with heated bovine milk (control), raw bovine milk, retail pasteurised milk, raw bovine skim milk and retail pasteurised low fat milk during the bactericidal sequestration assay maintained at 37°C.

2.4.4. Effect of fat reduction in human milk

Laboratory centrifugation (Patton and Huston, 1986) was used to defat (remove the upper cream layer) human milk since the available volume was too small for treatment by conventional dairy cream separation technology.

Similar to bovine milk, the full-fat form of human milk was significantly ($p < 0.05$) more effective than the defatted/skimmed human milk (Fig. 2.4), thus confirming the association between Complement and the fat phase of the milks of both species at a microstructure level.

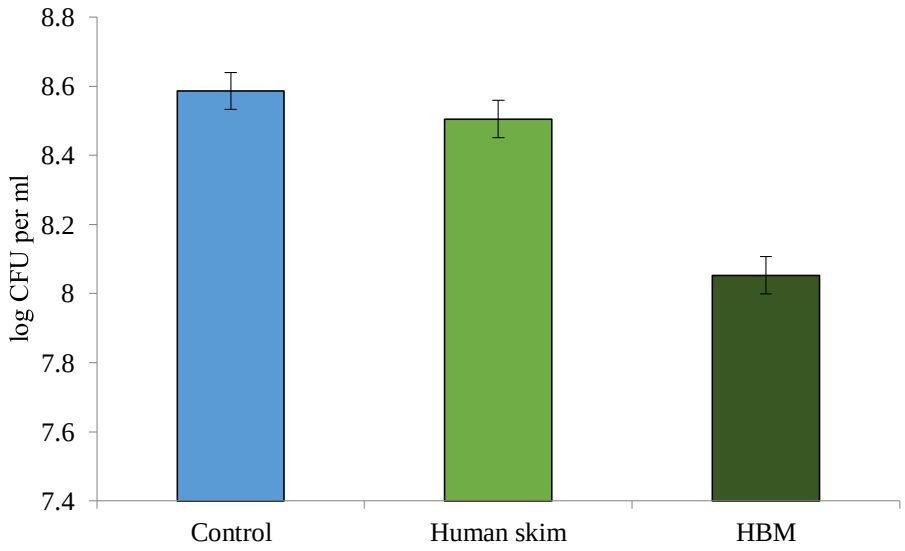


Figure 2.4 Total viable counts of *E. coli* O111 (log10 CFU/ml) after 2h incubation in (i) heat treated bovine milk (control), (ii) lab prepared skim human breast milk, (iii) human breast milk, in accordance with the bactericidal sequestration assay.

2.4.5. Effect of heat treatment on Complement activity

The bactericidal sequestration assay was performed on bovine milks which had been heated to each of the following temperatures: 42°C, 45°C, 65°C, 72°C, 85°C or 95°C with holding for 10 min and compared to that of bovine milk heated to 56°C for 30 min as control (Fig. 2.5). Optimum Complement activity (6.52 log CFU/ml) was observed at 42°C followed by a slight loss (6.76 log CFU/ml) as the temperature increased to 45°C. Based on assay counts of 7.83 - 7.92 log CFU/ml, a sizeable loss in Complement activity occurred when these milks were heated to either 65°C or 72°C for 10 min (Fig. 2.5). At the same time, the residual Complement activity associated with the latter two temperatures, which correspond to batch pasteurisation (65°C, 30 min) and continuous high temperature, short time (HTST) holding conditions (72°C, 15 s), is consistent with its low level in retail, commercially pasteurised milks referred

to earlier. Further increases in temperature (85°C or 95°C for 10 min) increased the levels of inactivation of the Complement activity. All samples were significantly different from one another χ^2 (6, n=21) = 28.566, $p < 0.001$ (Fig. 2.5). Further posthoc comparisons using the Mann-Whitney test to look at individual samples identified that there was no significant difference between heating 65°C and 72°C ($p < 0.05$).

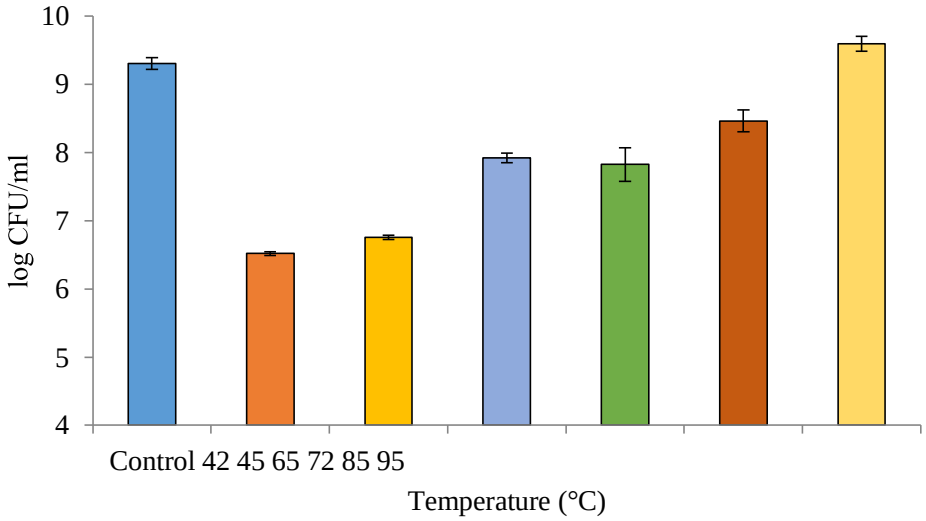


Figure 2.5 Total viable counts of *E. coli* O111 following 2h incubation in accordance with bactericidal sequestration assay for 2h, 37°C.

2.4.6. Comparison of cream and buttermilk samples

Buttermilk is known to be rich in milk fat globule membrane (MFGM) components (El-Loly, 2011). The Complement activity of buttermilks was significantly ($p < 0.05$) greater than that of the creams from which they were generated according to the Kruskal Wallis test (Fig. 2.6). The buttermilk prepared from the cream of raw milk was significantly different ($p < 0.01$) from the laboratory-prepared cream, retail cream and the washed buttermilk samples based on the Mann-Whitney test of comparing each group individually A loss of bactericidal sequestration activity in the buttermilks

generated from washed cream would suggest that Complement components were removed to some extent into the aqueous phase.

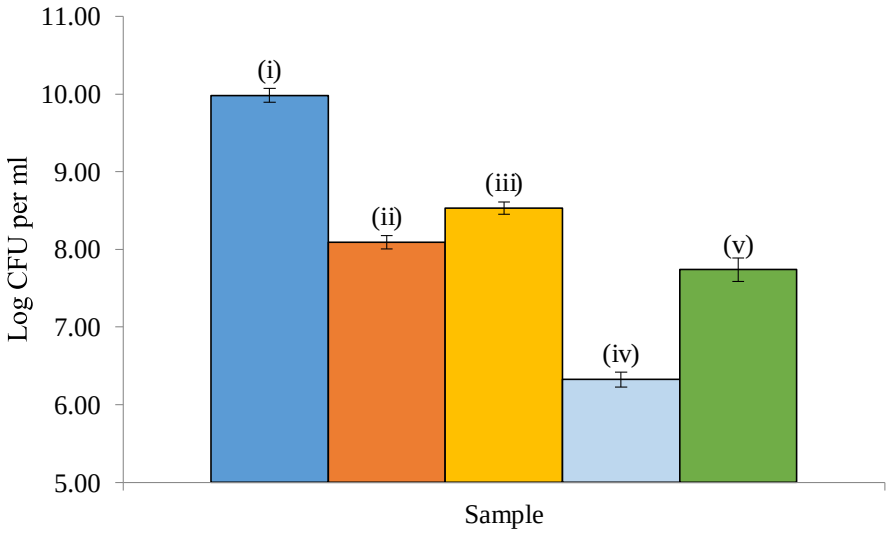


Figure 2.6 Total viable counts of *E. coli* O111 (log10 CFU/ml) following 2h incubation in (i) heated bovine milk, 56°C, 30 min (control), (ii) cream (~40% fat) prepared from freshly collected raw bovine milk, (iii) retail pasteurised cream, (iv) lab prepared buttermilk from cream of raw milk, and (v) lab prepared buttermilk made from thecream (washed twice at 37°C for 1h) of raw milk, after 2h incubation at 37°C according to the a bactericidal sequestration assay.

2.5. Discussion

This study confirms that Ogundele's (2002) bactericidal sequestration assay, which uses a specific strain of *E. coli* to detect the presence of Complement activity in human milk, may now be extended for similar assay detection in bovine milk. The bactericidal sequestration assay confirmed the presence and activity of Complement in bovine milk, which also suggests that both human and bovine milks are broadly analogous in terms of their sequestration response (Fig. 2.1 and Fig. 2.4). This is an interesting finding given the complexity of Complement, with its 30+ proteins and

protein fragments, i.e., that there should be considerable homology between human and bovine milks. Thus, the milks of both species would appear to share the same amplifying Complement cascade that builds up to the activation of a cell-killing membrane attack complex (Ogundele, 2001).

The results in Fig. 2.1 also highlight that the bactericidal sequestration assay was adaptable to allow for the direct comparison of human and bovine milk, thus making it possible to further investigate various bovine milk by-product streams and also milks subjected to different processing treatments.

Exposure to a range of thermal treatments of 42°C, 45°C, 65°C, 72°C, 85°C and 95°C for 10 min progressively inhibited Complement activity (Fig 2.5). The initial loss in Complement activity over the course of a 3°C rise during heating of bovine milk from 42 to 45°C highlights how heat labile these immune proteins are. Some residual Complement activity is still detectable in and around typical milk pasteurisation temperatures whether by batch heating or HTST methods. This concurred also with assay results generated with samples of commercially retailed pasteurised milks.

Testing of fresh untreated and retail pasteurised bovine milks in both full and low fat variants provided the first indication that Complement activity appeared to be greater in milks containing a higher fat content (Fig. 2.3). This was confirmed in the course of a follow-up study involving cream separation of milk by either gravity-induced or centrifugally-employed methods, which showed that greater Complement activity was detectable in the fat-enriched phase of both bovine and human milk. Attempts at cream dilution to ascertain if there was a relationship between Complement response and fat content were not conclusive. This may have been due to limitations with the microbiological assay, as a prozone-based inhibition effect has previously been

described (Rainard, 1984, Ogundele, 2001). The prozone effect, originally described by Neisser-Wechsberg (1901), describes the inhibition due to excess antiserum of the bactericidal reaction which is mediated by the complement system. This inhibition by excess antiserum may occur when the normal serum serving as a Complement source is either lacking in bactericidal activity itself or is only capable of limited activity due to the presence of specific antibodies (Muschel et al., 1969). As cream may contain more concentrated levels of antisera, in particular high concentrations of IgG, they may mask the bactericidal effect of cream. In any case, Complement-based sequestration of *E. coli* O111 was no longer observed in samples in which milk fat was depleted or substantially reduced in the case of raw and retail low-fat milks. Simulated butter-making of the prepared creams enabled Complement activity to be tracked during further partitioning between the fat and aqueous (buttermilk) phases. Potent Complement activity evident in buttermilk samples suggests that it most likely associates with the MFGM components present. MFGM, has already been shown to exhibit some antibacterial properties (Singh, 2006). MFGM as a tri-layer of both proteins and lipids from the endoplasmic reticulum membrane and mammary epithelium cells that envelopes the lipids in milk (Mather, 1999), is also a carrier of biologically active molecules like phospholipids, sphingolipids, cholesterol and other bio-functional proteins e.g. xanthine oxidase (Lopez 2011, 2012).

Overall, it can be seen that the Complement system is present in bovine milk and, while heat labile, a certain amount of activity was detectable after milk pasteurisation. Although not identical in response, the levels of Complement activity in bovine milk are comparable to that of human milk. Complement's association with the fat phase of milk has implications for the development of low fat variants of fresh dairy products,

and indeed where milk fat is replaced by non-dairy fat sources in infant formula. Future studies should aim to further investigate the factors that influence Complement activity in bovine milk and methods to retain its activity during processing.

2.6.References

ABBAS, A.K., LICHTMAN, A.H. AND PILLAI, S., 2014. *Cellular and Molecular Immunology*. Elsevier Health Sciences.

EL-LOLY, M. M. 2011. Composition, Properties and Nutritional Aspects of Milk Fat Globule Membrane-a Review. *Polish Journal of Food and Nutrition Sciences, 61*(1), pp. 7-32.

FARRELL, H.M., JIMENEZ-FLORES, R., BLECK, G.T., BROWN, E.M., BUTLER, J.E., CREAMER, L.K., HICKS, C.L., HOLLAR, C.M., NGKWAI-HANG, K.F. AND SWAISGOOD, H.E., 2004. Nomenclature of the proteins of cows' milk—sixth revision. *Journal of Dairy Science, 87*(6), pp.
1641-1674.

GALYEAN, M., PERINO, L. AND DUFF, G. 1999. Interaction of cattle health/immunity and nutrition. *Journal of Animal Science, 77*(5), pp. 1120-1134.

GOLDMAN, A., THORPE, L., GOLDBLUM, R. AND HANSON, L. 1986. Anti-inflammatory Properties of Human Milk. *Acta Paediatrica, 75*(5), pp. 689-695.

HANSSEN, F. S. 1924. The bacterial property of milk. *British Journal of Experimental Pathology*, 5(5), pp. 271.

HOGAN, J.S., TODHUNTER, D.A., SMITH, K.L. AND SCHOENBERGER, P.S., 1989. Serum susceptibility of coliforms isolated from bovine intramammary infections. *Journal of Dairy Science*, *72*(7), pp. 1893-1899.

ISO. 2004. Milk—Quantitative determination of bacteriological quality—guidance for establishing and verifying a conversion relationship between routine method results and anchor method results, EN ISO 21187. International Organization for Standardization, Geneva

ISO. 2013. Milk - Bacterial count - Protocol for the evaluation of alternative methods (Reference method), EN ISO 16297–1. International Organization for Standardization, Geneva.

KORHONEN, H., MARNILA, P. AND GILL, H. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition, 84,* pp. 75-80.

MATHER, I.H., 2000. A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *Journal of Dairy Science*, *83*(2), pp. 203-247.

MUSCHEL, L.H., GUSTAFSON, L. AND LARSEN, L.J., 1969. Re-examination of the Neisser—Wechsberg (antibody prozone) phenomenon. *Immunology, 17*(4), pp. 525.

NEISSER, M., AND F. WECHSBERG. 1901. Uber die Wirkungart bactericider sera. *Muench. Med. Wochenschr, 48,* pp. 697-700.

OGUNDELE, M.O., 2001. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. *Immunology and Cell Biology*, *79*(1), pp. 1-10.

OGUNDELE, M.O., 2002. Effects of storage on the physicochemical and antibacterial properties of human milk. *British Journal of Biomedical Science*, *59*(4), pp. 205.

OVIEDO-BOYSO, J., VALDEZ-ALARCON, J. J., CAJERO-JUAREZ, M., OCHOA-ZARZOSA, A., LOPEZ-MEZA, J. E., BRAVO-PATINO, A. AND BAIZABAL-AGUIRRE, V. M. 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of Infection, 54*(4), pp. 399-409.

PATTON, S. AND HUSTON, G. E. 1986. A method for isolation of milk fat globules. *Lipids, 21*(2), pp. 170-174.

RAINARD, P., POUTREL, B. AND CAFFIN, J.P., 1984. Assessment of hemolytic and bactericidal complement activities in normal and mastitic bovine milk.

Journal of Dairy Science, *67*(3), pp. 614-619.

REITER, B. AND BROCK, J. 1975. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype O111. *Immunology, 28*(1), pp. 71.

RICKLIN, D. AND LAMBRIS, J.D., 2007. Complement-targeted therapeutics. *Nature Biotechnology*, *25*(11), pp. 1265-1275.

SAMBROOK, J., RUSSELL, D. W. AND RUSSELL, D. W. 2001. *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press Cold Spring Harbor, New York.

SARMA, J. V. AND WARD, P. A. 2011. The complement system. *Cell and Tissue Research, 343*(1), pp. 227-235.

SINGH, H., 2006. The milk fat globule membrane—A biophysical system for food applications. *Current Opinion in Colloid and Interface Science*, *11*(2), pp. 154-163.

VAN HOOIJDONK, A. C. M., KUSSENDRAGER, K. D. AND STEIJNS, J. M. 2000. In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *British Journal of Nutrition, 84*(1), pp. S127-S134.

Chapter III

Establishment of a relationship between globular milk fat and active Complement proteins

3.1. Abstract

Recent work by the authors has identified the presence of Complement proteins in bovine milk; furthermore, an association between active Complement and the fat fraction has also been recognised. These findings pointed us to study the linkage further, such that the level of association between the milk fat separation and the Complement activity could be quantified. Thus, the present study encompasses the development of a novel method for the fractionation of bovine milk; this new technique restricts the levels of damage or interference to the natural activities and composition of the milk sections. Milk was systematically collected from each section of a graduated cylinder using a sterile syringe over a 24h period. Following this, each sample was assessed for fat content, somatic cell counts, fat globule size and total bacteria counts. The somatic cells and bacteria rose to the top of the milk, along with the fat; the average fat globule size (FGS) started initially in between 3.28 μm – 3.91 μm and increased over 24h to a wider range of 7.28 μm -2.54 μm. Next, the fractions collected throughout the 24h study were tested with a bacterial sequestration assay and it was determined that, as fat content increased, the sequestration capacity of the fractions also increased, which appeared to confirm the relationship between complement activity and fat content.

3.2. Introduction

Milk is basically an oil-in-water emulsion; milk fat globules are stabilised by a naturally occurring membrane that functions both as an emulsifier and biological transporter, i.e., a milk fat globule membrane (MFGM) formed during milk secretion in the mammary gland.

The natural creaming process in milk occurs when larger sized milk fat globules rise to the surface. Such gravity-based separation was traditionally used to enable a sufficiently fat-enriched cream to facilitate ease of de-emulsification during churning when producing butter. This natural creaming process is also used in traditional cheese making, specifically, for Grana Padana and Parmigiano Reggiano raw milk cheeses as a milder bio-process treatment for bacterial reduction and cheese flavour enhancement (Fox et al., 2004). In the traditional Parmigiano-Reggiano cheese production process, raw milk is stored at 20°C overnight and the milk separates under gravity, following which some of the cream layer is removed and fresh raw whole milk is added until a final milk of 2.4 to 2.5% fat remains. A similar process is used for Grana Padana, with differences observed in milk storage temperature, 15°C, and milk standardised, 2.12.2 % fat (Fox et al., 2004). In a study of the effects of time and temperature on changes to fat globule size distribution and fat content in milk fractions during gravity separation, Ma and Barbano (2000) showed that the volume mean diameter of the top layer increased from 3.13 μm (without separation) to 3.48 and 3.64 μm at 4° and 15°C, respectively. Euber and Brunner (1984) postulated a theory of fat globule clustering involving Immunoglobulin M (IgM) interaction in an antigen-antibody mode simultaneously with a fraction extracted by the authors, termed 'skim milk membrane', and with milk fat globules through specific carbohydrate moieties. This clustering is due to the action of the IgM which is affected by pH, concentration and the valency of cations (Kelly and Huppertz, 2006). Cold agglutination of milk fat globules appears to follow similar physiological behaviour to human blood when erythrocytes agglutinate and cryo-precipitation to occur when temperature is lowered (Walstra, 1983). Cryoglobulin, a protein complex containing IgM, becomes insoluble and precipitates on particles in the cold. In milk, the immunoglobulin fraction euglobulin,

appears to be largely responsible for the cold agglutination phenomenon (Walstra, 1983). More recently, the presence of both somatic cells and immunoglobulins has been shown to be necessary for gravity separation of fat, bacteria, and spores in whole milk (Geer and Barbano, 2014). The same authors showed that the presence of immunoglobulins alone without somatic cells was not sufficient to cause bacteria, fat, and spores to rise to the top. It was speculated that
IgM functions as a cold agglutinin rather than as a cryoglobulin.

Previous work reported by the authors indicated that a lower incidence of Complement activity is evident in fat-reduced and skimmed milks which points towards some form of association between Complement and creaming. Hence, the objective of this study was to examine the effect of creaming induced by gravity separation on Complement activity of milk sampled simultaneously from different points on a column of milk held stationery at 15°C over a 24h period. Additional analyses of each sample included characterisation of fat globule size distribution, somatic cell counts and total bacteria counts.

3.3. Materials and Methods

3.3.1. Sample preparation -Bovine Milk

Fresh whole bovine milk was aseptically collected from the bulk tank in the Moorepark Dairy Production Centre (MDPC) farm using a Milk Sampling Dipper which had been sterilised with 70% ethanol. Experimental work was carried out immediately following collection in order to prevent any unmonitored gravitational separation.

3.3.2. Bacterial Strain

Escherichia coli O111 (*E. coli* NCTC 8007, serotype O111 K58(B4)) a pathogenic Complement sensitive strain was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections Porton Down Salisbury Wiltshire, SP4 0JG UK). This strain was routinely grown in Luria-Bertani (LB) medium at 37°C with shaking. Standard LB broth and agar was prepared as described (Sambrook et al., 2001).

3.3.3. Time-monitored sampling during gravitational separation of cream

The schematic in Fig 3.1 displays a 1000 ml graduated cylinder. Five holes were drilled into the graduated cylinder and a small rubber seal was placed into each hole to prevent leakage. At sampling times, a sterile needle pierced the seal and 10 ml samples were withdrawn into a 10 ml syringe. The apparatus was maintained at 15°C for 24h and milk was withdrawn from each sampling port at 0h, 4h, 8h and 24h. All the equipment was either autoclaved for sterilisation at 121°C for 15 min or wiped with 70% ethanol.

The study was conducted in a laminar flow hood in order to minimise the risk of environmental contamination.

The adapted graduated cylinder had holes drilled at the 200 ml, 400 ml, 600 ml, 800 ml and 1000 ml points on the cylinder. A total of 1100 ml of milk was added to the cylinder with an approximate height of 26.5 cm. Fraction 1 was the section with the milk above 800 ml, the hole was drilled 4.5 cm from the bottom of the cylinder. The next fractions are 2 (800 - 600 ml) and 3 (600 – 400 ml), the holes were drilled at the heights of approximately 4.2 cm and 4.4 cm respectively, while the bottom two fractions 4 (400 - 200 ml) and 5 (200 - 0 ml) have heights of 4.8 cm and 4.6 cm, respectively.

3.3.4. Fat globule size

The fat globule size (FGS) of the bovine milk was routinely measured using Malvern Mastersizer 3000. This equipment uses the laser diffraction principle whereby particles passing through a laser beam will scatter light at an angle that is directly proportional to their size: large particles scatter at low angles, whereas small particles scatter at high angles. The samples were slowly added drop by drop under moderate stirring at room temperature. Each sample was analysed in triplicate. The size distribution of fat globules was characterised by the volume-weighted diameter D[4,3]

(μm) value as it is regularly referenced in the literature to evaluate size distribution. This value, D[4,3], is calculated by the integrated software it may be described simply as the mean whenever the result is displayed as a volume distribution.

The results were considered as Dv10, Dv50 and Dv90; however, results for Dv90 provided the clearest insight into particle size, thus, this is reported below. The Dv90 value signifies that the particle diameter corresponding to 90% cumulative are under this fat globule size value.

3.3.5. Milk composition - Fat content

An important marker for this new method is the fat content and so the accuracy of the measurement was significant, thus, the fat content was measured by the ISO reference method, Rose Gottlieb (ISO, 2010).

3.3.6. Somatic cell count

The SCCs were assessed using a Bentley Somacount 300® (Bentley Instruments Inc., Chaska MN, USA) (ISO, 2006, ISO 2008).

3.3.7 Total bacteria counts

The TBCs were measured using the 3M™ Petrifilm™ Aerobic Count Plates. A serial dilution was carried out on each sample and placed on the petrifilm. The films were then incubated at 30°C for 3 days. The viable cells can be enumerated with a specialised Plate Reader which detects a red dye in all viable colonies.

3.3.7. Bactericidal sequestration assay

E. coli O111 was prepared for overnight growth at 37 °C on Luria Betani (LB) agar. An isolated colony from replicate plates was inoculated into 3 tubes of LB broth (Merck KGaA, Darmstadt, Germany) and grown overnight at 37°C. The overnight cultures were centrifuged at 5000 rpm, a pellet was formed, the supernatant was removed and the pellet was re-suspended in phosphate buffer saline solution (PBS solution); this step was repeated twice. On the final step the pellet was suspended in LB broth and adjusted to 3 x 10^8 colony forming units (CFU) per ml using the McFarland Method (Goldman et al., 1986). Using 96-well plates 3 x 20 µl of each of the three replicate cultures was added to each round bottomed well (SARSTEDT Ltd., Wexford, Ireland) and 80 µl of the sample to be tested was then added. The plate was incubated at 37°C for 2h shaking at 200 rpm (Model Mini 4450 SHKA4450-1CE, Fischer Scientific, Ballycoolin, Dublin). A 20µl sample was taken from each well after the incubation time and total viable counts enumerated on LB agar using the pour plate method.

3.3.9. Reproducibility and statistical analysis

Throughout the study all work was carried out in triplicate using three separate cultures (i.e., three biological repeats) and repeated on three other separate days. Examination of the results was done using the SPSS program (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.).

3.4. Results

The laboratory method developed for the purpose of this study exploited the natural tendency of fat in fresh un-treated milk to rise to the surface under the influence of gravity according to Stokes law - which exploits the density differential between globular milk fat and surrounding serum as the driving force behind the physical separation of the two phases. The rapid rate of creaming in milk is due to the rise of the smaller individual globules which cluster and, thus, rise to the larger diameter spheres. Conventional centrifugation approaches, on the other hand, accelerate the force due to gravity in the course of generating cream. Hence, by opting for a natural process of cream separation over a 24h time period, it was possible to sample from a column of milk at 5 ports along its vertical volumetric scale at time intervals of 0h, 4h, 8h and 24h (Fig 3.1). In the course of monitoring for gradation in fat concentration with increasing height, all milk samples were analysed for fat content, somatic cell counts (SCC), fat globule size (FGS), total bacteria counts (TBCs) and bacterial sequestration capacity.

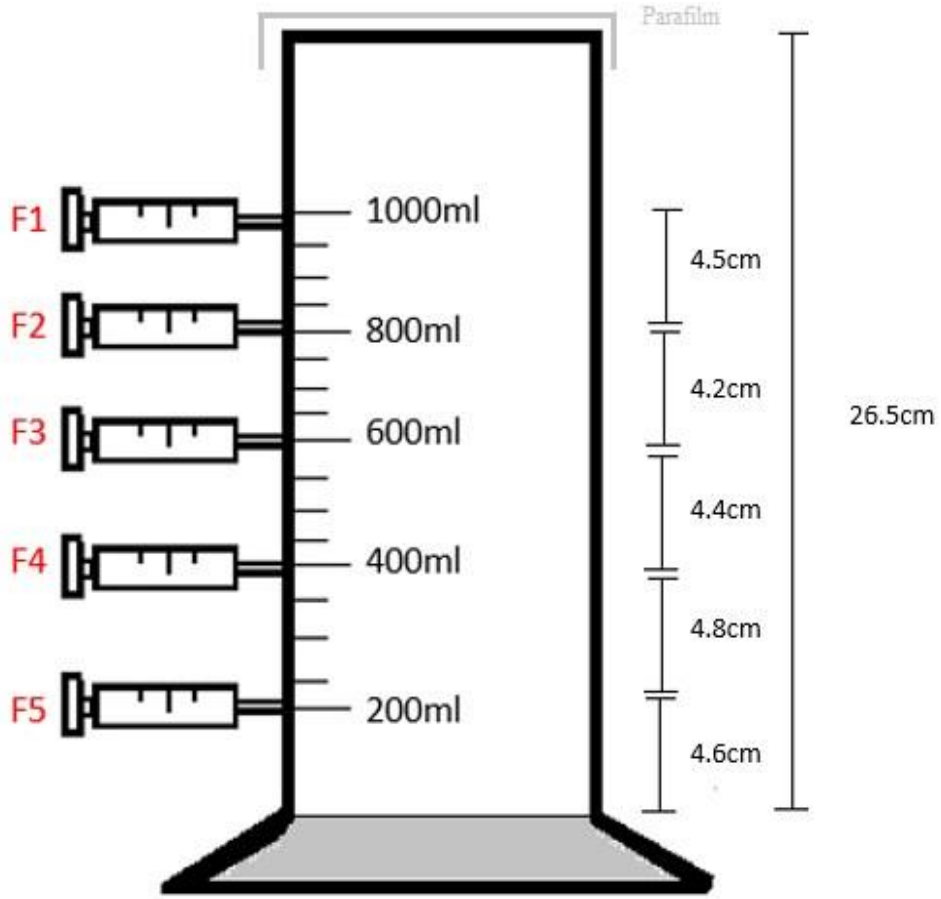


Figure 3.1 Schematic drawing of gravity separation cylinder closed using rubber seals; intermittent holes allowed the fractions 1 – 5 to be removed using a sterile syringe (10 ml) at time points over 24h incubation at 15°C.

3.4.1. Fat globule size

Initially, the mean FGS, expressed as D[4,3], of all 5 fractions were comparable with that of raw milk (3.61 μm). Indeed, fractions 1 (800-1000 ml, 4.5 cm), 2 (600 - 800 ml, 4.2 cm) and 5 (0 - 200 ml, 4.6 cm) reported an average D[4,3] value of 3.28 μm, 3.41 μm and 3.36 μm respectively (Fig 3.2). However, fractions 3 (400 - 600 ml, 4.4 cm) and 4 (200 - 400 ml, 4.8 cm) had a slightly higher D[4,3] value (3.87 μm and 3.91 μm, respectively) than that of the raw milk. Over time, FGS increased to 7.28 μm in

fraction 1, in keeping with the expectation that larger milk fat globules rise more quickly through the milk. FGS increases in fraction 2 and fraction 3 initially at the 4h sampling time and then declined. This was most likely due to the progression of larger fat globules to the upper fraction 1 level during the course of the remaining 20h. The FGS of fraction 4, on the other hand, decreased over time (2.32 µm at 24h) and, although it increased initially in fraction 5, at 24h the D[4,3] value decreased to 2.54 µm.

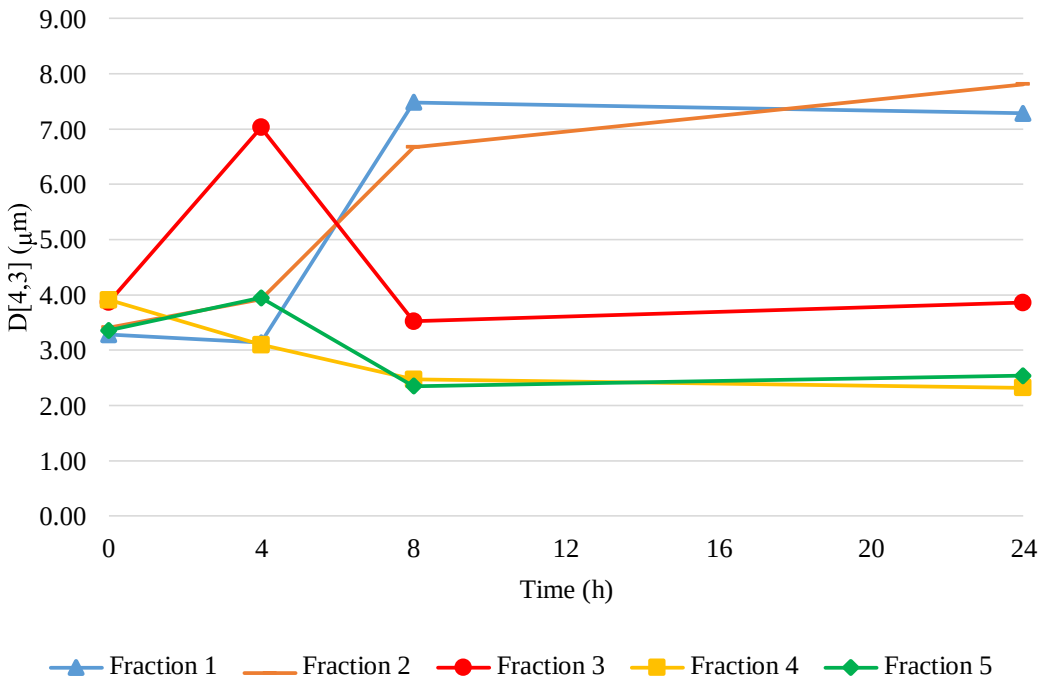


Figure 3.2 D[4,3] measurements from the Malvern Mastersizer of the fat globule size distribution. A sample was collected from all 5 fractions of the 1000 ml graduated flask at set time points over a 24h time period; 0h, 4h, 8h and 24h.

3.4.2. $D_v0.9$

The $D_v0.9$, representing the 90% of fat globules that fall below the indicated size, was initially higher in fractions 3 (6.37 µm), 4 (6.40 µm) and 5 (6.27 µm) (Fig 3.3). As fat travels through the milk sample to the top, the largest D_v90 is found in

fractions 1, 2 and 3 with values of 7.84 µm, 7.22 µm and 6.64 µm, respectively. The highest D_v90 value occurred in fraction 1 (800 -1000 ml, 4.5 cm) and was closely followed by fractions 2 (600 – 800 ml, 4.2 cm) and 3 (400 – 600 ml, 4.4 cm).

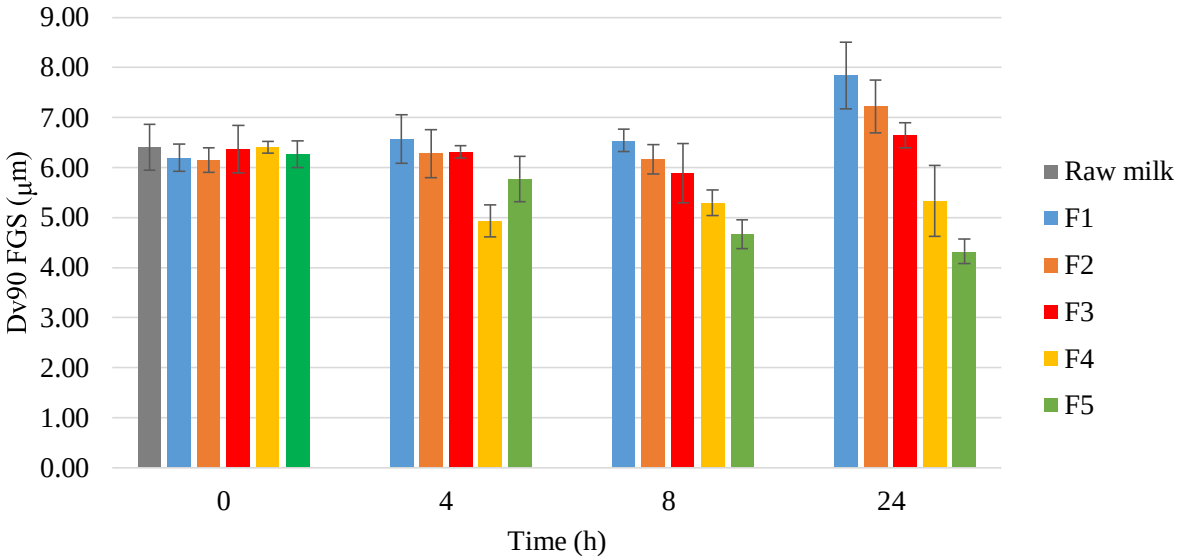


Figure 3.3 Results of the Malvern Mastersizer measurements for the D_v90 value. Samples were collected from each fraction over a 24 time period at 0h, 4h, 8h, and 24h

3.4.3. Fat content

At the start of the study, the fat content of all 5 fractions were similar (4.39%, 4.40%, 4.41%, 4.24%, and 4.13% fat in fractions 1-5, respectively) to that of raw milk (4.4% fat) (Fig 3.4). According, as cream rose to the top, the fat content of fraction 1 increased over time to reach a maximum of 14.63% fat after 8h, the fat content of fraction 2 increased gradually to an intermediate level of 8.18% over the same time period. As expected, the respective fat content in fractions 3 (400 – 600 ml,4.4 cm), 4 (200 – 400 ml, 4.8 cm) and 5 (0 – 200 ml, 4.6 cm) declined over time: 3.34% fat,

2.17% fat and 1.86% fat at 24h due to the upward mobility of fat globules to the creaming zone fractions 1 and 2.

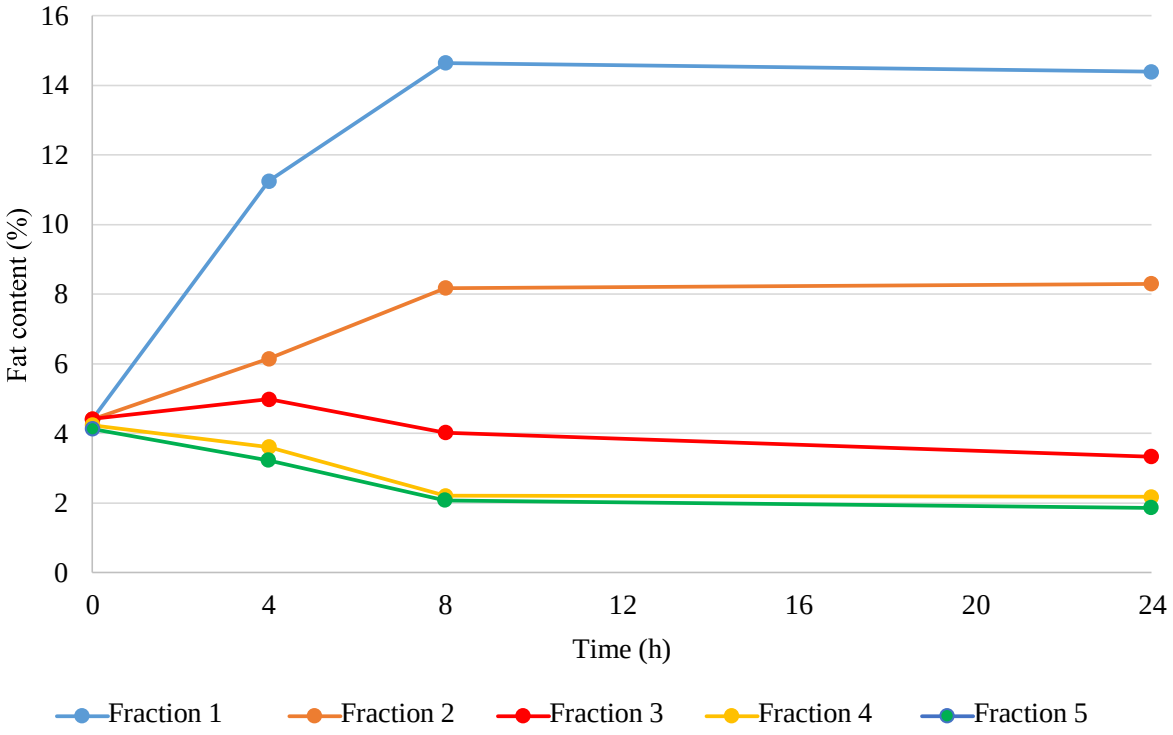


Figure 3.4 Results of fat content (%) analysis using the Rose Gottlieb method, each sample was collected from the 1000 ml graduated cylinder at set time points over 24h: 0 h, 4h, 8h and 24h

3.4.4. Somatic cell count

Throughout the time intervals of this study, the SCC of fraction 1 (800 - 1000 ml at 4.5 cm) increased with changes in fat content (Fig 3.5). Indeed, the SCCs increased in fraction 1 to ~57,000 cells per ml after 24h. A similar trend was evident with fraction

2 (600 – 800 ml, 4.2 cm) where a final SCC count of ~37,000 cells per ml was recorded at 24h. Fractions 3 (400 - 600 ml, 4.4 cm) and 4 (200 – 400 ml, 4.8 cm) SCC increased initially to ~31,000 and 37,000 cells per ml, respectively, in the following 8h fraction

3 increased to ~28,000 and fraction 4 reduced to ~19,000 cells per ml. Over the next

16h SCC reduced to ~28,000 and 19,000 cells per ml for fractions 3 and 4 respectively. Accordingly, as the fat content reduced in fraction 5, the SCC also declined and reached the lowest recorded value by 24h (19,000 cells per ml).

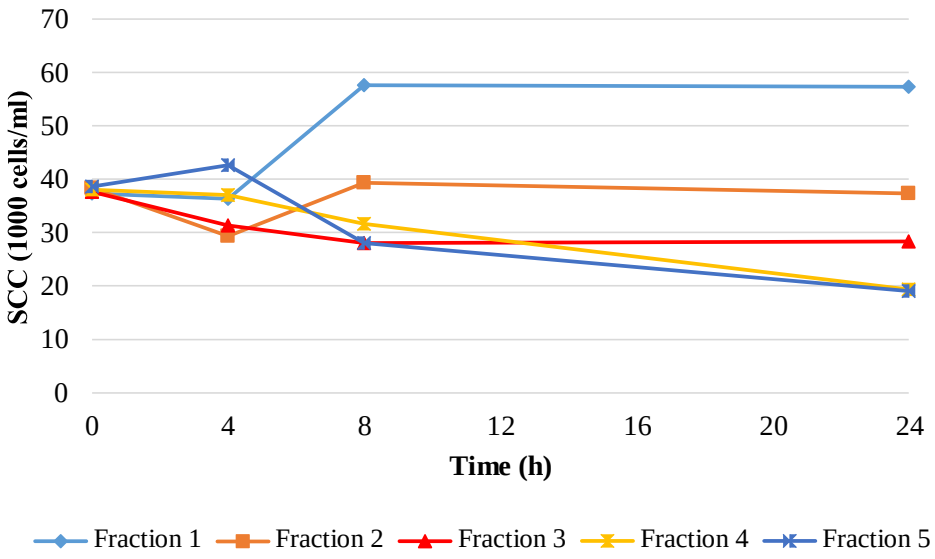


Figure 3.5 Somatic cell counts (1000 cells/ml) from the Bentley somacount equipment, each sample was collected from fractions 1-5 at set time points over a 24h time frame; 0h, 4h, 8h and 24h

3.4.5. Total bacterial count

The TBC in fraction 1 increased over time as bacteria rose to the top of the milk sample (Fig 3.6). The initial TBC of 3.54 log CFU per ml in fraction 1 (800 - 1000 ml at 4.5 cm) reached a final concentration of 4.06 log CFU per ml after 24h. TBC also increased in fraction 2 (600 - 800 ml, 4.2 cm) from 3.6 log CFU per ml to 3.78 log CFU per ml after 8h before dropping off over time to 2.8 log CFU per ml. Fractions 3 (400 - 600 ml, 4.4 cm), 4 (200 – 400 ml, 4.8 cm) and 5 (0 - 200 ml, 4.6 cm) shared a similar decline of approx. 1 log unit with time to 2.54, 2.36 and 2.17 log CFU per ml.

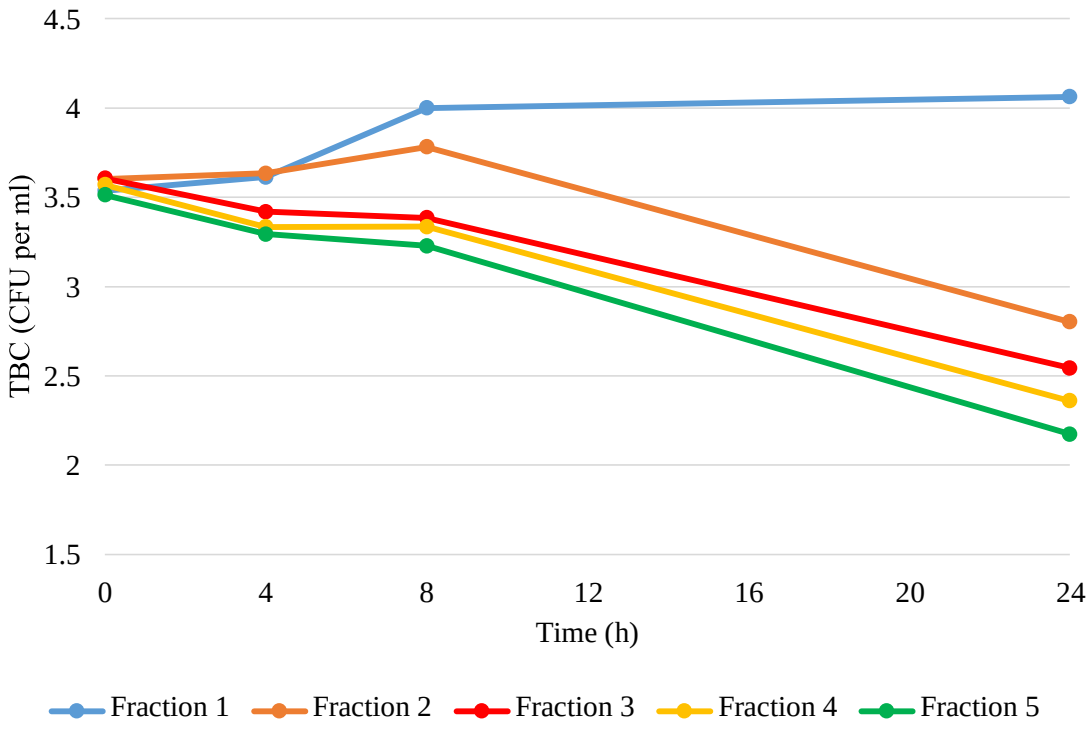


Figure 3.6 Total bacteria counts (TBC) in the milk samples collected from the 5 sampling ports were completed at each time point (0h, 4h, 8h and 24h). The values are represented as log CFU per ml.

3.4.6. Bactericidal sequestration assay

The bactericidal sequestration capacity of fractions 1 (800 – 1000 ml at 4.5 cm) and 2 (600 - 800 ml, 4.2 cm) increased as reflected in the reduction of viable cells of the target microorganism (*E. coli* O111). This finding also aligns with the increasing fat content in fraction 1 (Fig 3.7). The viable cell counts reduced from 7.93 log to 7.49 log CFU per ml in fraction 1 over 24h, while in fraction 2 (600 – 800 ml, 4.2 cm) this reduced to 7.64 log CFU per ml. However, in the milk samples tested from fraction 3 (400 - 600 ml, 4.4 cm) the numbers of *E. coli* remained stationary and even increased slightly by approximately 0.06 log CFU per ml in the fractions where the fat had been somewhat reduced. The bactericidal capacity of each fraction against *E. coli* O111 declined as the fat content reduced in fractions 4 and 5 over time.

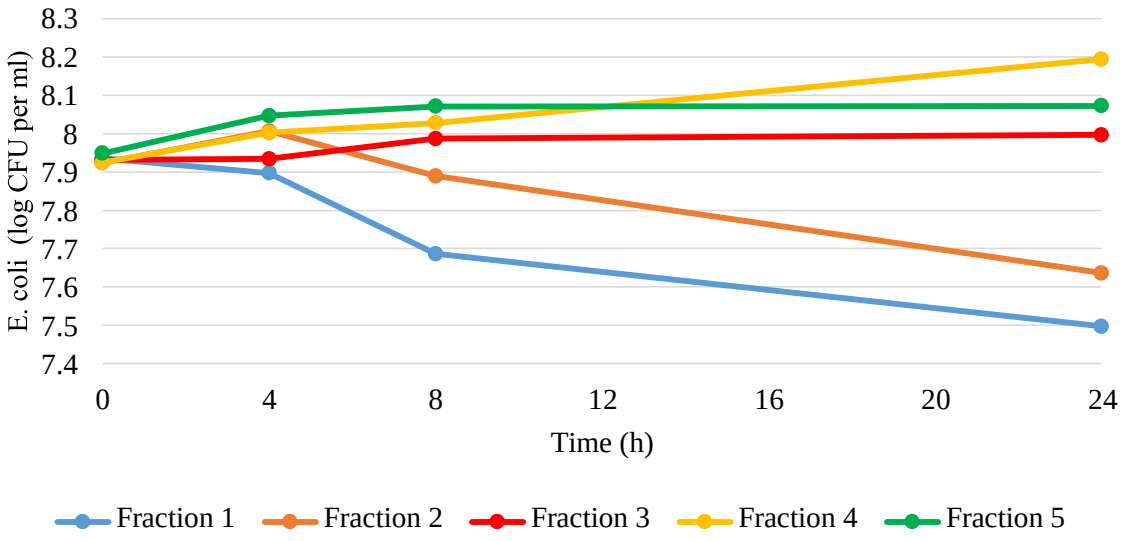


Figure 3.7 The bactericidal sequestration assay was also carried out on all samples collected. Results of the bactericidal sequestration assay are expressed in the log CFU per ml of the *E. coli* O111 strain. The experiment was completed over a 24h period and the samples were collected at 0h, 4h, 8h and 24h.

3.5. Discussion

The diminished levels of Complement observed in low-fat milks during the early part of the study prompted a need to investigate further this phenomenon. Natural creaming of milk according to gravity separation was observed in a 1000 ml graduated flask fitted with sampling ports at designated points on the vessel (Fig. 3.1) in order to allow milk samples to be extracted with minimum disturbance of the vessel's contents. An advantage of this separation method is that any associations taking place simultaneously between the globular milk fat and serum phases would not be damaged as a result of the increased shear applied by the centrifugal force. The slow separation was effectively achieved after 24 h at 15°C, as the bulk of the fat in the milk had risen to the top of the flask. The temperature of 15°C chosen for this study, was initially designed to facilitate a moderate rate of creaming over a 24 h period without major levels of bacterial spoilage taking place. This proved later to be fortuitous when it came to data interpretation, i.e., it was possible to differentiate from other phenomena taking place at the lower temperature of 4°C.

As the milk fat rose in the container, a fat globule size gradient emerged whereby larger fat globules occurred in the top layer i.e., FGS fraction 1 (800 -1000 ml, 4.5 cm) > Fraction 2 (600 – 800 ml, 4.2 cm) > Fraction 3 (400 – 600 ml, 4.4 cm). This physical phenomenon is underpinned by Stoke's Law, which predict that larger milk fat globules rise faster than their smaller counterparts due to a density differential and the velocity effect being proportional to the square of particle diameter (Mulder and Walstra, 1974). However, biochemical phenomena are also implicated, due to mechanisms which promote *inter alia* clustering of smaller fat globules. The

phenomenon of agglutination occurring during creaming of milk is attributed to the intervention of particular immunoglobulins (Walstra et al., 1983). Immunoglobulins are better known for displaying similar behaviour in blood during the course of antigen-antibody reactions. Furthermore, the association of IgM in the aggregation and gravity separation of milk is fundamental to the promotion of clustering (Walstra et al., 1983). A temperature-related variation of this, known as cold agglutination, involves a similar mechanism whereby a non-specific reaction occurring at ca. 4°C results in an insoluble protein complex, comprising of IgM,that induces other particles to flocculate (Kelly and Huppertz, 2006). Described as a cryoglobulin, due to its propensity to undergo cold-induced aggregation and precipitation, IgM appears to induce temperature-dependent cluster formation, which was indicated by the formation of a precipitate from cold whey containing cluster-promoting components
(Euber and Brunner, 1984).

However, recent research has shown that both SCC and immunoglobulins are required for normal gravity separation of milk to occur at 4°C in pasteurised (72°C, 17.31s) milk but not following pasteurisation at higher temperature (76°C, 7 min; High temperature pasteurisation) (Geer and Barbano, 2014). This confirms that the clusterpromoting functionality of immunoglobulins is lost as a result of heat induced inactivation during High temperature pasteurisation and no creaming occurs. These findings by Geer & Barbano (2014) suggest that the application of Stoke's Law to describe the natural creaming phenomenon in milk is conditional on the biological involvement by somatic cells and native immunoglobulins present. Increased Complement-activity in the upper layers observed during the current creaming study conducted at 15°C confirms that some form of association between Complement and

globular milk fat exists, and would appear to be independent of the cryo-agglutination phenomena that occur at the lower temperature of 4°C. It should also be noted that freshly drawn raw milk samples were used in the current study, in contrast with the use of Low or High temperature pasteurisation conditions for the pre-heat treatments of milk samples by Geer & Barbano (2014). Such heat treatments would have considerably diminished native Complement activity. As no preheat treatment control was employed in the current study, it is not possible to categorically state that the milk fat creaming phenomenon observed was due to the activity of immunoglobulins at ambient-like conditions (15°C), or purely due to the physical forces of Stoke's Law at play, or even a combination of both. Bacteria and somatic cells did concentrate in the upper cream layers, which suggests that agglutination/clustering may be occurring. One advantage of conducting this study at 15°C was that it eliminated the simultaneous involvement of the cryoagglutination phenomenon (induced at 4°C), so that it is possible to conclude that the association of Complement with globular milk fat acted independently. Somatic cells were present, albeit at low concentrations, in the current study but, as no test control was included, it is not possible to verify whether the SCs participated actively or passively in creaming.

3.6. References

EUBER, J.R. AND BRUNNER, J.R., 1984. Re-examination of fat globule clustering and creaming in cow milk. *Journal of Dairy Science*, *67*(12), pp. 2821-2832.

FOX, P. F., MCSWEENEY, P. L., COGAN, T. M. AND GUINEE, T. P. 2004. *Cheese: Chemistry, Physics and Microbiology: General Aspects*, Academic Press.

FOX, P.F., AND MCSWEENEY, P.L.H. 2001. Advanced Dairy Chemistry: 1. Proteins. 2nd ed., Gaithersburg, MD: Aspen Publishers, Inc.

GEER, S.R. AND BARBANO, D.M., 2014. The effect of immunoglobulins and somatic cells on the gravity separation of fat, bacteria, and spores in pasteurized whole milk. *Journal of Dairy Science*, *97*(4), pp. 2027-2038.

GOLDMAN, A., THORPE, L., GOLDBLUM, R. AND HANSON, L. 1986. Anti-inflammatory Properties of Human Milk. *Acta Paediatrica, 75*(5), pp. 689-695.

HUPPERTZ, T. L., AND KELLY, A.L, 2006. Physical Chemistry of Milk fat globule globules. *In:* FOX, P. F., AND P. L. MCSWEENEY (ed.) *Advanced Dairy Chemistry*, *Volume 2, Lipids* (pp. 173-212). Springer.

ISO. 2008. Milk—Enumeration of somatic cells—part 1: microscopic method (Reference method), EN ISO 13366–1. International Organization for Standardization, Geneva

ISO, E. 2010. Milk–Determination of fat content-Gravimetric method (Reference method). EN ISO 11870-1. International Organization for Standardization, Geneva

MA, Y. AND BARBANO, D.M., 2000. Gravity separation of raw bovine milk: fat globule size distribution and fat content of milk fractions. *Journal of Dairy Science*, *83*(8), pp. 1719-1727.

MAYE, S., STANTON, C., FITZGERALD, G. F. AND KELLY, P. M. 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research, 82*(3), pp. 328-333.

MULDER, H. AND WALSTRA, P., 1974. *The milk fat globule. Emulsion science as applied to milk products and comparable foods* (No. 4). Wageningen, Netherlands, Centre for Agricultural Publishing and Documentation.

OGUNDELE, M.O., 1999. Inhibitors of complement activity in human breast-milk: a proposed hypothesis of their physiological significance. *Mediators of Inflammation*, *8*(2), pp. 69-75.

SAMBROOK, J., RUSSELL, D. W. AND RUSSELL, D. W. 2001. *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press Cold Spring Harbor, New York.

WALSTRA, P., 1983. Physical chemistry of milk fat globules. In *Developments in*

Dairy Chemistry—2, pp. 119-158. Springer Netherlands.

Chapter IV Transformation of serum susceptible *Escherichia coli* O111 with p16*S*lux plasmid to allow for real time monitoring of Complement-based inactivation of bacterial growth in bovine milk

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2016. Transformation of serum-susceptible *Escherichia coli* O111 with p16*S*lux plasmid to allow for real-time monitoring of complement-based inactivation of bacterial growth in bovine milk. *Journal of Dairy Science*, *99*(1), pp. 112-119.

4.1. Abstract

Complement activity has only recently been characterised in raw bovine milk. However, the activity of this component of the innate immune system was found to diminish according as milk was subjected to heat or partitioning during cream separation. Detection of Complement in milk relies on a bactericidal sequestration assay. This assay exploits the specific growth susceptibility of *E. coli* O111 to the presence of Complement. Practical application of the assay was demonstrated when a reduction in Complement activity was recorded in the case of pasteurised and reduced fat milks. This presented an opportunity to improve the functionality of the bactericidal sequestration assay by incorporating bioluminescence capability into the target organism. Following some adaptation, the strain was transformed by correctly integrating the p16*S*lux plasmid. Growth properties of the transformed strain of *E. coli* O111 were unaffected by the modification. The efficacy of the strain adaptation was verified by correlating (r = 0.966; SEy = 0.957) bioluminescence with that of bactericidal sequestration assay total plate counts within the range 7.5 – 9.2 log CFU/ml using a combination of raw and processed milk samples significantly, the transformed *E. coli* O111 p16*S*lux strain could be identified in milk and broth samples using bioluminescence measurement, thus enabling the bacterial assay –viability test to be monitored in real time throughout incubation.

4.2. Introduction

Bovine milk is substantially adapted during the production of humanised infant milk formula (IMF) in order to align its composition with that of human breast milk. However, while much progress has been made in addressing nutritional needs of the

neonate; little attention has been given to the activity of milk's defence systems. Complement, a part of the innate immune system, is made up of approximately 30 distinct plasma proteins that interact to opsonize pathogens and induce a series of inflammatory responses to help fight infection by killing invading microorganisms (Rainard, 2003). Complement is known to be present and anti-pathogenic in human breast milk (Ogundele, 2001) and its presence and activity in bovine milk has also been reported (Maye et al., 2015, Rainard, 2003, Barrio et al., 2003, Rainard P, 1984). Recent efforts to characterise Complement activity in milk have relied on the use of a bactericidal sequestration assay which exploits the sensitivity of a specific strain of *E. coli* O111 (*E. coli* NCTC 8007, serotype O111 K58 (B4) H2) to this innate immune protein system (Ogundele, 2002, Monteiro-Neto et al., 1997). Thus, the growth of added *E. coli* O111 is hindered as a result of the active Complement in milk the extent of growth inhibition of the pathogen may be used as an indirect measure of Complement activity (Reiter and Brock, 1975a). Among examples of where this bacterial assay technique has been applied include the measurement and identification of Complement activity in mastitic and human milks (Rainard, 1984, Barrio et al., 2003).

Previous work by the authors succeeded in detecting residual levels of Complement in milks after processing, lower levels being associated with more intense exposure of milk to heat, e.g., pasteurisation temperatures, and also in the case of low fat milks.

Thus, it was felt that there was a need to adapt and improve the bactericidal sequestration assay (viability test) by lux tagging of the target microorganism in order to elaborate changes taking place to Complement during simulated milk processing steps.

The authors were recently successful in applying the bactericidal sequestration assay to establish Complement activities in freshly drawn bovine milks as seen in Chapter 2. Furthermore, it was also discovered that the Complement response was considerably reduced during exposure of bovine milks to simulated milk processes such as heating and preparation of low-fat milks. Inactivation of indigenous enzymes in milk such alkaline phosphatase is a commonly used bio-marker to prove the efficacy of milk pasteurisation. This study was less preoccuppied with the use of Complement as a bio-marker for calibrating the effectiveness of thermal processes, but more interested in the preservation, where possible, of milk's innate immune properties.

As bovine milk is subjected to considerable processing during manufacture of infant milk formula, i.e., pasteurisation, homogenisation, evaporation and spray drying, it was felt that an improved bacterial assay would be more useful for real time monitoring during operations that give rise to reduced Complement activity. Lux tagging of the target microorganism was deemed to be an appropriate strategy which would allow bioluminescent signals to reflect growth and bioluminescence imaging to visualise the magnitude of bacterial inhibition speedily during the viability test. Thus, the initial step was to develop a method to introduce the plasmid, p16*S*lux (Morrissey et al., 2011; Riedel et al., 2007a) containing the lux operon derived from *Photorhabdus luminescens* into the *E. coli* O111 strain. One challenge with this approach was a lack of available data on successful genetic modifications to *E. coli* O111. In addition, previous work aimed at rapid detection of this strain identified the existence of a number of phenotypes of the O111 organism (Schmidt and Karch, 1996). However, there is some support available in the reporting of successful adaptations of different

strains and microorganisms involving the p16 *S*lux plasmid, e.g., lux-tagging of Gram-negative strains such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, *Brucella melitensis*, *Pseudomonas aeruginosa*, and *Citrobacter rodentium* (Riedel et al., 2007a).

Thus, the study's two-fold objective was, firstly, to perform strain transformation to ensure consistent expression of the p16*S*lux in *E. coli* O111. Secondly, it was necessary to compare and contrast the growth behaviour of the transformed *E. coli* O111 strain alongside that of the original unmodified culture according to the protocol of the regular bactericidal sequestration assay.

4.3. Materials and methods

4.3.1. Bacterial strains, media, and chemicals

E. coli O111 (*E. coli* NCTC 8007, serotype O111 K58 (B4)) a pathogenic Complement sensitive strain, was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections Porton Down, Salisbury Wiltshire, SP4 0JG UK). The strain was routinely grown in Luria-Bertani (LB) medium 37°C with shaking. When required, the antibiotic erythromycin (Sigma Chemical Company, St. Louis, MO) was added at a concentration of 500 µg/ml.

4.3.2. Preparation of competent cells and electroporation

Originally, a method described by Dagert and Ehrlich (1979) was undertaken which required the withdrawal of 2 ml from a flask containing 200 ml sterile LB broth and

its replacement with 2 ml of an overnight culture grown in LB medium. The inoculated medium was grown at 37°C for approx. 2h or until the OD at 600 nm was within the range 0.6 – 1.0. At this point, the 200 ml was equally distributed into 4 sorvall tubes and the cells were harvested by centrifugation at room temperature for 15 min at 15,000 g. A cell pellet was collected and washed twice with distilled water. The cell pellet was then washed using glycerol and finally re-suspended in glycerol which contained on average $10^9 – 10^{10}$ viable bacteria.

Unfortunately, this method was unsuccessful and no viable electrocompetent cells were established. A second method was applied which called for 2 ml of the overnight cultures to be added to 200 ml of LB broth before growing to an OD at 600 of 0.2 - 0.4 in approx. 2.5h. The cells were harvested by centrifugation for 5 min at 5000 g and washed twice using $CaCl_2$. The cells were incubated on ice for 2h between washes. The cell pellet was finally collected and re-suspended in glycerol and stored at -80°C until required. This method proved to be effective on *E. coli* O111 and produced electrocompetent cells.

The plasmid p16*S*lux, kindly donated by Professor Colin Hill's research group in University College Cork, Ireland, was prepared using the Qiagen mini-prep kit as previously described, and integrated into the *E. coli* O111 bacterial chromosome by homologous recombination (Riedel et al., 2007b). For electroporation, 7 µl of the p16*S*lux was purified using the Qiagen plasmid mini-prep kit and was mixed with 50 µl of the electrocompetent *E. coli* O111. This mixture was transferred to a 5 mm gap width electroporation cuvette and placed in a BTX Electroporation System (Harvard Apparatus, Holliston, USA).

4.3.3. Sample preparation

Fresh whole milk was collected from the Moorepark Dairy Production Centre (MDPC) farm on the day of testing. An Armfield disc bowl centrifuge (Armfield, Ringwood, UK) was used in the laboratory to generate cream samples (approximately 40% fat) and skimmed milk (approximately 1% fat) from the fresh raw milk. Commercially pasteurised bovine milk was purchased from local supermarkets on the day of testing.

All samples were analysed for TBC using the reference ISO methods ((ISO 2004, ISO 2013). The SCC of the raw, skimmed and retailed milks was determined using a Bentley Somacount 300® (Bentley Instrument Inc., Chaska MN, USA). Milks with SCC < 200,000 cells per ml were selected for bacterial assay in order to avoid false positive results being contributed by the high levels of other immune factors like immunoglobulins and leukocytes triggered in the wake of mammary infection.

4.3.4. Control preparation

Freshly collected raw milk was heat treated at 56°C for 30 min to ensure complete inactivation of the complement protein (Korhonen et al., 2000). All samples were screened using the ISO methods (ISO 2004, ISO 2013) and also the milks were streaked on Nutrient and Luria Bertani agar.

4.3.5. Bactericidal sequestration assay – viability test

Wild type *E. coli* O111 and the transformed *E. coli* O111 was prepared for overnight growth at 37°C on Luria-Bertani (LB) agar as described (Sambrook et al., 2001). Isolated colonies was picked from replicate plates and inoculated into three tubes of LB broth (Merck KGaA, Darmstadt, Germany) and grown overnight at 37°C at 200 rpm. After approx. 18 – 24h, the overnight cultures were centrifuged at 5000 rpm, followed by re-suspension of the resulting pellet in phosphate buffer saline solution (PBS solution) - this step was replicated twice. Following this, the pellet was resuspended in LB broth and adjusted to 3 x 10^8 colony forming unit per ml (CFU per ml) using the McFarland method. Using 96-well plates, 3 x 20 µl of both strains were added to each round-bottomed well (Sarstedt Ltd. Wexford, Ireland) and 80µl of the sample to be tested was then added. The plate was placed in a shaking incubator (Model Mini 4450 SHKA4450-1CE, Fischer Scientific, Ballycoolin, Dublin) at 200 rpm for 2h at 37°C. A 20 µl sample was taken from each well after incubation; total viable counts were enumerated on LB agar.

4.3.6. Monitoring of growth in LB broth by performing viable plate counts and measuring bioluminescence

Overnight cultures of both the wild type (WT) and transformed *E. coli* strain were grown in LB broth. Erythromycin was included in the LB broth in order to support the transformed strain from reverting to its original form. These were centrifuged at room temperature for 15 min at a speed of 5000 g, followed by washing with PBS and re-suspension in LB broth. Twenty µl of inoculum was then added to fresh LB broth

and grown in a shaking incubator at a speed of 200 rpm and temperature of 37°C. Samples were collected at 1h intervals and viable plate counts were carried out to by plating dilutions onto LB agar. Two hundred microliters of the freshly inoculated LB broth was added to a 96 - well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark), and optical density was measured hourly using a Synergy HT plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Simultaneously, bioluminescence was also measured in photons per second per square centimetre with an IVIS Xenogen Imaging 100 system (Xenogen, Alameda, CA) with a binning of 16 and an exposure time of 1 min.

4.3.7. Evaluation of minimum inhibitory concentration of the WT and transformed *E. coli* O111 in erythromycin

Both strains of *E. coli* O111 were tested according to the M.I.C.E strips (Ovoid Ltd, Basingstoke's Hants, RG24 8PW, England) method which gives an accurate minimum inhibitory concentration (MIC) over the range 256 – 0.015 μg/ml. This was to confirm that tolerance to erythromycin of p16*S*lux plasmid was conferred on *E. coli* O111 following transformation. (Riedel et al., 2007a)

4.3.8. Molecular biology experiments

PCR reactions were performed on the wild type *E. coli* O111 and transformed *E. coli* O111 using Biomix red (Mybio, Kilkenny, Ireland), primers were purchased from Eurofins (MWG, Ebersberg, Germany)

16S fwd (5'-ACACTGGAACTGAGACACGGTCCAGACTCC-3') and

16S rev (5'-TTGTAAAACGACGGCCAGTGAGCGCGCG-3')

to verify that the p16*S*lux plasmid had correctly integrated at the preferred location. The PCR reactions were carried out using a T3000 Thermocycler (Biometra, Gottingen, Germany) as previously described (Morrissey et al., 2011) with the addition of a preliminary "hotstart" step. Gel electrophoresis was performed using the Power pack 200 (Bio-Rad, Hercules, CA, United States of America) - 4 µl of the PCR products were routinely run on 1% agarose gels (Invitrogen, Carlsbad, CA) at 90 V and 2 A for 60 min.

4.4.Results

4.4.1. Transformation of the *E. coli* O111 strain

Following the utilisation of two separate methods to successfully transform *E. coli* O111, the mutation was ultimately effective. Finally, the p16*S*lux plasmid became well established within *E. coli* O111 following electroporation (Riedel et al., 2007a). The exact location of plasmid integration was confirmed by means of PCR using primers specifically designed to address the target integration site. Gel electrophoresis carried out on PCR products e.g. the identification of a 1,163-bp band produced confirmed the correct positioning of the plasmid (Fig 4.1).

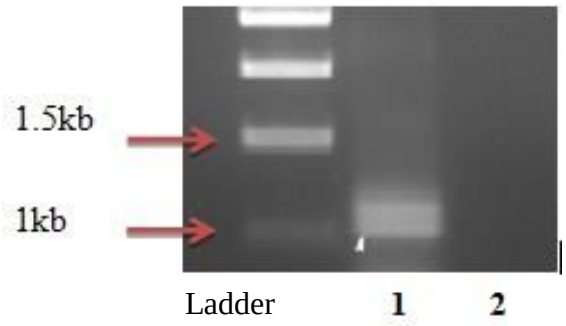


Figure 4.1 Electrophoresis gel showing the approx. 1,163bp band which confirms the presence of the p16 Slux plasmid which integrates into the 16S rRNA gene of Gram-negative bacteria. Lane 1 is the p16 transformed strain, lane 2 is the wild type strain; as expected, there is a band present in lane 1 indicating the successful integration of the plasmid in the correct position in the transformed *E. coli* O111 strain. Furthermore, PCR and gel electrophoresis performed on the unmodified *E. coli* O111 strain revealed that no PCR product was amplified from this strain.

4.4.2. Observation of growth properties of both strains

The WT and genetically modified strains of *E. coli* O111 were grown overnight on LB agar, it was found that both forms of *E. coli* were similar in terms of size, shape (round) and colour (cream).

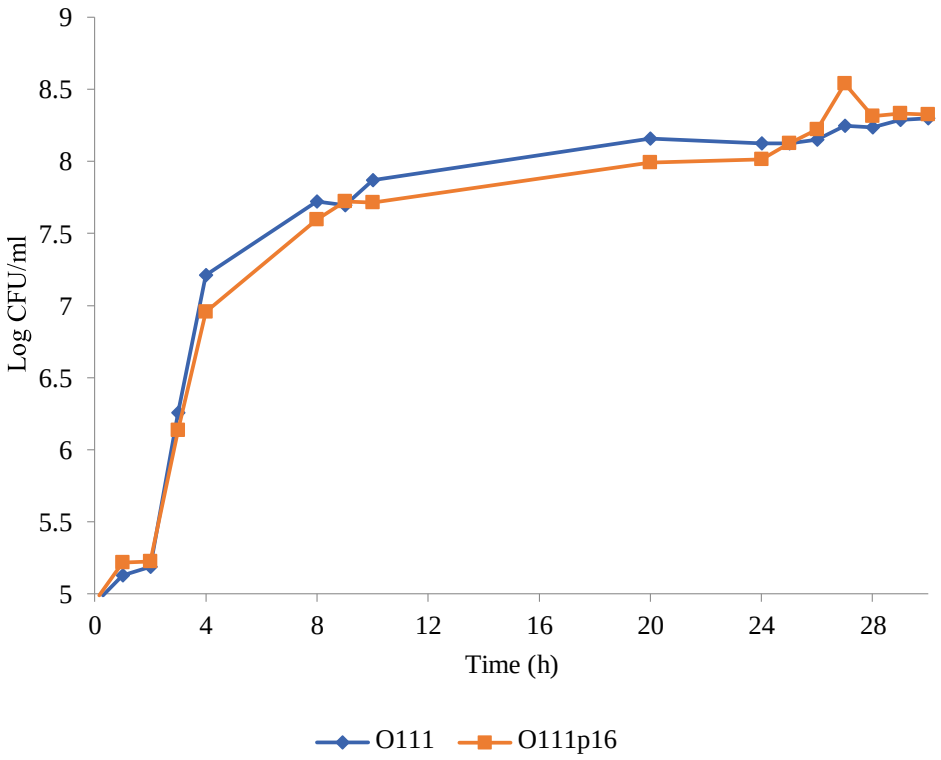


Figure 4.2 Total viable count of WT *E. coli* O111 compared to the transformed p16*Slux* *E. coli* O111 in LB broth. The measurements are represented as log CFU per ml. Both strains were grown over night in a shaking incubator at 37°C and 200 rpm for 27h in LB broth.

Importantly, there was no significant difference ($p < 0.05$) identified through enumeration of total viable counts during overnight growth of the isolated colonies in LB broth at 37°C, with values of 8.13 and 8.01 log CFU per ml for the WT and transformed cultures respectively (Fig 4.2); moreover, hourly measurement of absorbance readings at 600 nm identified similar growth as seen in Fig 4.3.

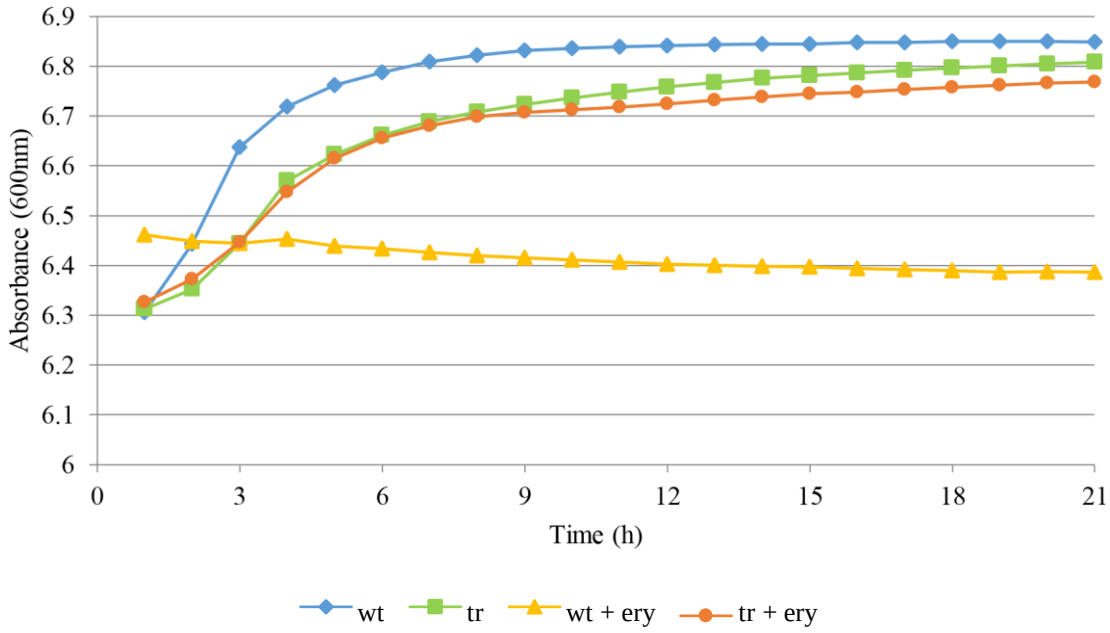


Figure 4.3 Absorbance readings (600 nm) of *E. coli* O111in LB broth, genetically modified *E. coli* O111 in LB broth, wild type *E. coli* O111 in LB broth with 500 µg/ml erythromycin, (X) genetically modified *E. coli* O111 in LB broth with 500 µg/ml erythromycin, (log10 CFU/ml) during incubation at 37°C for 21h.

Furthermore, the luminescence remained relatively stable throughout the entire growth curve, as was seen in previous studies (Riedel et al., 2007a); this was confirmed by the correlation between the reduction in luminescence ROI and respective CFU counts. Overall, it can be concluded that the growth properties of *E. coli* O111 strain remained intact after genetic modification involving plasmid insertion.

4.4.3. Confirmation of effects of the p16Slux plasmid.

Confirmation of acquired antibiotic resistance in the newly transformed strain to erythromycin was demonstrated during 21h incubation at 37°C in LB broth in the presence of erythromycin (Fig 4.3), while the WT *E. coli* O111 was unable to grow

under these conditions. In addition, the transformed strain produced strong bioluminescent readings in the images (Fig 4.4a and 4.4b) captured from the Xenogen IVIS, while the WT strain did not release any bioluminescence.

a. **b.**

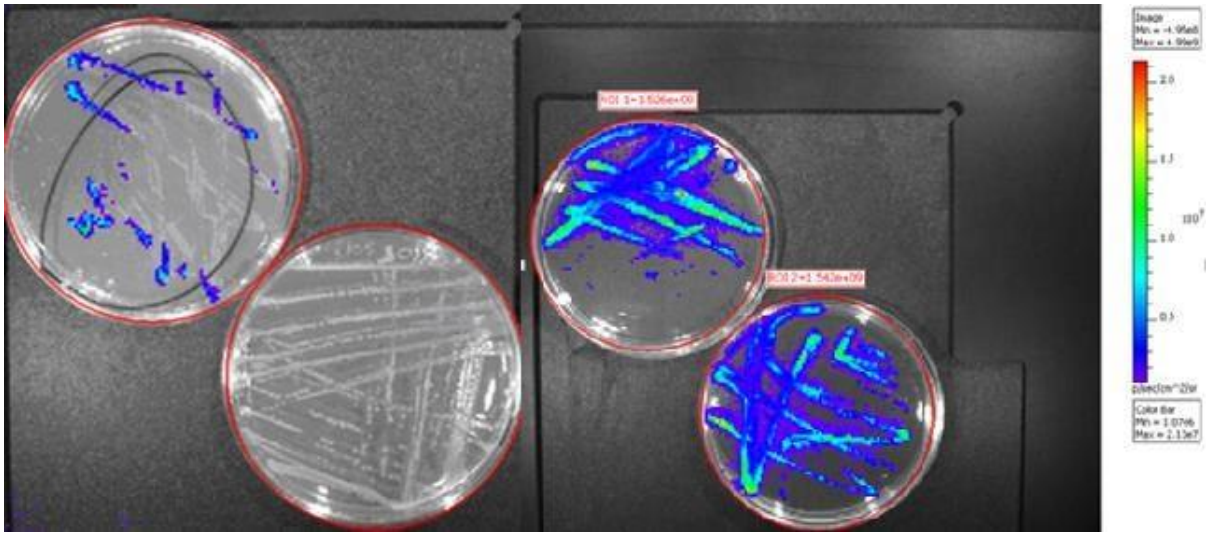


Figure 4.4a. Bioluminescence image of transformed *E. coli* O111 alongside the original unmodified wild type strain of *E. coli* O111 and **Figure 4.4b** representing the higher levels of transformed *E. coli* O111, the image was captured on a Xenogen IVIS 100 imager, culture was grown overnight on LB agar at 37°C.

Confirmation of tolerance to erythromycin of the transformed *E. coli* O111 strain is evident according to the lack of inhibition in the immediate zones of the M.I.C.E strips (Fig 4.5), thus, concurring with the functionality cited for the plasmid insert by Riedel, Casey et al., (2007). By contrast, a considerable area of clearance may be seen in the case of the WT strain.

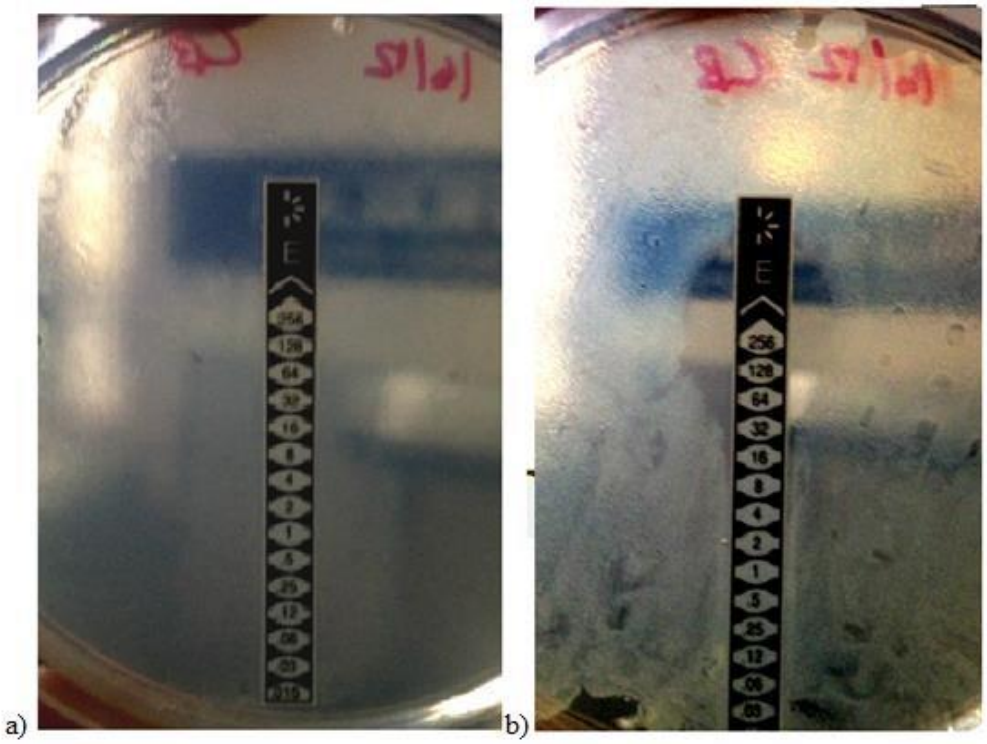


Figure 4.5 (a) Lack of inhibition in the immediate zones of the M.I.C.E strips provides confirmation of tolerance to erythromycin of the transformed *E. coli* O111 strain. (b) A considerable area of clearance may be seen in the case of the WT strain.

4.4.4. Application of transformed *E. coli* O111 p16Slux plasmid

Bioluminescent imaging was monitored in real time during the performance of the bactericidal sequestration assay involving the transformed *E. coli* O111 strain. The efficacy of strain adaptation was illustrated by the bioluminescent images captured, initially and after 2h (Fig 4.6) shaking incubation (37°C at 200 rpm) of p16Slux *E. coli* O111 in heat-treated (56°C, 30 min) milk, freshly-collected raw milk, skim milk and commercially retailed full-fat milk.

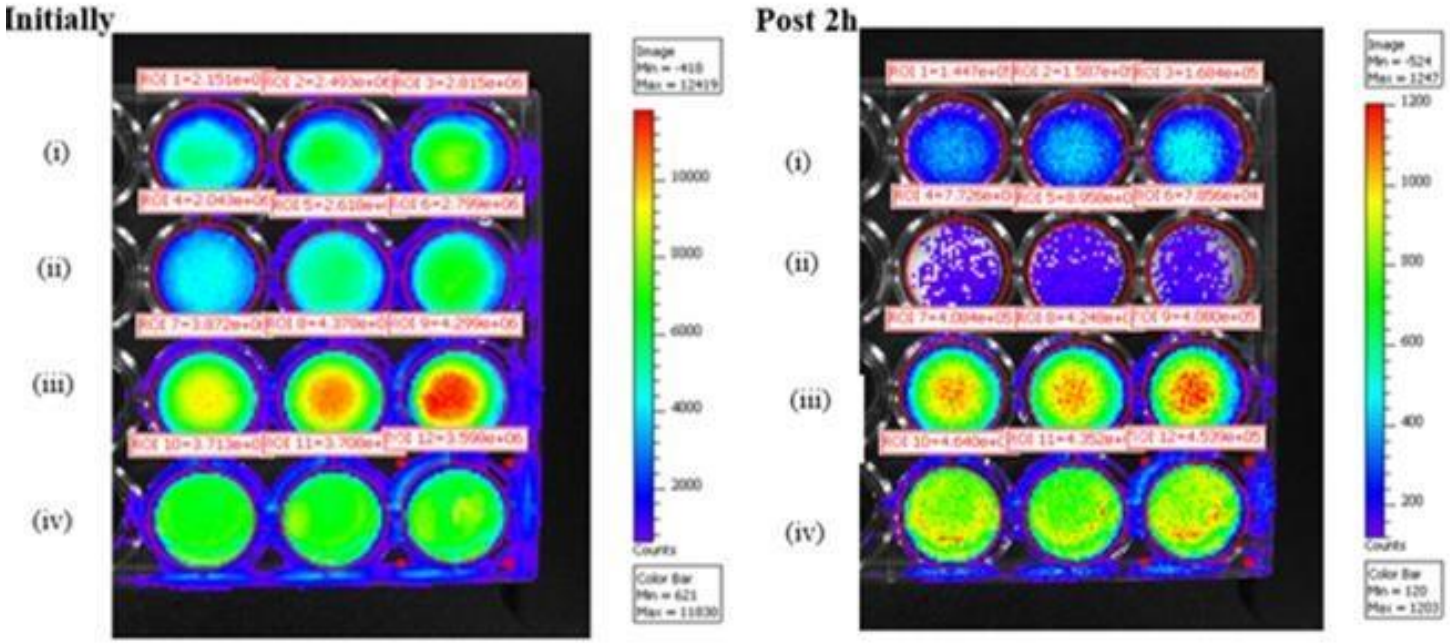


Figure 4.6 Growth of transformed *Escherichia coli* O111 at initial inoculation and following 2h incubation in (i) laboratory prepared skim milk, (ii) raw bovine milk, (iii) commercial pasteurized milk, and (iv) heated bovine milk (control) during the bactericidal assay for 2h at 37°C. Bacteria were counted in the red circled regions of interest (ROI).

The luminescent data overlaid on the photographic image of the clear 12-well plate (Fig 4.6) utilises a pseudocolor scheme which aids in the observation of light emission. In the brighter area of the image, more photons are detected and the photon intensity number is greater than in the less bright areas.

This is best described by tracking colour change from the time of initial inoculation to termination after 2h incubation of p16*S*lux *E. coli* O111 in a variety of treated milk substrates. Exponential growth of the culture in retailed pasteurised milk and heated bovine milk was represented by bright red and yellow light emission. However, the lighter blue coloured zones were a consequence of lower light emission when the

growth of transformed *E. coli* O111 was inhibited in skim milk, and most notably, in the raw bovine milk (due to Complement activity).

The corresponding results generated by the bactericidal sequestration assay using the genetically modified *E. coli* O111 p16 *S*lux strain for the control heat treated milk (56°C, 30 min), raw bovine milk, commercial retailed milk and lab-prepared skim milk were 9.18, 8.60, 7.70 and 8.58 log CFU/ml, respectively (Fig 4.7). This data coincided with the region of interest (ROI) photon measurements undertaken concurrently, in which the differences in bioluminescence intensity reflected changes in the aforementioned bacterial growth. The reduced total viable counts of *E. coli* O111 in raw bovine milk lower ($p < 0.05$) compared to skim, retail pasteurised and heat-treated control bovine milks reflected its stronger bacteriostatic effect due to Complement (Reiter et al., 1975a; Reiter and Brock, 1975b).

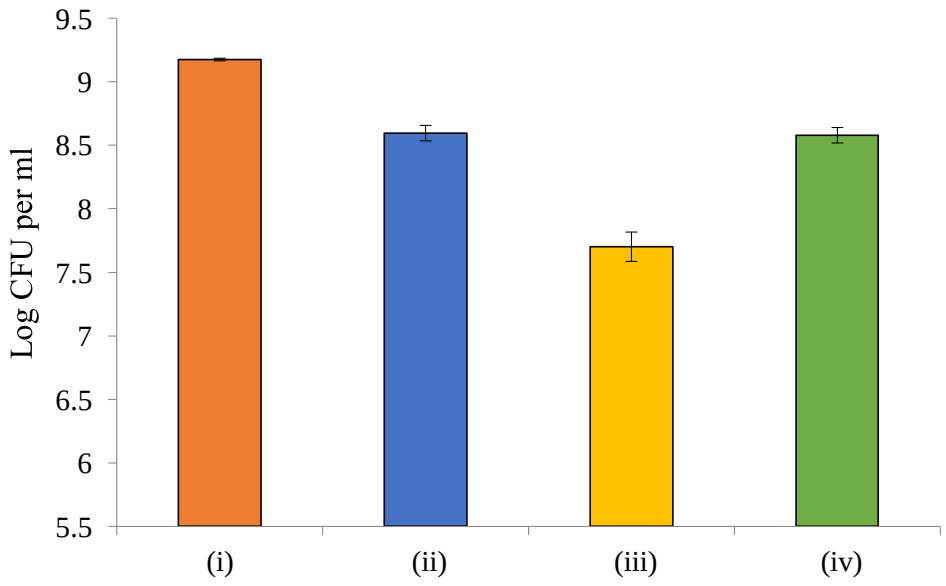


Figure 4.7 Growth of the transformed *Escherichia coli* O111 p16Slux (log10 CFU/ml) during bacterial sequestration assay at after 2h incubation with (i) heated bovine milk (control), (ii) commercial pasteurized milk, (iii) raw bovine milk, and (iv) laboratory prepared skim bovine milk. Error bars indicate standard deviation of triplicate assessments.

Hence, incorporation of bioluminescent imaging *via* genetic modification to enhance the functionality of the probe microorganism is an effective enhancement of the assay for monitoring Complement activity in various unprocessed and processed milks. According to the independent-samples t-test, there was a significant difference ($p < 0.05$) between all samples tested and the raw bovine milk, while statistically there was no significant difference ($p < 0.05$) between the lab-prepared skim milk and retail pasteurised samples.

4.4.5. Relationship between bacterial counts and bioluminescence of *E. coli* O111

The correlation between the traditional TBC and the adapted bioluminescent measurements the data was analysed (Fig 4.8). It can be seen that the adapted bioluminescent based sequestration method is strongly correlated to the unmodified assay according to the equation $y = 0.9748x + 0.2133$ with a correlation coefficient (R) 0.966, and a coefficient of determination (R^2) of 0.933. The standard error of prediction value is 0.957.

These studies confirm that the objective of inserting a p16 *S*lux plasmid into *E. coli* O111 in order to generate a bioluminescent response during subsequent growth was successfully accomplished. This transformed strain, which was originally used as an analytical probe in the bio-sequestration assay to detect the presence of Complement activity in human milk (Ogundele, 2002), and more recently in bovine milk (Maye et al., 2015) may now be utilized to measure antimicrobial response in real time throughout the bactericidal sequestration assay. This adds greater certainty to the test given the diversity of other innate bacterial strains present in non-pasteurised milk, in particular, strains which may lead to the risk of false positive identifications.

Furthermore, erythromycin resistance conferred on the transformed *E. coli* O111 also allows the strain to be selectively screened for by means of susceptibility tests with antibiotic-containing agars.

Finally, application of the now upgraded bactericidal sequestration assay confirmed the earlier findings of Maye et al. (2015) that pasteurisation (retail pasteurised milk) and cream removal (lab-separated skim milk) contributed to substantial loss of Complement activity but, importantly, some residual Complement activity is detectable in both the lab- prepared skim milk and pasteurised milk.

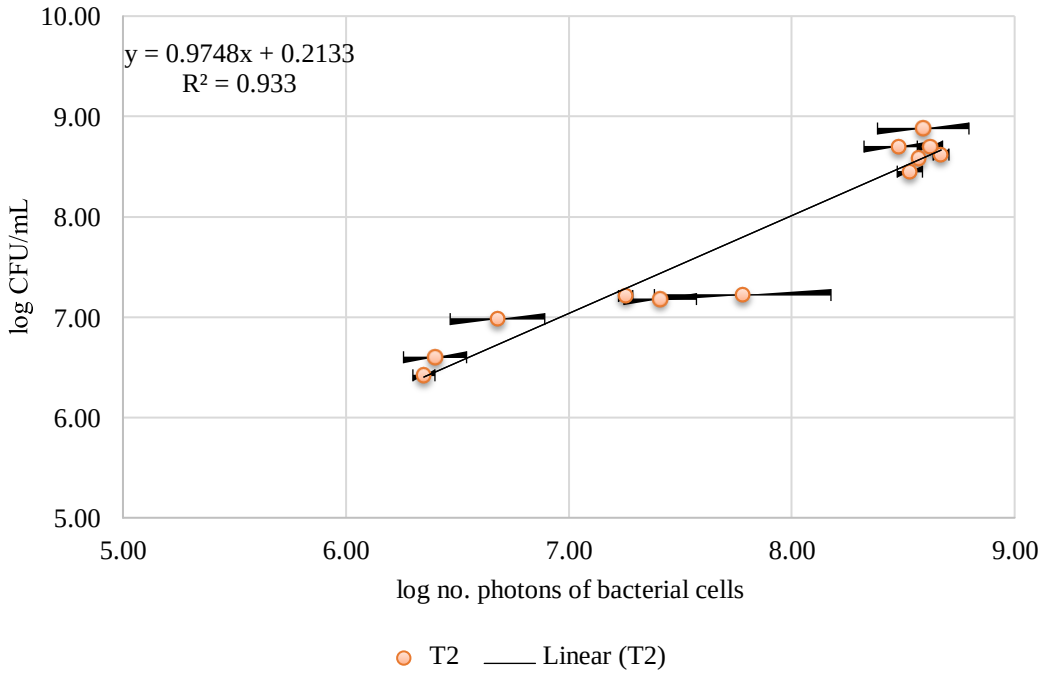


Figure 4.8 Assessment of the relationship between the bacterial counts of *E. coli* O111 measured by luminescence and total plate counts.

4.5.Discussion

The transformation of *E. coli* O111 proved to be challenging due to the relatively unknown response of *E. coli* O111 to genetic transformation and shortage of data in existence for the relevant strain. Moreover, a relevant study to rapidly identify *E. coli* O111 in food recognised much variance in phenotype within the O111 group, which suggested possible difficulty during attempts to modify the strain (Schmidt and Karch, 1996). For this reason, the initial method to prepare an electrocompetent strain was unsuccessful, while the second method, which featured additional adaptations such as the use of calcium chloride and altered temperature regimes, was ultimately effective. The insertion of the p16*S*lux plasmid transforms the strain and allows it to tolerate the antibiotic erythromycin; moreover, it affords the ability to emit bioluminescent light and in this way the strain can be measured quickly and allows real time monitoring. Finally, the p16*S*lux plasmid became well established within *E. coli* O111 following electroporation to the electrocompetent cells (Riedel et al., 2007a). The exact location of plasmid integration was confirmed by means of PCR using primers specifically designed to address the target integration site. It was confirmed that this modification did not affect the strains growth properties or appearance, further, serum susceptibility was maintained in the strain. Moreover, we also found that the luminescence was relatively stable throughout growth phases.

The next step was to include the transformed strain in the bactericidal sequestration assay. By considering the changes in the pseudocolour scheme, it could be seen that there was exponential growth of the culture in retailed pasteurised milk as well as in the heated bovine milk. In the skim and raw bovine milk there was restricted growth of the transformed strain. This was most significant in the raw bovine milk, which was further confirmed by the bactericidal sequestration assay whereby the raw milk was the most potent for restriction of transformed *E. coli* O111.

Hence, incorporation of bioluminescent imaging *via* genetic modification to enhance the functionality of the probe microorganism is an effective enhancement of the assay for monitoring Complement activity in various unprocessed and processed milks.

4.6.References

BARRIO, M. B., RAINARD, P. AND POUTREL, B. 2003. Milk complement and the opsonophagocytosis and killing of *Staphylococcus aureus* mastitis isolates by bovine neutrophils. *Microbial Pathogenesis*, *34*(1), pp. 1-9.

DAGERT, M., AND S. D. EHRLICH. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene*, *6*(1), pp. 23–28.

FRANK, M. M. AND FRIES, L. F. 1991. The role of complement in inflammation and phgocytosis. *Immunology Today*, *12*(9), pp. 322-326.

ISO. 2004. Milk—Quantitative determination of bacteriological quality—guidance for establishing and verifying a conversion relationship between routine method results and anchor method results, EN ISO 21187. International Organization for Standardization, Geneva.

ISO. 2013. Milk - Bacterial count - Protocol for the evaluation of alternative methods (Reference method), EN ISO 16297–1. International Organization for Standardization, Geneva.

KORHONEN, H., MARNILA, P. AND GILL, H. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition, 84*, pp. 75-80.

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research, 82*(3), pp. 328-333.

MONTEIRO-NETO, V., CARVALHO CAMPOS, L., PIANTINO FERREIRA, A. J., TARDELLI GOMES, T. A. AND TRABULSI, L. R. 1997. Virulence properties of *Escherichia coli* O111:H12 strains. *FEMS Microbiology Letters, 146*(1), pp. 123-128.

MORRISSEY, R., BEGLEY, M., OSHIMA, S., REA, M., ROSS, R. P. AND HILL, C. 2011. Real-time monitoring of luciferase-tagged *Cronobacter sakazakii* in reconstituted infant milk formula. *Journal of Food Protection®, 74*(4), pp. 573-579.

OGUNDELE, M. O. 2001. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. *Immunology and Cell Biology, 79*(1), pp. 1-10.

OGUNDELE, M.O., 2002. Effects of storage on the physicochemical and antibacterial properties of human milk. *British Journal of Biomedical Science*, *59*(4), pp. 205.

RAINARD, P. 2003. The complement in milk and defense of the bovine mammary gland against infections. *Veterinary Research, 34(*5), pp. 647-670.

RAINARD, P., POUTREL, B. AND CAFFIN, J.P., 1984. Assessment of hemolytic and bactericidal complement activities in normal and mastitic bovine milk. *Journal of Dairy Science*, *67*(3), pp. 614-619.

REITER, B. AND BROCK, J. 1975a. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype O111. *Immunology*, *28*(1), pp. 71.

REITER, B., BROCK, J. AND STEEL, E. D. 1975b. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. *Immunology*, *28*(1), pp. 83.

RIEDEL, C. U., CASEY, P. G., MULCAHY, H., O'GARA, F., GAHAN, C. G. AND HILL, C. 2007a. Construction of p16Slux, a novel vector for improved bioluminescent labeling of gram-negative bacteria. *Applied and Environmental Microbiology, 73*(21), pp. 7092-7095.

RIEDEL, C. U., MONK, I. R., CASEY, P. G., MORRISSEY, D., O'SULLIVAN, G. C., TANGNEY, M., HILL, C. AND GAHAN, C. G. 2007b. Improved

luciferase tagging system for Listeria monocytogenes allows real-time monitoring in vivo and in vitro. *Applied and Environmental Microbiology, 73*(9), pp. 3091-3094.

SAMBROOK, J., RUSSELL, D. W. AND RUSSELL, D. W. 2001. *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press Cold Spring Harbor, New York.

SCHMIDT, H. AND KARCH, H. 1996. Enterohemolytic phenotypes and genotypes of shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic-uremic syndrome. *Journal of Clinical Microbiology, 34*(10), pp. 2364-2367.

Chapter V Bovine milk Complement activity following intra-mammary challenge with *Streptococcus dysgalactiae*

Submitted Journal of Dairy research

5.1. Abstract

Recently published work as described in Chapter 2 highlighted the extent of Complement activity in bovine milk. Localised mastitic infection occurring in the mammary glands of dairy cows is readily detectable by the levels of somatic cells in milk. Thus, it is opportune to monitor Complement activity in milks in association with the animal's innate immune response to mammary infection.

Preliminary screening of milk samples taken randomly showed that milk with a high somatic cell count (SCC) reduced growth of the Complement-sensitive strain *E. coli* O111 to a greater extent ($p < 0.05$) than when the marker microorganism was grown in milk heated for the purpose of inactivating Complement. A follow-up study set out to determine the effect on Complement activity when a sub-clinical mastitic infection was induced in the mammary gland of four lactating dairy cows. The effect of *Str. dysgalactiae* spp. *dysgalactiae* inoculation into selected individual udder quarters of the mammary glands of each animal was followed by monitoring of SCC levels in the milks from the segregated udder samples during subsequent milking. At 72 and 96h post inoculation (PI), the SCCs for the challenged quarter increased to 6.73, 6.76, 6.84 and 6.71 log SCC per ml compared to normal values (approx. 4.38 log SCC per ml). At the same time, the bactericidal sequestration assay identified increased *E. coli* O111 inhibition that can be directly linked to greater Complement activity in those quarter milks affected by induced inflammation. The high SCC milks identified that all cows were more effective in limiting *E. coli* O111 growth PI, i.e., log CFU per ml

of *E. coli* O111 for all four cows numbered at day 2 was between 6.69 and 7.92, in contrast to the control quarters which had values ranging from 8.27 to 8.45 log CFU per ml. Milks from the unchallenged quarters in all four cows were significantly less effective at reducing growth of the assay strain ($p < 0.05$).

An ELISA assay targeting specific activation components of the Complement pathways confirmed that greater bacterial inhibition observed during the bactericidal sequestration assay was attributable to due to higher Complement activity in the milk samples from the affected quarters, i.e., with higher SCC. The induced infection was confirmed as self-limiting in three of the affected animals and their SCC returned to normal levels within 14 d PI, while the fourth cow required brief antibiotic intervention.

5.2. Introduction

There is considerable evidence to support the importance of passive immunity, present in breastmilk, in protecting of the neonate during the early stages of life (Goldman et al., 1986, Butler, 1979). As it requires some time for new-born babies immune system to be fully developed, mother's milk plays a crucial role in delivering protection and development. These innate immune components elicit a potent response against a large array of invading microorganisms. Important factors include;

antibodies, TNFα receptors, interleukin (IL)-1RA, partially digested lactoferrin, anti-inflammatory cytokines IL-10 and transforming growth factor. In addition, human breast milk also contains some antioxidants, protease inhibitors and prostaglandins. Beneficial *lactobacilli* and bioactive *bifidus* factors have also been isolated and measured in higher quantities in breast-fed infants compared to their formula-fed counterparts. Thus, the absence or limited presence of these beneficial factors in infant formula is likely to lead to a higher incidence of health disorders of an enteric nature, including inflammatory bowel diseases (Newburg, 2007, Tasnim, 2014).

Complement is a major component of the innate immune response and is known to provide protection in human breast milk against invading microorganisms (Ogundele, 2001). Its role is believed to be bactericidal, a biological marker that is defined by the bactericidal sequestration assay used to determine its presence (Ogundele, 2002). Originating in the blood and tissues of the lactating mammal, precursor zymogens of the Complement system at the sites of infection are activated locally and trigger a series of potent inflammatory events. It is known that a number of the Complement proteins are classified as zymogens which are proteases activated by proteolytic cleavage. These zymogens act at specific sites of infection to trigger a series of potent inflammatory events and also contribute to the activation of some components of the Complement system (Janeway et al., 2001). Following initiation, a cascading mechanism made up of 30 serum and non-serum proteins stimulate auxiliary

components of the innate immune as well as working alongside and promoting the adaptive immune system. Complement activity has recently been detected in freshly drawn bovine milk with an approx. eighty per cent bactericidal efficacy compared to human milk against the assay target bacterium (*E. coli* O111) (Maye et al., 2015). This finding raised questions as to the origin of Complement in milk, particularly with respect to mammalian immunity and transmissibility of innate immune components to milk. The inflammatory response is a process in the body which activates the required components to eliminate invading organisms and also to initiate tissue repair. Some of these antibacterial activities include increased levels of lactoferrin and specific blood proteins such as immunoglobulins and complement components (Pyörälä and Mattila, 1987).

Mastitis is as an inflammation of the mammary gland that affects lactating mammalian species (Sordillo and Streicher, 2002) and is ranked highest (16.5% in 2007) in terms of morbidity (±0.5 SD mean) expressed as a percentage of all cows in the USA (Sordillo, 2009).

Mastitis is categorised as either clinical or subclinical according to the degree of severity. The subclinical form in most cases may only detected using laboratory techniques and is widely undetected at farm level. However, clinical cases of infection are accompanied by fever, swelling of the udders as well as milk exhibiting a watery appearance, flakes, clots, or pus. In more serious cases (mostly associated with *Escherichia coli*), it is accompanied by clinical signs such as a reduction in milk

yield, elevated body temperature, lack of appetite, sunken eyes and signs of diarrhoea and dehydration. A major cause of mastitis is known to be due to microorganisms, the most pertinent causative agents being of bacterial origin. *Staphylococcus aureus, Streptococcus agalactiae, Str. dysgalactiae, Str. uberis* and *Escherichia coli* are the common causes of bovine mastitis. Other strains have also contributed to occasional herd outbreaks of mastitis. In general, mastitis occurs following entry by pathogens into the teat canal of cows.

From a consumer perspective, it is valuable to know that innate immune components contribute to the overall biological value and anti-pathogenic effects of milk. At the same time, there is little knowledge of how such innate immune factors are affected by the health status of the lactating cow. Hence, it was opportune to explore mastitic infection as a health disorder with which to monitor changes in Complement activity in milks drawn from both healthy as well as udder quarters showing the first clinical signs of mastitis in the secreted milk, i.e., containing high SCC.

Given the association of Complement with the innate immune system, it was hypothesised as to how well the passive immune response could be activated in a controlled manner by inoculating *Streptococcus dysgalactiae* spp. *dysgalactiae* into individual udder quarters of mammary glands. In the immediate aftermath, it was necessary to quantify the level of active Complement partitioning into milk from infected and non-infected mammary quarters of treated cows. It was also opportune

to make a general assessment of the extent of the relationship between elevated SCCs and the bacteriostatic potential of milks produced during the trials.

5.3.Materials and Methods

The experimental procedures carried out in the following sections were performed under licence from the Irish Department of Agriculture and Food, Agriculture House, Kildare St. Dublin 2, Ireland.

5.3.1. Milk samples

Fresh whole milk was collected from the Moorepark Dairy Production Centre (MDPC) farm on the day of testing. For part one of this studu milk was collected from 3 individual animals on 3 consecutive days at the morning milking time. Each milk sample was screened for presence of pathogenic microbes and levels of bacteria present were measured using ISO 16917/IDF 161 method (ISO 2013). The SCC was also assessed using ISO methods using Bentley Somacount 300® (Bentley Instruments Inc., Chaska MN, USA). Milks were classified according to high SCC, i.e., >200,000 cells per ml, while low SCC milks were <100,000 cells per ml. For this part cows were selected with quarters exhibiting both high and low SCCs for the preliminary study. Milk was collected from the individual quarters of each cow

containing high and low SCC and assessed using the bactericidal sequestration assay. For the subsequent inoculation trials, milk samples were aseptically collected from individual quarters from cows who maintained the selection criteria outlined below. SCCs were monitored 7 days pre- and up to 12 days post inoculation (PI). Bulk milk samples were closely monitored up to 2 months following inoculation.

5.3.2. Control preparation

Freshly collected raw milk was heat-treated at 56°C for 30 min to ensure complete inactivation of the complement protein (Korhonen et al., 2000). All samples were screened using the ISO methods (ISO 2013, ISO 2004) and also the milks were streaked on Nutrient and Luria Bertani agar.

5.3.3. Animal selection

The animals selected were from the dairy production centre in Moorepark Fermoy, Co. Cork and were chosen based on their low SCC as well as long term and up-to-date infection information. Seven days pre intra-mammary challenge, all animals and individual quarters were inspected by trained staff; to be selected, each animal required a healthy appearance. SCC was monitored from all cow milk samples to ensure no increase in levels due to infection. The milks were also screened using

violet red and aesculin blood agar to monitor background bacteria (results not shown). Violet red agar is used to detect the levels of coliforms in food or dairy products. Blood aesculin agar selects for the growth of *Staphylococci*, *Streptococci* as well as *S.uberis* by means of the aesculin cleaving. The growth of coliform bacteria, pseudomonads and yeasts is also possible on non-selective agar. For the purpose of this study these agars were used to monitor any significant changes in the milk other than the *Streptococcus dysgalactiae*.

5.3.4. Bacterial strains

A strain of *Str. dysgalactiae* spp. *dysgalactiae* previously isolated and identified from an animal in the Dairy Production centre in Teagasc Food Research Centre, Moorepark, was used for the intra-mammary challenge.

Escherichia coli O111 (*E. coli* NCTC 8007, serotype O111 K58(B4)) a pathogenic Complement sensitive strain was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections Porton Down Salisbury Wiltshire, SP4 0JG UK). This strain was routinely grown in Luria-Bertani (LB) medium at 37°C with shaking. Standard LB broth and agar was prepared as described by Sambrook et al., (2001).

5.3.5. Intra-mammary challenge

S. dysgalactiae spp. *dysgalactiae* DPC 5435 described was grown at 37°C in tryptic soy broth (Difco Laboratories, Detroit, USA). Following this the overnight cultures were serially diluted and plated to determine CFU count. 200 µl of this culture was diluted with 1.8 ml of maximum recovery diluent (MRD, Oxoid) and this 2 ml suspension (containing 2500 CFU *S. dysgalactiae* spp. *dysgalactiae*) was used for the challenge. Infusions as well as milk sampling were performed under licence from the Irish Department of Agriculture and Food, and the cows' health was subsequently monitored by trained farm staff and veterinary personnel. For animal welfare purposes, where clinical signs of infection were observed, intra-mammary challenged cows received antibiotic treatment.

5.3.6. Bactericidal sequestration assay

E. coli O111 was prepared for overnight growth at 37°C on LB agar. An isolated colony from replicate plates was inoculated into 3 tubes of LB broth (Merck KGaA, Darmstadt, Germany) and grown over night at 37°C. The overnight cultures were centrifuged at 5000 rpm, a pellet was formed, the supernatant was removed and the pellet was re-suspended in phosphate buffer saline solution (PBS solution) this step was repeated twice. On the final step, the pellet was suspended in LB broth and

adjusted to 3 x 10^8 colony forming units (CFU) per ml using the McFarland Method (Goldman et al., 1986). Using 96-well plates 3 x 20 µl of each of the three replicate cultures was added to each round bottomed well (SARSTEDT Ltd. Wexford, Ireland) and 80 µl of the sample to be tested was then added. The plate was incubated at 37°C for 2h shaking at 200 rpm (Model Mini 4450 SHKA4450-1CE, Fischer Scientific, Ballycoolin, Dublin). A 20 µl sample was taken from each well after the incubation time and total viable counts enumerated on LB agar using the pour plate method.

5.3.7. C5a ELISA assay

A C5a ELISA kit (BlueGene, Shanghai, China) assay was purchased and used in parallel to the Bactericidal sequestration assay. The fresh milk samples were run in the ELISA assay on the day of collection. This assay was used to detect the levels of convergent C5a Complement protein in the milks.

5.3.8. Reproducibility and statistical analysis

Throughout the study all work was carried out in triplicate using three separate cultures (i.e., three biological repeats) and repeated on three other separate days. Examination of the results received was done using the SPSS program (SPSS Inc. Released 2009.

PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.)

5.4.Results

5.4.1. Complement activity in milks with high and low somatic cell counts

Preliminary screening was carried out to investigate the extent of the association between SCC and Complement activity. Three cows with elevated SCCs (> 200,000 cells per ml) in individual quarters were selected from the MDPC dairy herd. Milk was aseptically collected from the individual high and low SCC quarters of each cow and tested by the bacterial sequestration assay. Milks from those quarters of individual animals with high SCC inhibited most ($p < 0.05$) of the growth of the Complementsensitive strain, *E. coli* O111, compared to the control (heat-inactivated milk). However, when the high SCC milks from affected quarters of Cows 1, 2, and 3 were compared to milks from their healthy quarter (low SCC < 100,000), the difference was not significant ($p>0.05$), based on respective *E. coli* O111 growth values of 8.08, 7.60 and 8.12 log CFU/ml for high SCC and 8.21, 7.97 and 8.34 log CFU/ml (low SCC milks). In any case, it needs to be remembered that low SCC raw bovine milk also contain active Complement with bacteriostatic capabilities as it has not been heat treated (Maye et al., 2015).

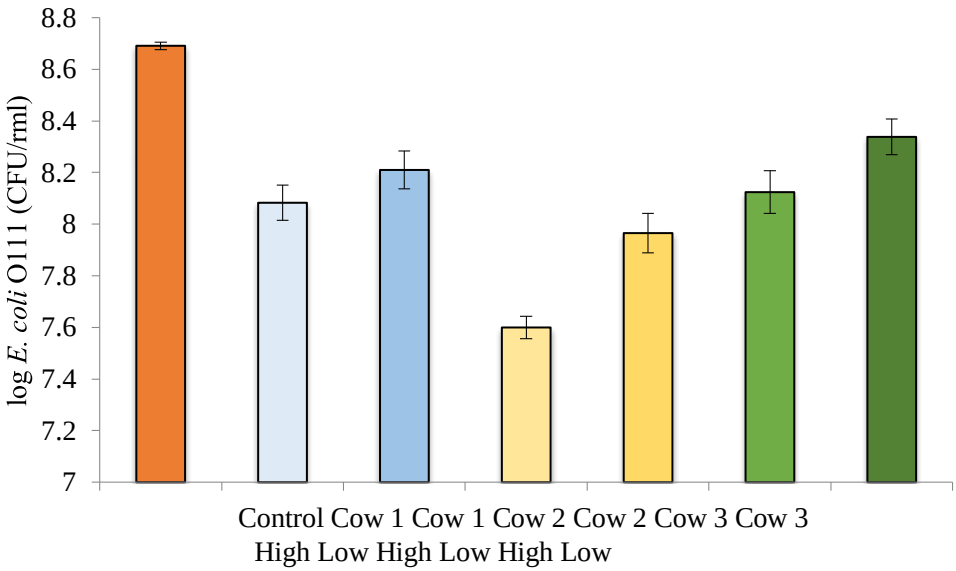


Figure 5.1 Results of the bacterial sequestration assay of the milk samples from individual cows in the MDPC herd. Each cow had a quarter producing both high and low SCC milks. The samples include; (i) heat treated raw bovine milk (control, 56^{o}C 30 min), (ii) Cow 1 qtr with high SCC, (iii) Cow 1 qtr with low SCC, (iv) Cow 2 qtr with high SCC, (v) Cow 2 qtr with low SCC, (vi) Cow 3 qtr with high SCC, (vii) Cow 3 qtr with low SCC.

Overall, the results of this pre-screening experiment indicated that innate immune components were evident in the high SCC milks according to the higher Complementbased bactericidal activity. As this was a preliminary study, it was not possible to identify the stage of inflammation in the case of high SCC milks, e.g., the cow may be at either the initiation stage or in the phase of recovery following infection. It was also evident that the innate immune response to infection in one quarter was not manifested in the adjoining healthier quarters, and so it may be

concluded that Complement activation would appear to be localised at the site of infection.

Additionally, it can be seen that high and normal SCC milks had significantly reduced *E. coli* O111 growth rates in comparison to the control ($p < 0.05$), i.e., a milk in which Complement has been heat-inactivated (56^{o}C for 30 min) as described by Korhonen et al., 2000a.

5.4.2. Intra-mammary challenge of individual quarters with *Streptococcus dysgalactiae*

In a follow-up study, the bovine immune system response was deliberately challenged by inoculating an infectious strain *Str. dysgalactiae* into one (healthy) quarter of 4 selected cows from the MDPC herd. Subsequently, milk was aseptically collected periodically at designated milking intervals (7h, 12h, 24h, 48h, 72h and 7d, 14d and 21d PI) from both an unchallenged control quarter and an infused quarter. During follow-up sampling and screening, viable *Str. dysgalactiae* were recovered at 7h and 24h from the infused quarters of all four treated cows. Importantly, there were no other pathogenic strains isolated from the milk drawn from the affected quarters throughout the trial. Equally, all of the uninfected control quarters remained free of pathogenic microorganisms throughout this period also.

5.4.3. Clinical response and milk characteristics

Following infusion, all 4 cows presented with signs of udder inflammation in the test quarters 24h PI. The cows were monitored individually for the anticipated signs of mastitis, e.g., inflammation and swelling of challenged quarters, increased levels of SCC and expressed milk with abnormal signs such a watery appearance, flakes, clots or pus. It was observed that the SCC of the tested quarters for all 4 animals peaked at times between 72 and 96h PI. At this time, clots were visible in the milk collected from the infused quarters, and the SCCs for the challenged quarter increased to 6.73, 6.76, 6.84 and 6.71 log SCC per ml for cows numbered 1 to 4, respectively (Fig 5.2). Hence, it was concluded that the *Str. dysgalactiae* infection was now established, so that the immune response in the inoculated quarters could be monitored. The control (uninfected, healthy) quarters did not show any significant change in SCC ($p < 0.05$). Close monitoring of udder conditions and milking from the four cows continued during the period following establishment of peak infection. The infection was selflimiting in the case of three of affected animals, and their SCCs returned to normal levels within 14d PI, while the fourth cow required brief antibiotic intervention. SCC surveillance continued for up to 30d PI in order to ensure that all cows returned to and maintained a healthy lactating status.

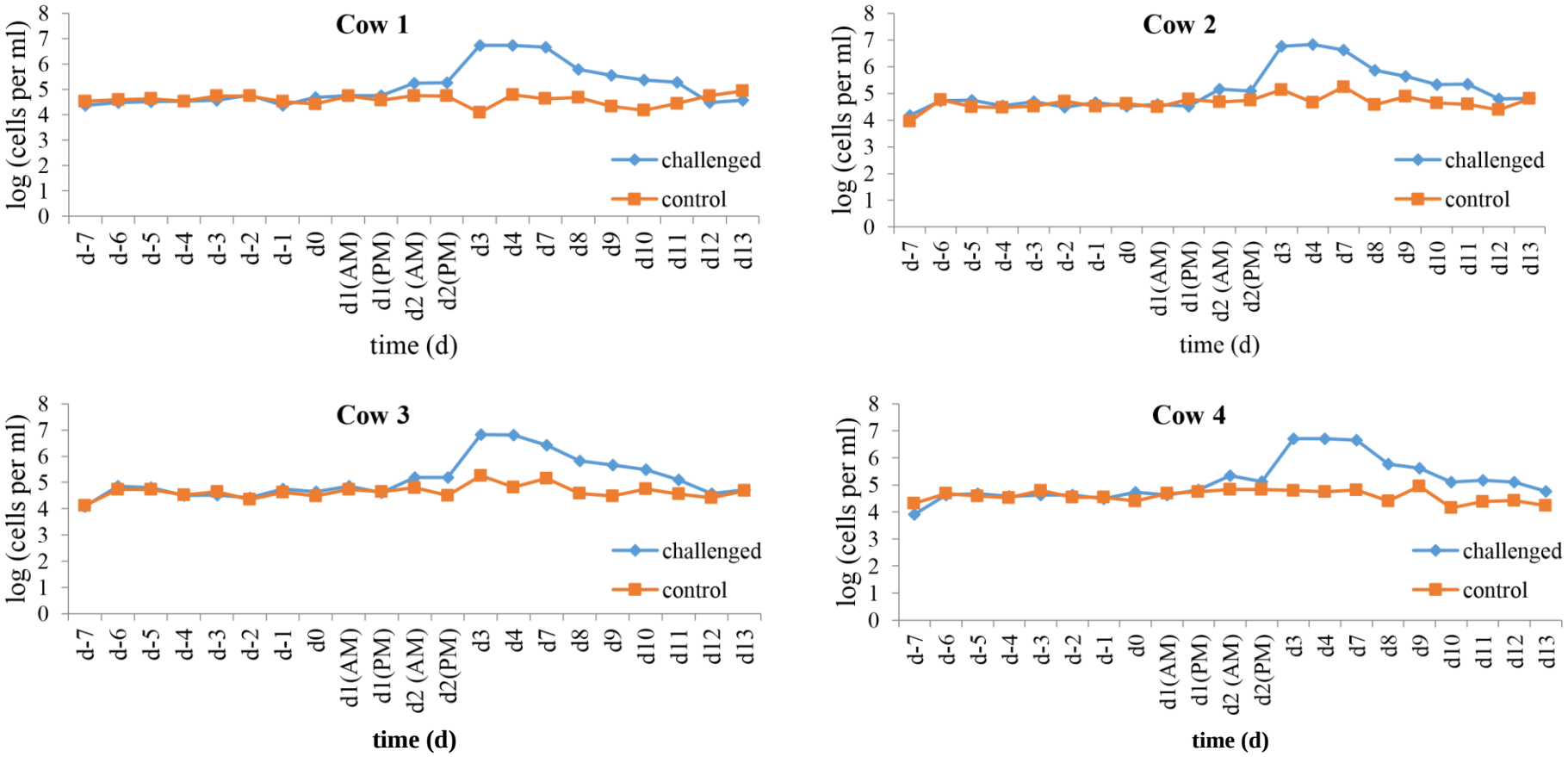


Figure 5.2 The individual SCCs (log cells per ml) for both the challenged quarter and the control. The SCC was measured from 7 d prior to intra-mammary challenge until d 18 PI

5.4.4. Viable bacteria enumerated from collected milk pre and post -inoculation

Milks were first screened using ISO reference methods (ISO, 2004) for bacterial growth in order to identify the rate of growth and survival of *Str. dysgalactiae* in the challenged quarters, as well as confirming the bacterial status of each cow's mammary gland. As expected the number of viable bacteria in the challenged quarter increased from the natural values in the days PI (Fig 5.3) while, at the same time, there was no significant effect on the number of viable bacteria in the control (uninfected) quarters throughout the study *(*$p < 0.05$). The bacteria shed in the milks from the infused quarters had the appearance and growth characteristics of *Str. dysgalactiae.* The bacterial counts peaked between days 2 and 4 PI, cow 1 peaked on day 3 at 8.72 log CFU per ml, cows 2 and 3 peaked on day 2 at 8.797 and 9.28 respectively, cow 4 reached its peak on day 3 with 8.7 log CFU per ml. For animal welfare reasons, the cows were monitored during the following 30 d PI (results not shown) to ensure that all animals were restored to regular udder health and normalised in terms of low bacterial levels in milk

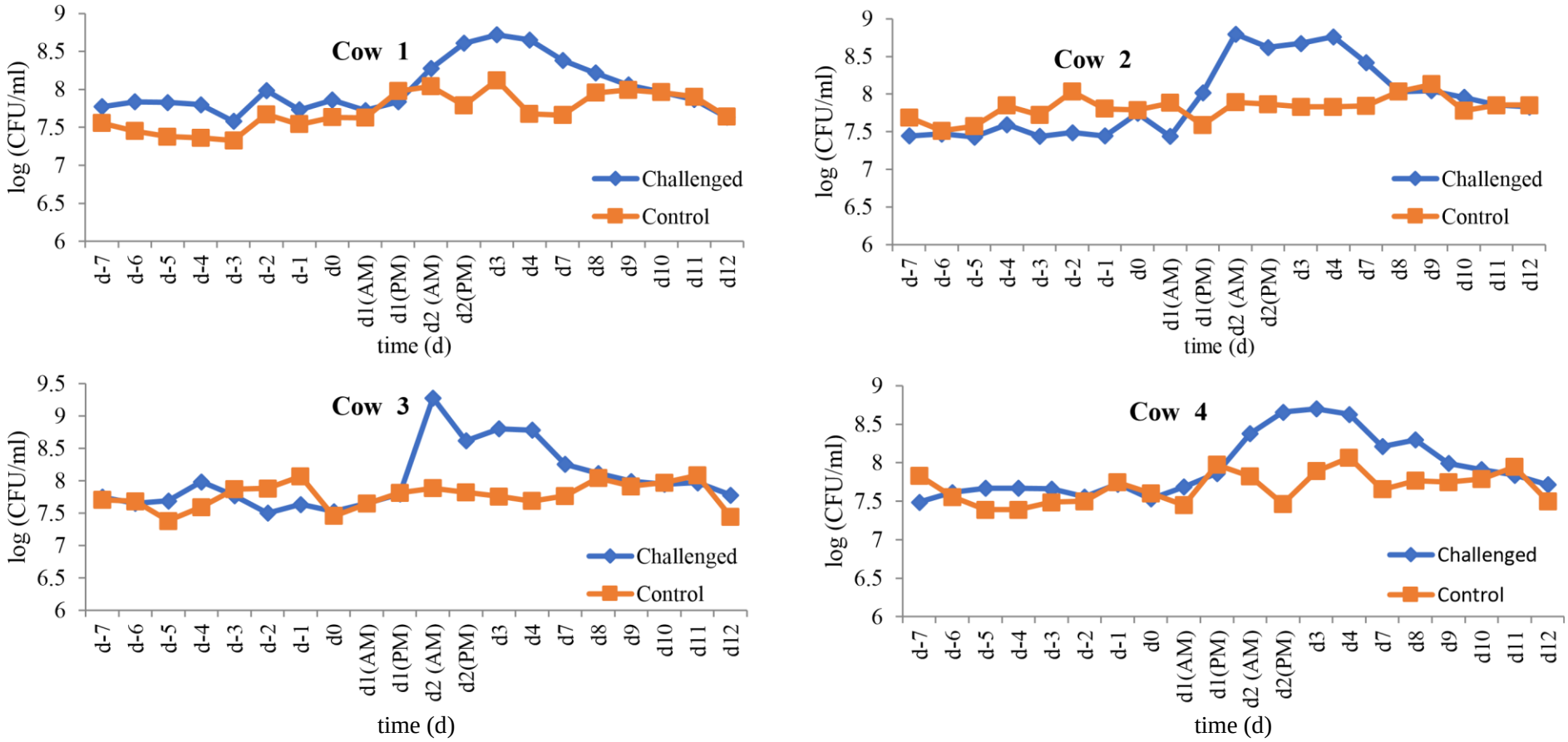


Figure 5.3 The individual colony forming units (log CFU/ml) of the *Str dysgalactae* for both the challenged quarter and the control. The CFU was measured from 7

d preceding to intra-mammary challenge until d 18 PI.

5.4.5. Bacterial sequestration assay

From the moment that infection had taken hold, as reflected in the higher milk SCCs, the bactericidal sequestration assay was undertaken on the milks from both the inoculated and control quarters. It was found that the milks from the challenged quarters of all cows were more effective at restricting *E. coli* O111 growth PI according to the reduced log CFU per ml for cows numbered 1 to 4 by 6.81, 6.69, 6.8, and 7.93 respectively. This effect was most notable between D1 and D7 PI. The corresponding control quarters in all four cows were significantly less effective at reducing growth of the assay strain *(*$p < 0.05$). These healthy (control) quarters of all animals remained stable in terms of Complement efficacy throughout the trial (Fig 5.4). Overall, it can be concluded that the increased effectiveness *(*$p < 0.05$) of the challenged quarters at restricting growth of the assay bacterium (*E. coli* O111) reflects an increased Complement response at a localised level, i.e., within the individual infected quarter, but not that of the mammary gland (udder) as a whole.

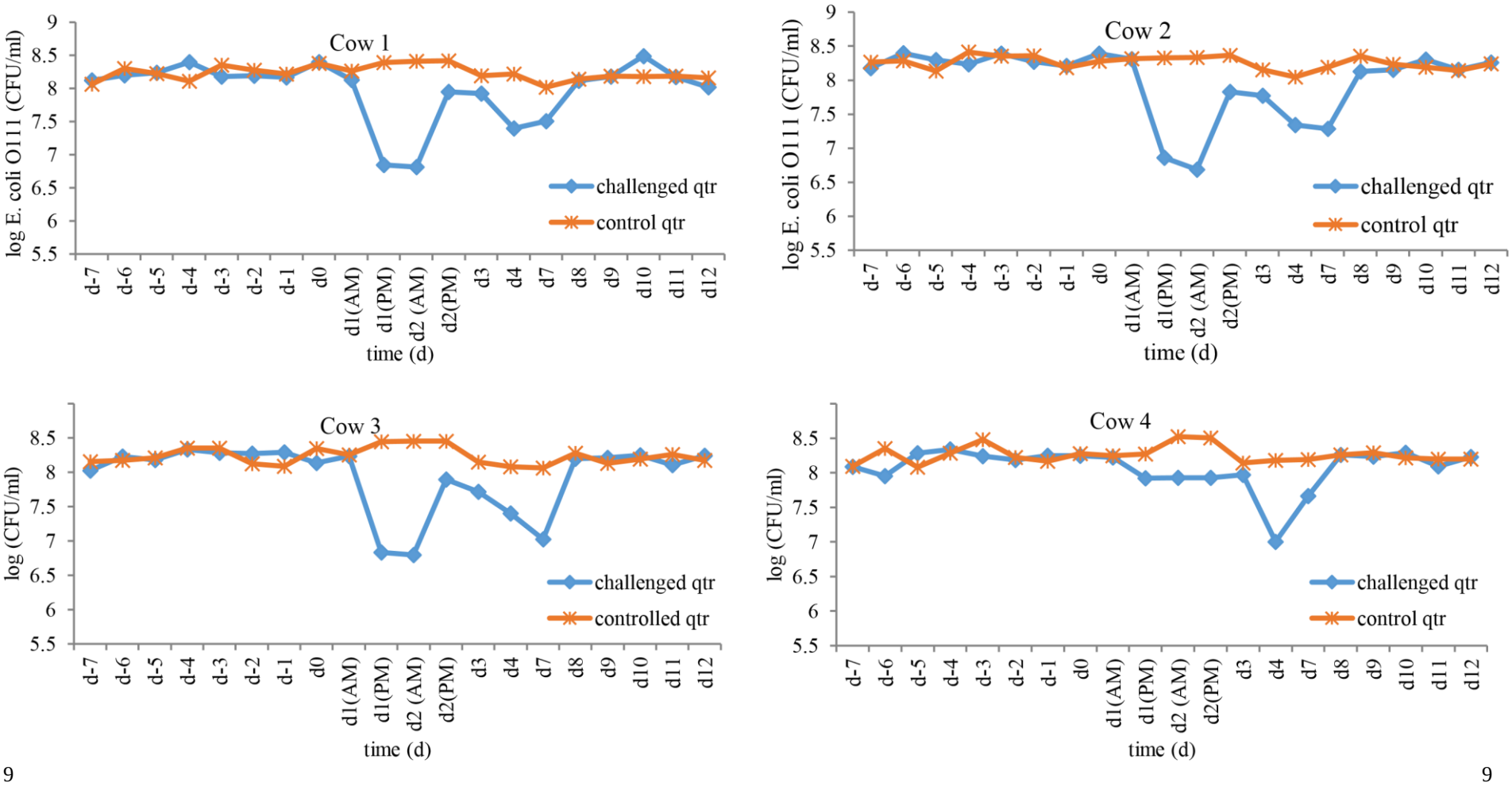


Figure 5.4 Complement activity in milks was assessed using the bactericidal sequestration assay from both challenged and control quarters. Measurement as

reflected by the total viable counts from (log CFU/ml) during performance of the bactericidal sequestration assay

5.4.6. C5a ELISA assay

Complement component C5a is an important inflammatory marker that is activated late in the cascading pathway during activation of the innate immune response. An Enzyme Linked Immunosorbent Assay (ELISA) was performed to quantify C5a in order to establish if elevated levels of this component coincided with greater inactivation of the bactericidal sequestration assay target microorganism (*E. coli* O111), and provide further evidence of increased Complement activity. Related studies indicate that C5 may be produced in the epithelial cells of the mammary gland; hence, it is an effective marker to allow the complement activity to be monitored at a key juncture of the Complement pathway (Stevens et al., 2012). The C5a ELISA assay indicated that there was significantly greater ($p < 0.05$) levels of C5a in the infected quarters compared to the control quarters (Fig 5.5), thus confirming that the Complement system is active in the individual infected udder quarters.

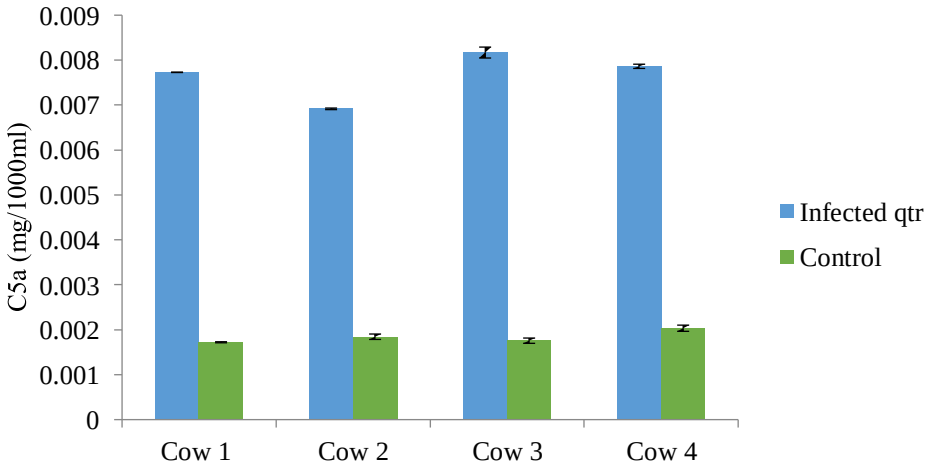


Figure 5.5 Levels of complement component C5a as determined by an ELISA assay completed on the milks from both the control and challenged quarters of the individual cows

5.5. Discussion

Inflammation of the mammary gland is mostly triggered when pathogenic microorganisms enter *via* the teat canal, following which both the innate and specific immune systems work in combination to defend the affected area (Tasnim, 2014). In this study, the mammary immune response of four dairy cows was challenged by inducing a controlled case of mastitis, using a known strain of *Str. dysgalactiae*. Mastitis is a persistent inflammatory condition which is categorised as either clinical or the sub-clinical in dairy cows. Subclinical mastitis is associated with increased SCC, can give rise to a reduced milk yield (ISO, 2006) and impact negatively on milk quality criteria. It is reported that mastitis cases cost the US dairy industry approximately $2 billion yearly due to the reduced milk production (Sordillo, 2009). Milk SCC is commonly linked to milk payment regimes for off-farm milk and ideally should not exceed 200,000 cells per ml, as otherwise financial penalties are frequently imposed. On the other hand, milk from a healthy quarter has been described as containing reduced bacterial growth and not exceeding 100,000 cells/ml (Pyörälä, 2003). The type of pathogen may not be known they can still induce less elevation in the SCC. High SCC affects the quality of the milk by contributing to increased rates of lipolysis and casein hydrolysis (Riollet et al., 2002). The somatic cells also known as leucocytes represent the specific immune response to infection as the mammary gland tries to contain the invading pathogen. Adaptive immunity involves highly specific constituents (e.g. T-cells, B-cells, leucocytes, phagocytes, antibodies and immunoglobulins) which detect specific molecular structures on the surface of the invading pathogens (Hannson et al., 2002). The significant increase in Complement activity in mammary quarters with high SCC as shown in this study confirms that the innate immune system works in parallel with the localised specific immune response in the mammary gland *(*$p < 0.05$).

Some cow to cow variation encountered during the study may be attributed to genetic variation which is known to be associated with differences in mastitic response (Rupp and Boichard, 2003). A genetic evaluation system was implemented in France in 2010, which assessed the occurrence of clinical mastitis in three dairy breeds – Montbéliarde, Normande and Holstein (HO) (Govignon-Gion et al., 2015). Low heritability of clinical mastitis has been documented, however, some correlations with other traits may hold some potential answers. The results indicate high correlations (often>0.50, in absolute value) between clinical mastitis and somatic cell score (SCS), longevity and some udder traits.

In challenge tests, the mastitis-inducing strain, *Str. dysgalactiae*, inoculated into the mammary gland of the four cows studied was based on a strain isolated previously from an incidence of mastitis in the MDPC herd. This Gram-positive strain is credited with a significant number of both clinical and subclinical infections (Hogan et al., 1989, Todhunter et al., 1990). The strain is considered to have characteristics of both a contagious and an environmental pathogen. Previously, it was isolated from mammary glands exhibiting the signs of infection, and may be transferred during milking (Marth and Steele, 2001).

As expected, SCC increased from base levels in the inoculated quarter in the hours and days following inoculation, thus confirming that the infecting *Str. dysgalactiae* pathogenic strain had triggered tissue inflammation in the affected mammary quarter. It has been previously reported that the duration of inflammation may be longer than the length of infection, and as Complement plays a major role in inflammation this would support the improved Complement response we have seen during the first 4d

PI (Pyörälä and Mattila, 1987). Moreover, bactericidal sequestration assay counts in the non-infected (control) quarter were largely unaffected throughout the trial period, thus confirming that the innate immune response was mainly localised.

No other pathogenic bacteria were tested from the milk samples, which confirmed that the increase in SCC was as a direct response to *Str. dysgalactiae* inoculation. Reference milk culture methods in combination with selective media supported the screening process to confirm the presence of *Str. dysgalactiae* (Hillerton, 1999).

The ELISA test based on its specific determination of Component C5a, a chemotactic protein known for its recruitment of inflammatory cells, in the freshly collected milk samples provided added confirmation that Complement was the key innate immune factor at work in combating the induced-mammary infection. Thus, the extent of association between activated Complement response and raised SCC as an inflammatory indicator of individual mammary quarters may be worthy of further research. Additional data would need to be generated to establish whether the differences between the inactive (healthy quarter) and activated (high SCC quarter) Complement levels may be correlated with SCC and / or provide an indirect measure of the animal's capacity to combat mammary infection.

It has been identified that the incidence of mastitis in cows is directly linked to variation in mammary gland physiology and the efficiency of the mammary gland defence system (Sordillo and Streicher, 2002). It is, thus, reassuring to know that milk drawn from healthy quarters during periods of mastitic infection in adjoining quarters is not exposed to transmission of elevated levels of immune components (assuming of course, that milk from the affected quarter is isolated from entering the

food chain, usually in conjunction with therapeutic intervention). Overall, the study provides key insights into the innate immune response in the mammary gland when triggered by *S. dysgalactiae* spp. *Dysgalactiae* inoculation. Hyperimmunisation of lactating dairy cows with specific antigens is frequently employed to induce increased antibody activity in the milks collected from these animals. One example is a commercial bovine colostral whey preparation containing enriched levels of immunoglobulins intended to enhance the immune and bacteriolytic responses of calves (Hammarström and Weiner, 2008). Oral administration of milks collected from hyper-immunised cows improved the incidence of acute colitis and maintained intestinal homeostasis in mice models (Wang et al., 2014). Unfortunately, there is little published information on how Complement activity in milk is affected following hyper-immunisation of selected dairy cows. The current induced-mastitis study proved that the Complement response was of a local nature i.e., confined to the affected milking quarter. However, it is reasonable to expect that a systemically-triggered response following hyperimmunisation is most likely to be manifested also by increased Complement activity in addition to the release of specific antibodies. Further research which monitors for changes in Complement as a result of hyper-immunisation of dairy cows is recommended. In exploring the potential for generating therapeutic preparations based on generated antibodies, there is considerable interest also in the release of cationic host defense peptides (HDP) which are known to be transcriptionally regulated and dependent on stimulus and cell type. Furthermore, HDP are regulated and/or coordinated in conjunction with the expression of other entities of innate immunity and acute inflammation.

5.6. References

BUTLER, J.E., 1979. Immunologic aspects of breast feeding, anti-infectious activity of breast milk. In *Seminars in Perinatology 3*(3), pp. 255-270.

GOLDMAN, A., THORPE, L., GOLDBLUM, R. AND HANSON, L. 1986. Anti-inflammatory Properties of Human Milk. *Acta Paediatrica, 75*(5)**,** pp. 689-695.

GOVIGNON-GION, A., DASSONNEVILLE, R., BALOCHE, G. AND DUCROCQ, V., 2016. Multiple trait genetic evaluation of clinical mastitis in three dairy cattle breeds. *Animal, 10*(04), pp. 558-565.

HANSSON, G.K., LIBBY, P., SCHÖNBECK, U. AND YAN, Z.Q., 2002. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circulation Research*, *91*(4), pp. 281-291.

HILLERTON, J. 1999. Redefining mastitis based on somatic cell count. *Bulletin-FIL-IDF (Belgium)*.

HOGAN, J.S., TODHUNTER, D.A., SMITH, K.L. AND SCHOENBERGER, P.S., 1989. Serum susceptibility of coliforms isolated from bovine intramammary infections. *Journal of Dairy Science*, *72*(7), pp. 1893-1899.

ISO. 2004. Milk—Quantitative determination of bacteriological quality—guidance for establishing and verifying a conversion relationship between routine method results and anchor method results, EN ISO 21187. International Organization for

Standardization, Geneva.

ISO. 2006. Milk—enumeration of somatic cells—part 2: guidance on the operation of fluoro-opto-electronic counters. EN ISO 13366-2 1–13. nternational Organization for Standardization, Geneva.

ISO. 2013. Milk - Bacterial count - Protocol for the evaluation of alternative methods (Reference method), EN ISO 16297–1. International Organization for Standardization, Geneva.

JANEWAY, C. A, TRAVERS, P., WALPORT M, AND SCHLOMCHIK, M. 2001 Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. *The Complement System and Innate Immunity*.

KORHONEN, H., MARNILA, P. AND GILL, H. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition, 84*, pp. 75-80.

MARTH, E. H. AND STEELE, J. 2001. *Applied dairy microbiology*, CRC Press.

MAYE, S., STANTON, C., FITZGERALD, G. F. AND KELLY, P. M. 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research, 82*(3), pp. 328-333.

NEWBURG, D. S. W., ALLAN.W. 2007. Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatric Research, 61*(1), pp. 2-8.

OGUNDELE, M.O., 2002. Effects of storage on the physicochemical and antibacterial properties of human milk. *British Journal of Biomedical Science*, *59*(4), pp. 205.

OGUNDELE, M.O., 2001. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. *Immunology and Cell Biology*, *79*(1), pp. 1-10.

PYÖRÄLÄ, S., 2003. Indicators of inflammation in the diagnosis of mastitis.*Veterinary Research*, *34*(5), pp. 565-578.

PYÖRÄLÄ, S. AND MATTILA, T., 1987. Inflammatory changes during experimental bovine mastitis induced by *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. *Journal of Veterinary Medicine Series A*, *34*(1-10), pp. 574-581.

RIOLLET, C., RAINARD, P. AND POUTREL, B., 2002. Cells and cytokines in inflammatory secretions of bovine mammary gland. In *Biology of the Mammary Gland.* pp. 247-258. Springer US.

RUPP, R. AND BOICHARD, D. 2003. Genetics of resistance to mastitis in dairy cattle. *Veterinary Research, 34*(5), pp. 671-688.

SAMBROOK, J., RUSSELL, D. W. AND RUSSELL, D. W. 2001. *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press

Cold Spring Harbor, New York.

SORDILLO, L.M. 2009 Current concepts on immunity and mastitis. WCDS *Adv. Dairy Technolog*y, *21*, pp. 111-119.

SORDILLO, L. M. AND STREICHER, K. L. 2002. Mammary gland immunity and mastitis susceptibility. *Journal of Mammary Gland Biology and Neoplasia, 7*(2), pp. 135-146.

STEVENS, M. G., DE SPIEGELEER, B., PEELMAN, L., BOULOUGOURIS, X. J., CAPUCO, A. V. AND BURVENICH, C. 2012. Compromised neutrophil function and bovine *E. coli* mastitis: Is C5a the missing link? *Veterinary Immunology and Immunopathology*, *149*(3), pp. 151-156.

TASNIM, S. 2014. Effect of breast feeding on child development: At birth and beyond. *South East Asia Journal Of Public Health, 4*(1), pp. 4-8.

TODHUNTER, D., SMITH, K. L. AND HOGAN, J. S. 1990. Intramammary Challenge of the Bovine Mammary Gland with Coliform Bacteria During Early

Involution. *Journal of Dairy Science, 73*(5), pp. 1217-1224.

WANG, Y., LIN, L., YIN, C., OTHTANI, S., AOYAMA, K., LU, C., SUN, X. AND YOSHIKAI, Y., 2014. Oral Administration of Bovine Milk from Cows Hyperimmunized with Intestinal Bacterin Stimulates Lamina Propria T Lymphocytes to Produce Th1-Biased Cytokines in Mice. *International Journal of Molecular Sciences, 15*(4), pp. 5458-5471.

Chapter VI Determination of bovine breed variation on Complement activity in freshly withdrawn milk

6.1.Abstract

The overall purpose of this study was to examine the Complement activity, a key marker of the innate immune system, present in milk of different breeds. In previous Chapters, the order to magnitude of antimicrobial response to the bacterial sequestration assay used to monitor Complement has been shown to be almost comparable in biological efficacy for both bovine and human milks (Maye et al., 2015). Specific studies conducted with bovine milk have identified that Complement activity is diminished following heat treatment or cream partitioning thus it was important to consider potential ways to increase this activity in the milk produced. It has been seen that there is some cow to cow variation which prompted the question as to whether cow breed may play a role to in the increased availability of this major humoral component (Kelsey *et al.*, 2003; Dillon and Veerkamp, 2001). In order to test this hypothesis, the complement levels between 7 cow breeds, to include pure- and crossbreeds, Norwegian Red, Jersey, Holstein, Kerry, Friesian, Norwegian Red crossbreed and Holstein Friesian crossbreed was assessed. A bactericidal sequestration assay was utilised for the detection of Complement in milk, whereby, the extent of growth inactivation of *E. coli* O111 was measured and the response was used as an indicator of presence and activity of Complement. The results show that the Norwegian Red purebred limited growth of the Complement sensitive strain to 6.249 log CFU/ml, which was closely followed by the Kerry purebred (6.922 log CFU/ml *E. coli* O111). Further to this a previously reported transformed *E. coli* O111 strain was transformed by correctly integrating the p16*S*lux plasmid which meant the strain could be monitored throughout growth using bioluminescent measurements. In addition, the levels of key complement pathway component, C5a, were quantified using an Enzyme Linked Immunosorbent Assay (ELISA). The results of this assay

identified that the Kerry cows had highest levels of active C5a with 0.00786 mg/1000ml, the Norwegian red purebred cows had levels of 0.00736 mg/1000ml closely followed by the crossbreed Norwegian red crossbreed with levels of 0.00647 mg/1000ml.

6.2 Introduction

Mastitis is one of the most prevalent diseases of cattle. Various studies have reported breed-dependent differences in the risk of developing this disease. Among two major breeds, Jersey cows have been identified as having a lower prevalence of mastitis than Holstein cows. It is well established that the nature of the initial innate immune response to infection influences the ability of the host to clear harmful bacterial pathogens. Whether differences in the innate immune response to intra-mammary infections explain, in part, the differential prevalence of mastitis in Holstein and Jersey cows remains unknown. When estimating heritability and selection, it was found that cases of clinical mastitis in first lactation Norwegian Cattle were affected by selection for other breeding traits (Heringstad et al., 1999). Due to the multifactorial nature of mastitis, management may include a series of activities, i.e., dry cow therapy, prevention of transmission of infection as well as the improvement of the immune system (Halasa et al., 2007).

Irish dairy farmers with the support of the research arm of the country's Agriculture and Food Development Authority (Teagasc) have combined cattle breeding programmes with improved husbandry practices in order to achieve better milk

production performance and increased resistance to disease in their dairy herds, particularly with respect to udder health and the incidence of somatic cell counts in milk (Beecher et al., 2010). Up to the mid-1980's, the predominant dairy cow breed in Ireland was British Friesian and its crossbreeds (Cattle breeds, 2014; Dillon and Veerkamp, 2001). Holstein Friesian was gradually introduced later in individual farms due to their high performance dairy production traits. A certain proportion of Jersey cows are frequently included in dairy herds because of their exceptionally high milk compositional averages of approx. 6% butterfat and 4% protein, along with milk yields of up to 8500 kg. The introduction of other European cattle breeds led to the identification of Norwegian Red for its overall milk production and udder health performances (Begley et al., 2009a; Begley et al., 2009b) under Irish dairy husbandry practices. Dairy cattle with enhanced and balanced immune responses are known to have a lower occurrence of disease including mastitis (Thompson-Crispi et al., 2014). Improved immune response to external and internal stimuli including pathogenic micro-organisms was strongly associated with improved response to vaccination and colostrum quality (Mallard et al., 2011). A particular focus was on exploiting the animal's own immune response genes. The genetic selection for mastitis is considered to be a potential strategy for improvement of overall udder health. Nordic countries have included mastitis in their genetic evaluation since 1978 with proven improvement in udder health (Govignon-Gion et al., 2015). Other genetic selection approaches currently used in the dairy industry include high immune response (HIR) technology, genomics to improve disease resistance or immune response, as well as the

ImmunityC™ sire have been deployed to enhance animal welfare and food quality while maintaining favourable production levels in order to feed a growing population (Thompson-Crispi et al., 2014). While these methods have addressed improving milk quality, composition and in conjunction with mastitis control programs, there is a concern that cattle may become more prone to infection due to the widening range of mastitis pathogens being encountered (Bradley, 2002). Sorg et al (2013) compared the innate immune response in vitro of two ancient and two modern dairy cattle breeds using RT-qPCR and ELISA techniques in an attempt to establish whether ancient cattle breeds not selected for high milk yield had a stronger phenotypical resistance to mastitis than modern high yielding breeds. They found a higher basal expression in the ancient breeds studied and that only parts of the toll-like receptor pathway (TLR) pathway had higher expression levels than modern breeds. Rainard and Riollet (2006) concluded that many questions need to be answered before the variability of the genes governing innate immunity can be harnessed to curb mastitis in dairy ruminants.

With previous work focussed mainly on characterising Complement markers generated by the innate immune system, it was opportune to compare milk samples from a cross section of Irish dairy cows selected according to breeding status (purebreds and crossbreds) for the presence of Complement activity and potential indication of the innate immune status of each breed. The milk sampling programme was undertaken with the following purebred dairy cows: Norwegian Red, Holstein, British Friesian, Jersey and Kerry, as well as crossbred Norwegian Red and Holstein-Friesian.

6.3 Materials and Methods

6.3.1. Milk collection and analysis

Freshly-drawn milk from individual cows selected from the Teagasc bovine genetics database were bulked according to breed following collection at a number of sites: Moorepark Dairy Production Centre (MDPC), Teagasc research farms and Teagasc associated dairy farmers in the south of Ireland during mid-lactation - May to July 2014, during which the animals were primarily grass-fed. The SCC of the freshly collected milk was determined using a Bentley Somacount 300® (Bentley Instruments Inc., Chaska MN, USA), and an SCC of ≤100,000 cells per ml was the considered the threshold value for healthy milk samples (ISO 2006; ISO, 2008). All samples were analysed for TBC using an ISO reference method (ISO, 2004; ISO, 2013).

6.3.2. Animal selection

Based on statistical power calculations, a minimum of 17 dairy cows from each of 7 breeds were required for milk sampling using the bovine genetics databases developed by Teagasc and the Irish Cattle Breeding Federation (ICBF). To ensure we would have the minimum amount at the end of the study 18 cows were included from each breed. The selection criteria for the breed of interest were set at >90% and ~50% for purebred and crossbred cows, respectively. A review of the recent health history of selected lactating cows took into consideration time of calving and any

intervention with antibiotic therapy. This was followed by monitoring of SCC for 3 weeks before initialisation of milk sampling. On 3 consecutive days, at morning milking time (approx. 08.00 h), milk samples were aseptically collected from 4 pure- and 2 crossbreeds: Norwegian Reds, Holsteins, Jerseys, Friesians, Kerry, Norwegian Red crossbreed and Holstein-Friesian crossbreed.

6.3.3. Control preparation

Freshly collected bulk raw milk was heat-treated at 56 °C for 30 min to ensure complete inactivation of the complement protein (Korhonen et al., 2000). All samples were screened using ISO methods (ISO 2013, ISO 2004), and also the milks were streaked on Nutrient and Luria Bertani agar.

6.3.4. Bacterial strains, media, and chemicals

Escherichia coli O111 (*E. coli* NCTC 8007, serotype O111 K58 (B4)) a pathogenic Complement sensitive strain, was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections, Porton Down, Salisbury Wiltshire, SP4 0JG UK). The strain was routinely grown in Luria-Bertani (LB) medium 37 °C with shaking. When required, the antibiotic erythromycin (Sigma Chemical Company, St. Louis, MO) was added at a concentration of 500 mg/ml to the broth to select for the growth of transformed *E. coli* O111.

6.3.5. Bactericidal sequestration assay

Escherichia coli NCTC 8007, serotype O111 K58(B4) was the serum susceptible strain selected – also known to be frequently isolated from neonates affected by gastroenteritis (Reiter and Brock, 1975a). This strain was stored as a stock culture and grown on LB agar and sub-cultured in LB broth. It was grown overnight at 37 °C and adjusted to $3x10^8$ CFU per ml for the assay. To the round bottomed 96-well plate sourced from Sartsedt (SARSTEDT Ltd., Sinnottstown Lane, Drinagh – Wexford Ireland). 20µl of the *E. coli* O111 culture was added to each well. To this 80µl of milk sample to be tested was added under aseptic conditions. The plate was then put into a shaking incubator (Model Mini 4450 SHKA4450-1CE, Fischer Scientific, Ballycoolin, Dublin) at 37°C and 100 rpm for 2h. After this time the plate was transferred to a laminar flow hood and 20 µl sample was taken from each well. These samples were tested for viable counts using LB agar (Merck KGaA, Darmstadt, Germany) and the pour plate method.

For the transformed strain, it was conducted in the same way; however, the strain was initially grown in overnight in LB broth with Erythromycin. When the growth reached $3x10^8$ CFU per ml, the culture was centrifuged and a pellet formed. The pellet was washed twice and then finally solubilised in LB broth. The remaining steps of the assay proceeded as described for the WT *E. coli* O111.

6.3.6. Real time monitoring of the *E. coli* O111 p16Slux in Milk

E. coli O111 NCTC 8007 described above was transformed with plasmid p16Slux as described in Maye et al., (2016) at the Biosciences Research Institute, University College Cork Ireland. Once the p16*S*lux plasmid was successfully inserted as previously described (Riedel et al., 2007) the strain was incubated with the individual milk samples which had been aseptically collected from the 6 breeds of cows. These were run alongside a complement-inactivated control to confirm that the reduction in *E. coli* O111 p16*S*lux numbers were attributable solely to the activity of Complement present.

6.3.7. C5a ELISA assay

C5a ELISA kit (BlueGene, Shanghai, China) assay was used to detect the convergent C5a Complement protein in the milks. This kit was specifically prepared to detect for Bovine Complement C5a. Phosphate buffer saline (PBS) solution was used as a negative control.

6.3.8. IgM ELISA assay

An IgM ELISA kit designed specifically for bovine IgM (Bethyl laboratories, USA) was used to detect the presence of IgM in the different breed milks. Phosphate buffer saline (PBS) solution was used as a negative control.

6.3.9. Statistical Analysis

Statistical analysis was undertaken on data generated from analyses of triplicate milk samples from 18 cows per breed resulting from collection at 3 separate intervals over the time period referred to in Section 2.1.1.at other consecutive 3-day intervals. Data was analysed using an SPSS program (SPSS Inc., Released 2009. PASW Statistics for

Windows, Version 18.0. Chicago: SPSS Inc.).

6.4 Results

6.4.1 Comparison of Complement-associated bactericidal activity in different breeds of cows

The bactericidal sequestration assay used to determine Complement activity relies on the unique sensitivity of the *E. coli* O111 strain to the presence of Complement. Consequently, a reduction in colony forming units (CFU) is observed. Hence in the context of this study, a lower survival of *E. coli* O111 would indicate a breed with higher levels of active Complement. There was a significant difference ($p < 0.05$) in growth of the *E. coli* sequestration strain in the milks of the 7 cow breeds when compared to the heat-treated control (Fig 6.1), notably the milk produced by the purebred Norwegian Red (followed by the Kerry purebred) cows was effective at restricting the growth of the bacterial sequestration strain. The results (Table 6.1)

indicate that the control (heat-treated milk, 56°C for 30 min) with a value of 9.113 is significantly higher than the milks of the seven breeds, whereby, the Norwegian Red

(purebred) restricted *E. coli* O111 to an average growth of 6.249 log CFU/ml, next the

Kerry (purebred) had 6.922 log CFU/ml, which was closely followed by the Norwegian red (crossbreed) with a measurement of 7.072 log CFU/ml, the final four breeds; Holstein (purebred), Friesian (purebred), Jersey (purebred), Holstein Friesian (crossbreed) exhibited growth levels of 7.600, 7.364, 8.257, 7.162 log CFU/ml respectively - the lower value indicating a more effective Complement response. Enhanced cell reduction factors of 2.864 and 2.191 ($p < 0.05$) were measured following incubation of the *E. coli* O111 culture with the freshly collected milks from Norwegian Red and Kerry cows, respectively, both of which were significantly different ($p < 0.05$) from the control. It was also observed that the Norwegian Red cross-bred appears to conserve a significant amount of the bactericidal capability ($p < 0.05$) of its parent pure-breed. A one-way analysis of variance test confirmed that all samples were more effective at limiting the growth of the *E. coli* O111 when individually compared to the negative control. Due to this significant result for all 7 milks, it was necessary to conduct post hoc tests which allow further exploration of the differences among the means. The post-hoc statistical analysis comparing the individual data sets provided further confirmation that the Kerry and Norwegian red were significantly more effective at impeding viable *E. coli* O111 when compared to the other 5 breeds.

Cow	Control	Norwegian Red	Norwegian Red X	Holstein	Friesian	Jersey	Holstein Friesian	Kerry
1	9.000	6.200	7.009	7.652	7.484	7.812	7.145	6.944
2	9.125	6.204	7.071	7.845	7.252	7.822	7.098	7.008
3	9.321	6.279	7.141	7.781	7.236	7.922	7.252	6.948
4	8.988	6.259	6.749	7.660	7.419	8.038	7.202	6.803
5	9.259	6.208	6.900	7.629	7.432	8.136	7.106	7.073
6	9.621	6.537	6.949	7.460	7.313	8.146	7.337	6.988
7	8.255	6.129	6.953	7.966	7.213	8.215	7.166	7.089
8	8.951	6.350	6.940	7.760	7.466	8.237	7.137	7.126
9	9.235	6.399	7.021	7.173	7.581	8.309	7.132	7.029
10	9.148	6.350	7.177	7.714	7.373	8.364	7.298	7.062
11	8.956	6.266	7.106	7.721	7.391	8.367	6.980	7.002
12	9.258	6.204	7.370	6.905	7.234	8.375	7.194	7.168
13	8.785	6.278	7.274	7.491	7.331	8.379	7.234	7.069
14	8.899	6.184	7.128	7.705	7.484	8.385	6.979	6.874
15	9.259	6.207	7.225	7.553	7.252	8.408	7.040	6.740
16	9.855	6.330	7.076	7.516	7.236	8.491	7.257	7.075
17	8.890	6.059	7.065	7.681	7.419	8.581	7.203	5.726
18	9.230	6.043	7.151	7.599	7.432	8.646	7.162	6.868
Average	**9.113**	**6.249**	**7.072**	**7.600**	**7.364**	**8.257**	**7.162**	**6.922**
Standard Dev	**0.339**	**0.119**	**0.147**	**0.244**	**0.109**	**0.240**	**0.099**	**0.319**

Table 6.1 Results of the Bactericidal sequestration assay performed on the milks from each of the 18 cows from 7 breeds – Milk was hygienically collected on three consecutive morning milking and the assay was completed immediately after collection.

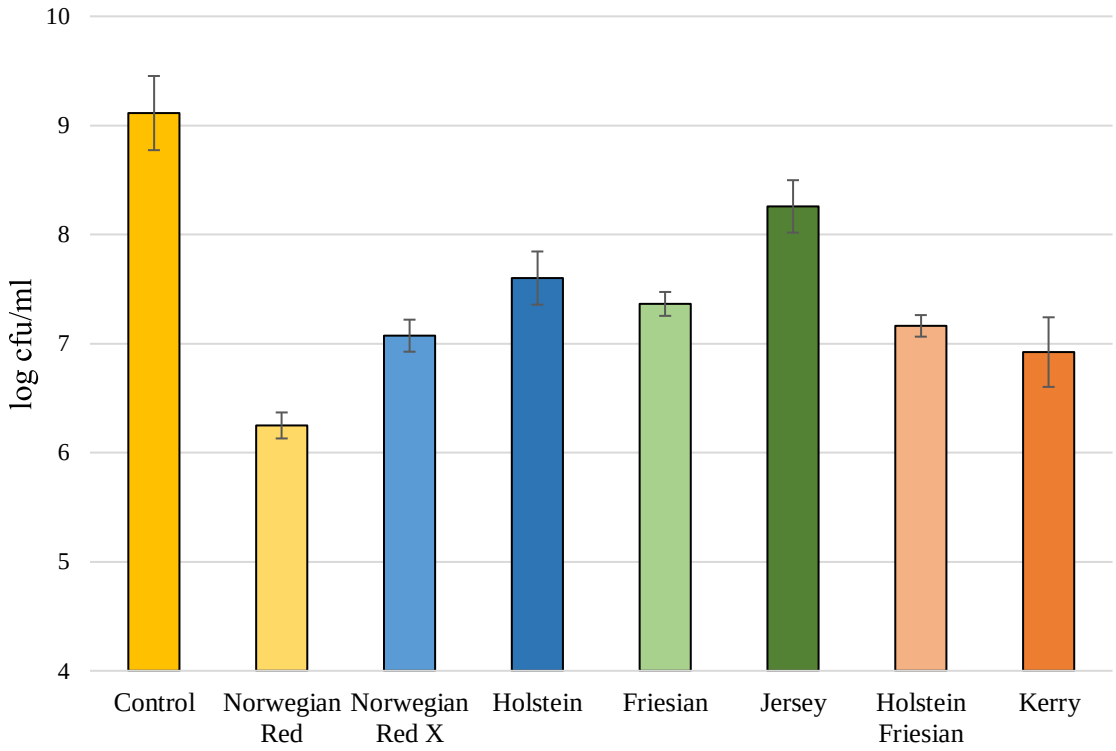


Figure 6.1 Total viable counts of *E. coli* O111 (log10 CFU/ml) following 2h incubation in the bacterial sequestration assay with samples, Control (heated bulk bovine milk 56°C, 30 min), after 2 h incubation at 37°C and 200 rpm.

6.4.2 Measurement of Complement pathway component C5a

An additional ELISA assay was carried out with the objective of quantifying levels of active C5a of the Complement cascade. Component C5a is known to be an inflammatory mediator which is intimately involved in neutrophil recruitment, hence it was selected in this study as a biological marker to confirm Complement system activity (Riollet *et al.*, 2000). This assay was carried out on freshly collected milk

samples on the day of collection. The ELISA analyses of all 7 breeds (Fig 6.2) confirm that the C5a component was dependent on breed of cow.

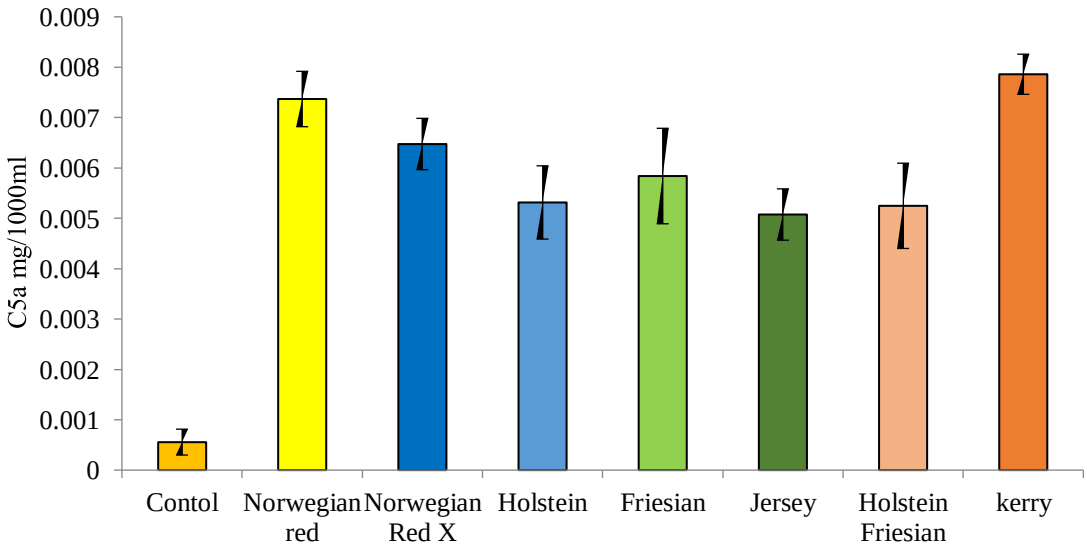


Figure 6.2 Average C5a ELISA assay results for the 18 individual milk samples from each of the breeds as well as one control (bulk milk heat treated at 56°C for 30 min).

Moreover, a one-way analysis of variance test of the data show that individually each breed was significantly different from the control ($p < 0.05$), which implied all breeds had some C5a component present. Post-hoc statistical analysis confirmed that analogous to the bactericidal sequestration assay the Kerry and Norwegian red purebreds had significantly more C5a present when compared to the other pure- and crossbreds ($p < 0.01$). Yet again the Norwegian red crossbreed maintained a significant level of C5a and was not significantly different from its purebred equivalent, consequently, it may be considered that there is a level of stability of these qualities of the Norwegian Red breed ($p < 0.05$). This analysis appears to confirm

that the increased *E. coli* O111 inhibition can be accredited to increased activity of this key Complement component among these two breeds.

The relationship between the C5a ELISA assay and the bactericidal sequestration assay was investigated using the Pearson product-moment correlation coefficient. This test recognised a strong negative correlation between the two variables, i.e., higher Complement C5a levels corresponded with greater inhibition of *E. coli* O111, thus confirming that the milks of the Norwegian red and Kerry breeds stood out in terms of higher Complement activity.

6.4.3 Measurement of Immunoglobulin M using ELISA assay

Immunoglobulins are strongly associated with the antimicrobial capabilities of bovine milk; in order to confirm that the antimicrobial effect seen in the 7 breed milks was due to increased levels of Complement instead of Immunoglobulins it was decided to assess the levels of IgM in the milk samples. An IgM ELISA completed on the milk samples collected from the 7 breeds and control samples (Fig 6.3) confirmed that IgM levels for all 7 breeds were comparable.

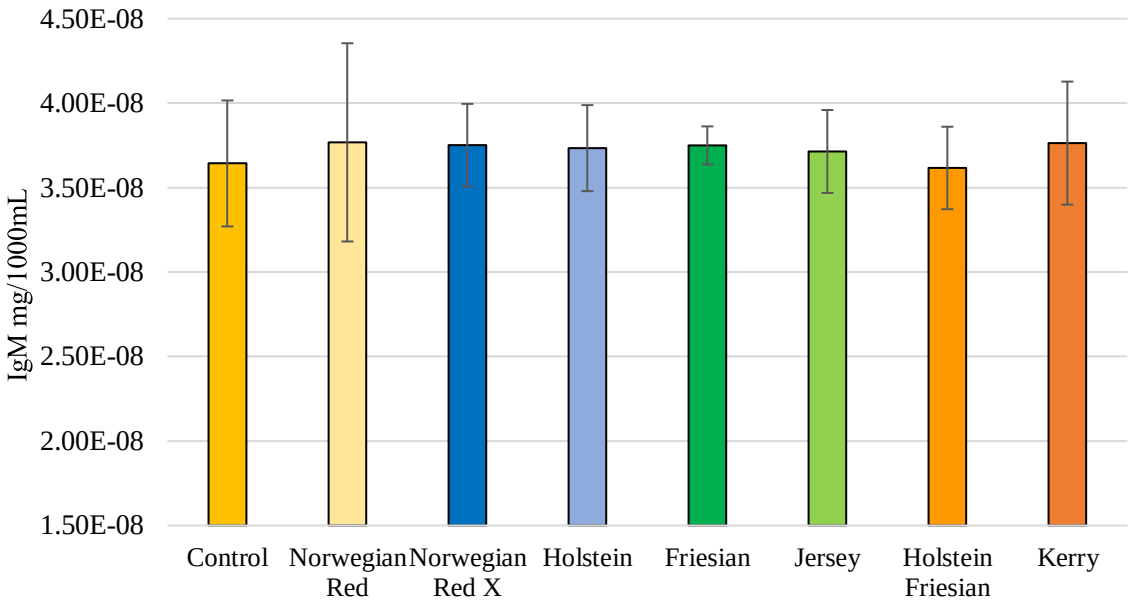


Figure 6.3 IgM ELISA assay results for the milks collected from the 7 pure- and cross-breeds as well as from the Control sample which is produced by heat treating bulk tank milk at 56°C for 30 min.

6.4.4 Somatic cell counts of milk samples

SCCs remained low (< 200,000 cells) throughout the trial, and there was no significant difference ($p < 0.05$) between each breed (Fig 6.4). Given that such low SCC levels reflect positively on the udder health status of the animals, the observed responses can therefore attributed to Complement present in the milk and not due to other immune components (Schukken et al., 2003). There was no significant difference among individual cows within breeds.

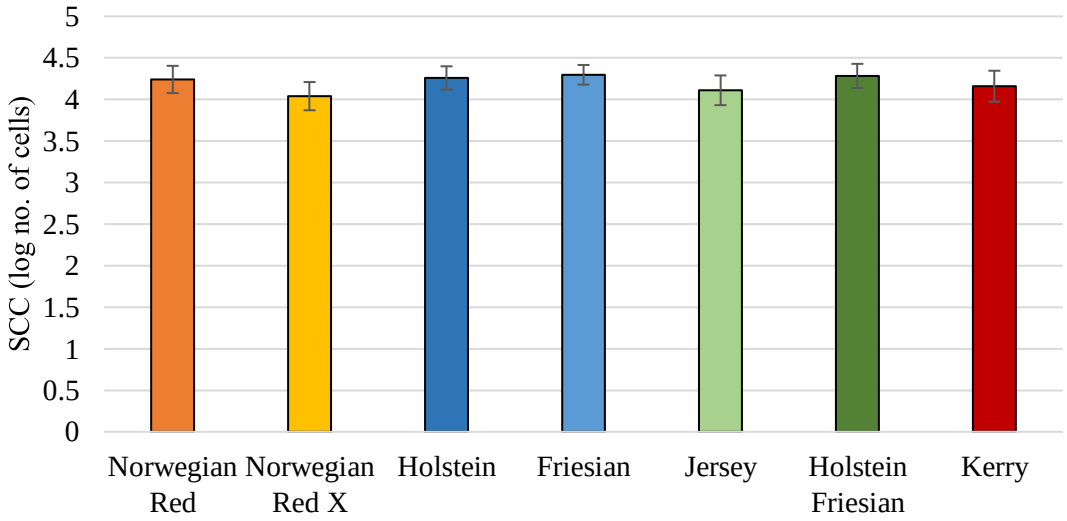


Figure 6.4 The average somatic cell counts (log) for all 7 breeds was monitored throughout the trial, all remained at normal levels and there was no sign of sub- clinical or clinical infection in any animal included in the study.

6.4.5 Use of transformed *E. coli* O111 for real time monitoring in breed variation raw bovine milks

Previous work by the authors successfully transformed *E. coli* O111 with the p16 *S*lux plasmid. This adaptation allows specification of the sequestration technique by lux tagging of the target microorganism in order to elaborate more substantively changes taking place in Complement activity due to breed variation. This test adaptation by the authors provides supplementary information in relation to the direct performance of the breed-specific variation, e.g., incorporation of the p16 *S*lux plasmid ensures that the Complement response may be measured throughout incubation and for a longer time (24h), and thus provide detailed monitoring of the viability of *E. coli*

O111, the assay marker microorganism. The lux-tagged *E. coli* O111 strain was inserted into a modified bactericidal sequestration assay whereby the amount of bacteria was reduced (i.e., 3 X 10^5 CFU per ml) to slow the growth of *E. coli* O111 and avoid the death phase due to lack of nutrients available. As seen in Fig 6.5 the control (heat treated raw milk, 56°C 30 min) allows the bacteria to grow and thus, increase bioluminescence over time.

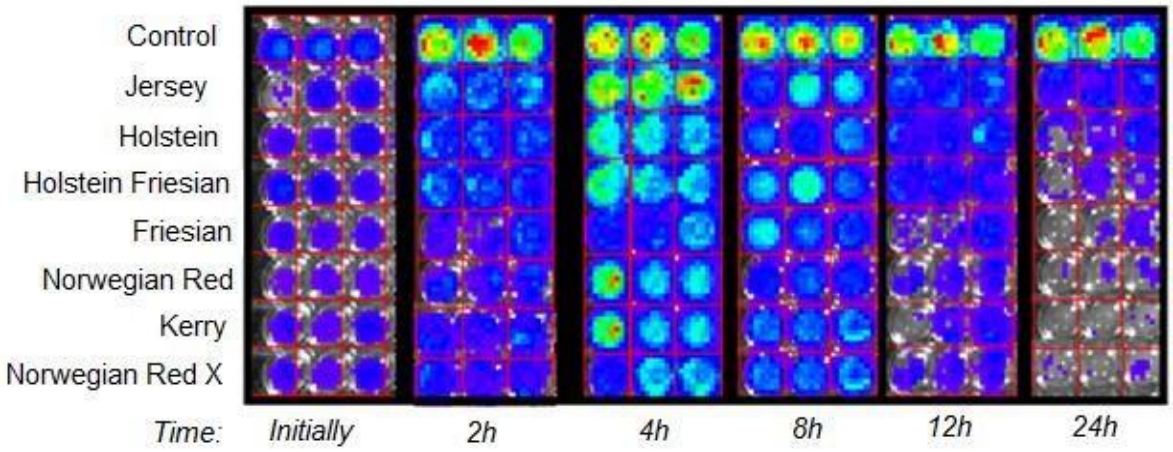


Figure 6.5 The bioluminescent image captured of the 96 well plate monitoring growth of *E. coli* (ROI) during bactericidal sequestration assay of after inoculation with transformed *E. coli* O111 and incubated over 24h

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Without exception, all 7 breeds reduced the bioluminescence over time; however, the Norwegian Red pure-breed and the Kerry pure-breed animals were capable of reducing the growth of *E. coli* O111, and thus, the bioluminescence emitted most efficiently ($p < 0.05$). After 24h, there was little or no bioluminescence remaining in the wells containing milks produced by these breeds.

When lux tagged *E. coli* O111 was employed, region of interest (ROI) values were monitored in order to quantify the effect of Complement; ROI was estimated by selecting an area of particular interest in individual wells of 96-well plates. As expected, the control (raw bovine milk, heated 56°C, 30 min) allowed the bacteria to grow and attain a stationary growth phase from approx. 7h until 24h (Fig 6.6).

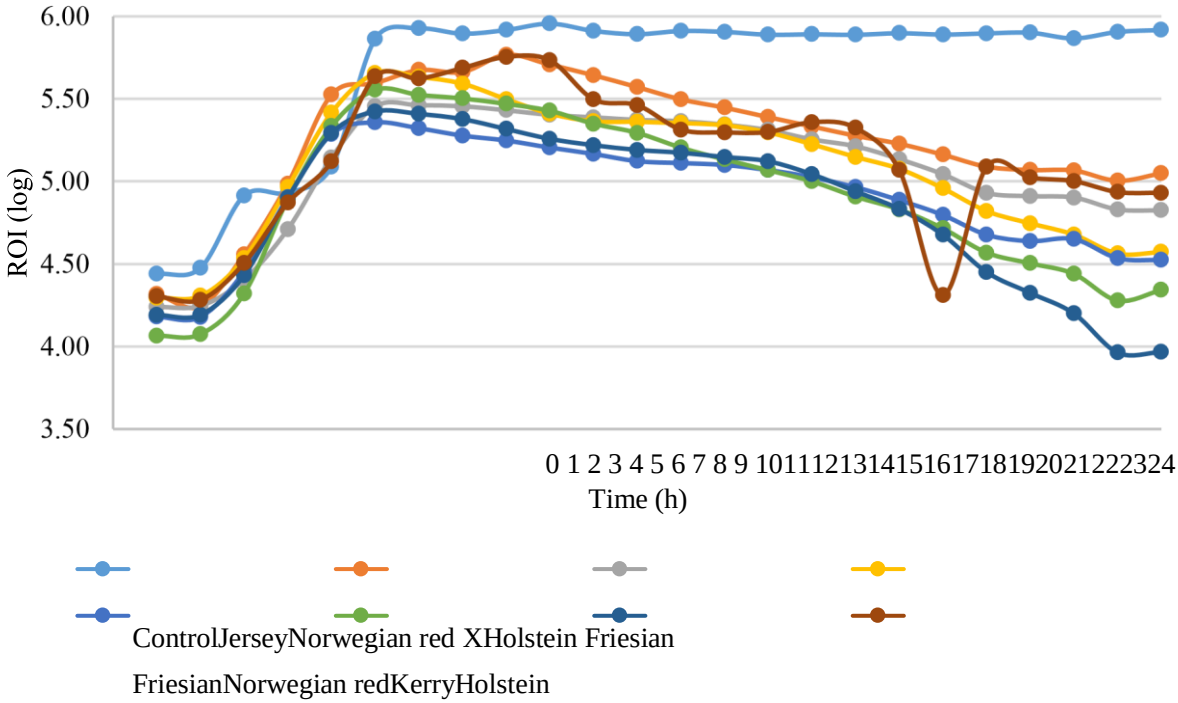


Figure 6.6 Region of interest readings from Xenogen IVIS of *E. coli* O111 (log) during incubation at 37°C for 24h in the Control and the 7 different breed milks following inoculation with transformed *E. coli* O111

However, all milks from the seven breeds slowed growth at an earlier 5h stage, leading to eventual decline of the culture numbers from 7h onwards. The milk of the Kerry breed was more efficient at reducing growth to 3.96 (log) according to the ROI measurement. Overall, the bioluminescence signal for all breeds was reduced, this result correlated well with the other testing methods described above; the ROI

readings had reduced to 4.28, 4.83, 4.94, 4.56, 4.53 and 5.00 following incubation with Norwegian Red purebred, Norwegian Red crossbred, Holstein purebred, Holstein crossbred, Friesian purebred and Jersey purebred, respectively.

6.5 Discussion

Overall, the milks of Norwegian red and Kerry purebred dairy cows exhibited better Complement response when challenged by the bactericidal sequestration assay, thus confirming that these breeds possess more robust innate immune systems – assuming that the migration of Complement across the mammary tissues into milk reflects its concentration in the blood sera. In addition, it was observed throughout the study that the Norwegian red crossbreed maintained strong Complement activity, which would suggest that the genes relevant for this protein system are stable and well maintained within the Norwegian Red breed. The superior immunity of the Norwegian Reds was evident during a study which compared Norwegian crossbreeds to purebred Holstein calves - the Holsteins had reduced antibody-mediated immunity compared with Norwegian Red-Holstein crossbred calves (Cartwright et al., 2011). Norwegian red cows have improved immunological and genetic resistance to disease. This has been associated with specific MHC genes which were found to be essential in eliciting a suitable immune response against invading pathogens (Kulberg et al., 2007). As we know, there has been increased emphasis placed on clinical mastitis studies and the effect on milk production (Seegers et al., 2003). Breeding programs based on this selection criteria have affected the total merit index of the Norwegian red breed (Heringstad et al., 2003), therefore, it may be that the elite-sires that have been selected in recent years, on average, are genetically less susceptible to infection.

Furthermore, this genetic improvement of mastitis resistance *via* selection is feasible, which may also imply that improved Complement activity could be selected for using similar breeding programmes.

The Complement activity measurements reported in this study may help to provide an underlying explanation for why superior overall cow health has been observed in the Norwegian Red pure and crossbreeds (Begley et al., 2009b). The strong Complement activity performance of the Kerry breed alongside the Norwegian Reds correlates with the awareness of a high level of genetic conservation within the former breed. Geographically, the breed enjoys considerable isolation in the south-west of Ireland, where it appears to have maintained a stable set of genes to maintain the integrity of the breed (MacHugh et al., 1998). Norwegian Red, on the other hand, is a dairy breed that was established in 1935 by the crossing of dairy breeds with several Scandinavian breeds, including the Norwegian Red and -White, Red Trondheim and the Red Polled Østland (Bai 2011). Complement activity data according to the bactericidal sequestration assay is further corroborated by an ELISA measurement of component C5a where the Norwegian red purebred and the Kerry purebred again performed the best, with levels of 0.00737 and 0.00787 mg/1000ml respectively. The IgM ELISA assay also confirmed there was no significant difference in IgM levels between the 7 breed milk samples; furthermore, there was no significant difference between the 7 milk samples and the control. The control value was similar to the other milks which is consistent with those reported by Mainer *et al*., (1997). The effects of different heat treatments on IgG, IgA and IgM studied by these authors showed that denaturation increased with higher temperature and longer holding times. Furthermore, IgM would not be denatured when exposed to the Complement

inactivation temperature (56ºC for 10 min). Bannermann et al. (2008) found that the temporal response of a wide range of physiological markers following infection was similar between Holstein and Jersey cows, and concluded that the innate immune response at udder health level between these two dairy breed is highly conserved despite previously reported differences in mastitis prevalence, as well as genotypic and phenotypic traits. However, innate immune capabilities have been observed using comparative genomics to identify heritability of the candidate genes associated with the specific innate immune response (Jann, 2009). The selected genes are being sequenced in different cattle populations to discover novel single nucleotide polymorphisms (SNP) in order to differentiate genetic differences between individual animals.

This study indicates that the selection of bovine phenotypes for breeding, based on studies of immunology and other functional characteristics could assist in improving the heritability of mastitis resistance and improved overall health. This supposition has already been found to have a positive effect on mastitis resistance in the NR dairy cow population (Heringstad, et al., 2003). Furthermore, epidemiological modelling, quantitative trait loci detection and expansion of additional functional genomic testing could provide valuable tools in understanding the genetic determination of mastitis resistance. Along with this, this study could also provide improved characteristics for fertility and protein yield.

6.6.References

BAI, G.T., 2011. Establishment and characterization of bovine oviductal epithelial cells in culture and study of sperm binding capacity:" Feasibility studies towards the development of a semen fertility assessment assay for Norwegian red (Master's Thesis).

Retrieved from: https://brage.bibsys.no/xmlui/handle/11250/132353

BANNERMAN, D.D., KAUF, A.C.W., PAAPE, M.J., SPRINGER, H.R. AND GOFF, J.P. 2008. Comparison of Holstein and Jersey innate immune responses to *Escherichia coli* intra-mammary infection. *Journal of Dairy Science*, *91*(6), pp. 2225-2235.

BEECHER, C., DALY, M., CHILDS, S., BERRY, D.P., MAGEE, D.A., MCCARTHY, T.V. AND GIBLIN, L., 2010. Polymorphisms in bovine immune genes and their associations with somatic cell count and milk production in dairy cattle. *BMC genetics*, *11*(1), pp. 1.

BEGLEY, N., BUCKLEY, F., BURNSIDE, E. B., SCHAEFFER, L., PIERCE, K. AND MALLARD, B. A. 2009a. Immune responses of Holstein and Norwegian Red x Holstein calves on Canadian dairy farms. *Journal of Dairy Science, 92*(2)**,** pp. 518-525.

BEGLEY, N., BUCKLEY, F., PIERCE, K. M., FAHEY, A. G. AND MALLARD,

B. A. 2009b. Differences in udder health and immune response traits of Holstein-Friesians, Norwegian Reds, and their crosses in second lactation. *Journal of Dairy Science*, *92*(2), pp. 749-757.

BRADLEY, A.J., 2002. Bovine mastitis: an evolving disease. *The Veterinary Journal*, *164*(2), pp. 116-128.

CARTWRIGHT, S., BEGLEY, N., SCHAEFFER, L., BURNSIDE, E. AND MALLARD, B. 2011. Antibody and cell-mediated immune responses and survival between Holstein and Norwegian Red× Holstein Canadian calves. *Journal of Dairy Science*, *94*(3), pp. 1576-1585.

HALASA, T., HUIJPS, K., ØSTERÅS, O. AND HOGEVEEN, H., 2007. Economic effects of bovine mastitis and mastitis management: A review. *Veterinary quarterly*, *29*(1), pp.18-31.

HERINGSTAD, B., KLEMETSDAL, G. AND RUANE, J., 1999. Clinical mastitis in Norwegian cattle: Frequency, variance components, and genetic correlation with protein yield. *Journal of Dairy Science*, *82*(6), pp. 1325-1330.

HERINGSTAD, B., REKAYA, R., GIANOLA, D., KLEMETSDAL, G. AND WEIGEL, K.A., 2003. Genetic change for clinical mastitis in Norwegian cattle:

A threshold model analysis. *Journal of Dairy Science*, *86*(1), pp. 369-375.

ISO. 2004. Milk — Quantitative determination of bacteriological quality—guidance for establishing and verifying a conversion relationship between routine method results and anchor method results, EN ISO 21187. International Organization for Standardization, Geneva.

ISO. 2006 . Milk—enumeration of somatic cells—part 2: guidance on the operation of fluoro-opto-electronic counters. EN ISO 13366-2 1–13. nternational Organization for Standardization, Geneva.

ISO. 2008. Milk—Enumeration of somatic cells—part 1: microscopic method (Reference method), EN ISO 13366–1. International Organization for Standardization, Geneva.

ISO. 2013. Milk - Bacterial count - Protocol for the evaluation of alternative methods (Reference method), EN ISO 16297–1. International Organization for Standardization, Geneva.

JANN, O.C., KING, A., CORRALES, N.L., ANDERSON, S.I., JENSEN, K., AITALI, T., TANG, H., WU, C., COCKETT, N.E., ARCHIBALD, A.L.

AND GLASS, E.J., 2009. Comparative genomics of Toll-like receptor signalling in five species. *BMC Genomics*, *10*(1), p.216

KELSEY, J. A., CORL, B. A., COLLIER, R. J. AND BAUMAN, D. E. 2003. The Effect of Breed, Parity, and Stage of Lactation on Conjugated Linoleic Acid (CLA) in Milk Fat from Dairy Cows. *Journal of Dairy Science*, *86*(8), pp. 2588-2597.

KORHONEN, H., MARNILA, P. AND GILL, H. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition*, *84*, pp. 75-80.

KULBERG, S., HERINGSTAD, B., GUTTERSRUD, O.A. AND OLSAKER, I., 2007. Study on the association of BoLA-DRB3. 2 alleles with clinical mastitis in Norwegian Red cows. *Journal of Animal Breeding and Genetics*, *124*(4), pp. 201-207.

MACHUGH, D., LOFTUS, R., CUNNINGHAM, P. AND BRADLEY, D. 1998. Genetic structure of seven European cattle breeds assessed using 20 microsatellite markers. *Animal Genetics*, *29*(5), pp. 333-340.

MAINER, G., SANCHEZ, L., ENA, J.M. AND CALVO, M., 1997. Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. *Journal of Food Science*, *62*(5), pp. 1034-1038.

MALLARD, B.A., ATALLA, H., CARTWRIGHT, S., HINE, B.C., HUSSEY, B., PAIBOMESAI, M., THOMPSON-CRISPI, K.A. AND WAGTER-LESPERANCE, L., 2011. Genetic and epigenetic regulation of the bovine immune system: Practical implications of the high immune response technology. In *Proc National Mastitis Council 50th Annual Meeting* (pp. 53-63). National Mastitis Council, Verona WI.

MAYE, S., STANTON, C., FITZGERALD, G. F. AND KELLY, P. M. 2015. Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay. *Journal of Dairy Research, 82*(3), pp. 328-333.

MAYE, S., STANTON, C., FITZGERALD, G.F. AND KELLY, P.M., 2016. Transformation of serum-susceptible *Escherichia coli O111* with p16Slux plasmid to allow for real-time monitoring of complement-based inactivation of bacterial growth in bovine milk. *Journal of Dairy Science*, *99*(1), pp. 112-119.

DILLON. P. AND VEERKAMP R.F. 2001. *Breeding Strategies* [Online]. Available: http://www.teagasc.ie/publications/2001/ndc/ndc-dillon.asp [Accessed 08 July 2014].

RAINARD, P. AND RIOLLET, C. 2006. Innate immunity of the bovine mammary gland. *Veterinary Research, 37*(3), pp. 369-400.

REITER, B. AND BROCK, J. 1975. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype O 111.

Immunology, 28(1), pp. 71.

RIEDEL, C. U., MONK, I. R., CASEY, P. G., MORRISSEY, D., O'SULLIVAN, G. C., TANGNEY, M., HILL, C. AND GAHAN, C. G. 2007. Improved luciferase tagging system for Listeria monocytogenes allows real-time monitoring in vivo and in vitro. *Applied and Environmental Microbiology, 73*(9), pp. 3091-3094.

RIOLLET, C., RAINARD, P. AND POUTREL, B. 2000. Differential Induction of Complement Fragment C5a and Inflammatory Cytokines during Intramammary Infections with *Escherichia coli* and Staphylococcus aureus. *Clinical and Diagnostic Laboratory Immunology, 7*(2), pp. 161-167.

SCHUKKEN, Y. H., WILSON, D. J., WELCOME, F., GARRISON-TIKOFSKY, L. AND GONZALEZ, R. N. 2003. Monitoring udder health and milk quality using somatic cell counts. *Veterinary Research, 34*(5), pp. 579-596

SEEGERS, H., FOURICHON, C. AND BEAUDEAU, F., 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary*

Research, *34*(5), pp.475-491.

SORG, D., FANDREY, E., FRÖLICH, K., MEYER, H. AND KLIEM, H. 2013. Mammary immunity of White Park and Highland cattle compared with Brown Swiss and Red Holstein. *Animal Genetic Resources/Ressources Génétiques Animales/Recursos Genéticos Animales, 52*, pp. 91-104.

Cattle Breeds - Friesian (2014, July 8th) retrieved from http://www.thecattlesite.com/breeds/dairy/24/friesian/overview.

THOMPSON-CRISPI, K., ATALLA, H., MIGLIOR, F. AND MALLARD, B. 2014. Bovine Mastitis: Frontiers in Immunogenetics. *Frontiers in Immunology, 5*, pp. 493.

Chapter VII General Discussion

From the outset, the experimental work described in this thesis aimed to confirm the existence of Complement in bovine milk and to establish some comparability with the biological efficacy of that occurring in human milk. Scientific interest over the years has been preoccupied primarily with the role of Complement in human milk (Ogundele, 1998, Ogundele, 1999), but there is little documented research about its

presence in normal bovine milk since Reiter and Brock (1975a) monitored Complement-mediated bactericidal activity of antibodies to a serum-susceptible strain of *E. coli* of the serotype O111 in colostrum and post-colostral milks. The milks screened as part of this initial study established that Complement is active in raw bovine milk and that the magnitude of its bacterial sequestration activity appears to approximate closely that of human milk. This finding is encouraging, because it puts a spotlight on the fate of Complement during the course of processing bovine milk, hence leading to the subsequent study which investigated the effects of a range of thermal treatments. It was clear from this that Complement was relatively easily inactivated in milk under moderate heating conditions (56^{o}C, 30 min) i.e., at less than batch pasteurisation temperatures, as reflected by the diminished capacity ($p < 0.05$) of milk to inactivate the serum-sensitive strain (Korhonen et al., 2000). Protein-based milk antimicrobial agents generally tend to be easily inactivated by heat treatment (Oram and Reiter, 1968). Thus, it is not surprising to encounter relatively low levels of Complement activity in retail pasteurised milk, a product normally subjected to regulatory high-temperature, short-time (HTST) heating requirement of 72^{o}C with holding time of 15s.

Equally revealing was that the residual Complement activity levels were lower in reduced-fat variants of retailed pasteurised milks, which pointed towards a possible association between milk fat and Complement. However, the reduced activity due to the fat removal indicates a fat-associated component. Of the antimicrobials with known associations to the fat fraction and MFGM such as lactoferrin, lactoperoxidase, immunoglobulins, lysozyme, not all components are as significantly affected at 56°C

for 30 min (Ford et al., 1977, Bullen and Rogers 1972, Ludikhuyze et al., 2001 and Dominguez 1997) .

A gravity-induced creaming study established that, as fat content increased in the upper cream layer, bactericidal capacity increased as monitored by the sequestration assay. This appeared to be analogous to the agglutination phenomenon that occurs during creaming in cow's milk whereby its cryoglobulin content forms clusters of milk fat globules, bacteria and somatic cells that migrate upwards. The distinguishing feature in the case of Complement is that the study was undertaken at 15^{o}C instead of the lower temperature (4^{o}C) where cryo-agglutination normally occurs. However, it confirms that Complement has some form of physical association with globular fat, particularly its MFGM, which was also evident from an additional study which revealed a greater bacteriostatic effect of buttermilk compared to skim milk during laboratory simulation of the butter-making process - buttermilk is known to be enriched with MFGM components released during cream churning (Corredig et al., 2003). Geer & Barbano (2014) reported that the interaction between somatic cell's and immunoglobulins play an important role in inducing aggregation of fat, somatic cell's, bacteria, and spores to rise throughout gravity separation. These authors postulate that the SCC improves buoyancy of the aggregates, facilitating their rise to the top.

A transformation of *E. coli* O111 with the p16 *S*lux plasmid enabled the strain to emit bioluminescent light, so that the bacterial sequestration assay could be measured quickly and also facilitates a high throughput method for assessing Complement activity in milk.

The immune status of the lactating animal and the corresponding Complement level detectable in milk puts the spotlight on such as factors as animal health status and breed.

The inflammatory response induced in the individual mammary immune reactions of four cows using a known strain of *Str. dysgalactiae* indicated that Complement activity increased only in the milks of the affected quarters, thus confirming that the innate immune system was acting at the site of local infection and not systemically in the form of increased Complement permeation into milk even from healthy quarters.

The study of cow breeds identified that Norwegian Red and Kerry cows exhibited greater Complement activity. Interestingly, there is considerable contrast in the lineage of both breeds. The Norwegian Red originated in 1935 following the crossbreeding of dairy breeds with Scandinavian breeds in order to improve health and fertility, while the Kerry is an ancient Irish breed that is now exceptionally rare. In the latter case, innate immunity appears to be well conserved, while in the case of the Norwegian Red, targeted crossbreeding succeeded in the creation of a healthy dairy cow with a more robust immune system. This is one step removed from targeting the genes and pathways responsible for Complement activation in conjunction with a breeding programme to increase Complement activity in milk. An investigation of the manner in which the Complement migrates into milk can improve our knowledge about its mode of action and provide an opportunity for the preservation of biological effect and development of a new functional product with immune-enhancing benefits.

As described previously, other antimicrobial constituents are also present in bovine and human milk, and while the specificity of the bacterial sequestration assay confirms the effects on Complement, it is also worthwhile to consider any parallel effects of these other constituents. Lactoferrin also has a role in the maintenance of the inflammatory response and is capable of inhibiting the classical Complement pathway (Samuelsen et al., 2004). In addition to its antimicrobial activity *in vivo* through ironsequestration, specific peptides derived from the N-terminal region from both human and bovine lactoferrin, lactoferricin H and lactoferricin B, respectively, inhibit the classical complement pathway in the course of controlling inflammation triggered by bacterial infection (Samuelsen, et al., 2004). Interestingly, the *E. coli* O111 strain that is susceptible to Complement in bovine serum is equally inhibited in iron-saturated sera (Reiter et al., 1975b). Another serum-resistant strain, *E. coli* O101, was equally uninhibited by unheated and heat-treated serums. During a preliminary study carried out by the authors (results not reported here) it was also observed that the serum resistant *E. coli* O125 was not inhibited to the same levels as the Complement sensitive *E. coli* O111.

Another antimicrobial constituent in milk, which has been reported to be bactericidal for specific pathogenic strains in the presence of IgA and Complement component C3. Curiously, there is known to be an increase in lysozyme activity on heating to 60°C which may be due to its release from the larger components in the milk (Evans et al.,

1978), however, the loss of Complement activity following heat treatment at 56°C for 30 min clearly confirms that this anti *E. coli* O111 activity is not due to lysozyme.

Xanthine oxidase is another milk constituent that increases in activity when fresh milk is subjected to different processing treatments, i.e., cooling, heating, homogenization, and enzymatic activity. While initially bound in some form to the fat phase, increased enzyme activity is always accompanied by a rise in activity in the skim milk phase. A hypothesis for this activity may be that the increased activity is associated to the redistribution of the enzyme. There appears to be a low association with the fat fraction and holding milk for 4h at 60°C does not affect the enzyme activity at all (Gudnason et al., 1962).

Bovine colostrum and milk have been recognised as a rich source of immunoglobulins which may provide protection when transferred to young infants – so-called 'immune milks' (Hurley and Theil, 2011). The effect of heating is immunoglobulin specific, e.g., in the case of IgG it is possible to heat treat colostrum at 60°C for one to two hours without loss of efficacy (Hurley and Theil, 2011). Existing knowledge on hyperimmunised milks is of particular importance for specific microbial diseases which cannot currently be treated using chemotherapy, e.g., rotaviruses, antibiotic-resistant enteropathogens and cryptosporidium. Milk with increased Complement activity and immunoglobulins may act as intervention agents in the prevention or treatment of enteric infection (Early et al. 2001). Considerations for the use of these milks include issues due to regulation, ethical reasoning which are mainly due to the

use of immunepotentiating adjuvants and frequency of immunisation doses. The immune-

supplementation of clinical diets and special infant formulas with specific antibodies appears, therefore, to be a challenging future approach. (Hurley and Theil, 2011). Other studies should aim to further investigate the factors that influence Complement activity in bovine milk and methods to retain its activity during processing. Treatment with milder heat processes could be a potential way to improve activity of the heat labile constituents; however, it is essential that these processes fulfil the food safety assurance equivalent to pasteurisation. A new technology which has been recently applied to other foods is the use of microwave technologies and, although critical control points would need to be maintained, this equipment would appear to have the potential to substitute ultra-high heat treatments which are currently used to process ready-to-feed liquid infant milk products. Current patented microwave volumetric heating technologies have the capacity to apply microwaves in a flow process, thus, enabling the sterilisation of milk products at temperatures up to 12^{o}C lower than conventional temperatures (Zadyraka et al., 2015). Moreover, this could also reduce the holding times for the milk product. Such a process may be applicable for milk products and formulations that aim to preserve the original Complement activity of its starting materials.

References

BULLEN, J.J. AND ROGERS, H.J., 1969. Bacterial iron metabolism and immunity to *Pasteurella septica* and *Escherichia coli*, *Nature*, 224, pp. 380.

CORREDIG, M., ROESCH, R.R. AND DALGLEISH, D.G., 2003. Production of a novel ingredient from buttermilk. *Journal of Dairy Science*, *86*(9), pp. 2744-2750.

DOMINGUEZ, E., PEREZ, M.D. AND CALVO, M., 1997. Effect of heat treatment on the antigen-binding activity of anti-peroxidase immunoglobulins in bovine colostrum. *Journal of Dairy Science*, *80*(12), pp. 3182-3187.

EARLY, E.M., HARDY, H., FORDE, T. AND KANE, M., 2001. Bactericidal effect of a whey protein concentrate with anti-Helicobacter pylori activity. *Journal of Applied Microbiology*, *90*(5), pp. 741-748.

EVANS, T.J., RYLEY, H.C., NEALE, L.M., DODGE, J.A. AND LEWARNE, V.M., 1978. Effect of storage and heat on antimicrobial proteins in human milk. *Archives of Disease in Childhood*, *53*(3), pp. 239-241.

FORD, J.E., LAW, B.A., MARSHALL, V.M. AND REITER, B., 1977. Influence of the heat treatment of human milk on some of its protective constituents. *The*

Journal of Pediatrics, *90*(1), pp.29-35.

GEER, S.R. AND BARBANO, D.M., 2014. The effect of immunoglobulins and somatic cells on the gravity separation of fat, bacteria, and spores in pasteurized whole milk. *Journal of Dairy Science*, *97*(4), pp. 2027-2038.

GUDNASON, G.V. AND SHIPE, W.F., 1962. Factors affecting the apparent activity and heat sensitivity of xanthine oxidase in milk. *Journal of Dairy Science*, *45*(12), pp. 1440-1448.

HURLEY, W.L. AND THEIL, P.K., 2011. Perspectives on immunoglobulins in colostrum and milk. *Nutrients*, *3*(4), pp. 442-474.

KORHONEN, H., MARNILA, P. AND GILL, H. S. 2000. Milk immunoglobulins and complement factors. *British Journal of Nutrition*, *84*, pp. S75-S80.

LUDIKHUYZE, L.R., CLAEYS, W.L. AND HENDRICKX, M.E., 2001. Effect of temperature and/or pressure on lactoperoxidase activity in bovine milk and acid whey. *Journal of Dairy Research*, *68*(04), pp. 625-637.

OGUNDELE, M. O. 1998. A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation. *Mediators of Inflammation*, *7*(5), pp. 363-365.

OGUNDELE, M. O. 1999. Cytotoxicity by stored human breast-milk: possible contribution of complement system. *Cell Biology International*, *23*(8), pp. 585-588.

ORAM, J.D. AND REITER, B., 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *170*(2), pp. 351-365.

REITER, B. AND BROCK, J. 1975a. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. I. Complement-mediated bactericidal activity of antibodies to a serum susceptible strain of *E. coli* of the serotype O111. *Immunology*, *28*(1), pp. 71.

REITER, B., BROCK, J.H. AND STEEL, E.D., 1975b. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. *Immunology*, *28*(1), p. 83.

SAMUELSEN, Ø., HAUKLAND, H.H., ULVATNE, H. AND VORLAND, L.H.,

2004. Anti-complement effects of lactoferrin-derived peptides. *FEMS Immunology & Medical Microbiology*, *41*(2), pp. 141-148.

ZADYRAKA, Y.V., GRITSININ, S.I., MISAKYAN, M.A., KOSSYL, I.A. AND BARKHUDAROU, E.M., Advanced Microwave Technologies Ltd, 2015. *Apparatus for Treating a Fluid with Microwave Radiation*. U.S. Patent 9,034,194.