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The detection and prediction of mastitis in dairy cows by particle analysis

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The Detection and Prediction of Mastitis in Dairy Cows by Particle Analysis

By

Iwona Anna Janik

***A thesis submitted in partial fulfilment of the University's
requirements for the Degree of Doctor of Philosophy***

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**Coventry University
in association with the Royal Agricultural University**

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Abstract

This study investigated the hypothesis that the particulate content of milk, as monitored with particle counters, is correlated to the health status of lactating dairy cows, in particular the condition mastitis. Twenty Holstein cows were monitored from the very first day of clinical mastitis outbreak until complete recovery from the disease. During the experiment, the changes in particle behaviour in all four quarters and mixture of milk from all of them were measured. For each sample the following parameters were measured: somatic cell count, fat content, lactose and protein concentration, number and size distribution of milk particles, electric conductivity and diameter of milk fat globules. In total over thirty mastitis outbreaks were observed and monitored throughout, including the first phase of this study when over three thousand samples of foremilk were collected and examined.

An operational protocol and particle monitoring device were designed with the help of a commercial company Facility Monitoring Systems Ltd (FMS), Malvern. A particle counter and Peak Height Analyser (PHA) were used to monitor particulate content of milk and a compound phase contrast microscope was used to identify milk particles by photographic visualisation and to establish their diameter.

It was observed that the number of particles, milk fat globule diameter and somatic cell counts were stable during periods without udder inflammation. Mastitis caused great changes in these parameters. Both milk particulate size and number were significantly affected by clinical and subclinical form of inflammation (change to the particulate behaviour). It was observed that the changes to the volume median diameter (VMD) of fat globules became evident a few days before clinical signs were present. Results obtained from a particle counter and the PHA were in agreement with data obtained by microscopy. Major changes were recorded in the number of total particles in milk before and during the outbreak of mastitis. Further research showed that changes took place in the pattern of particulate behaviour without visible signs of disease; additional data established that subclinical mastitis can be also identified through the monitoring of particles in milk. In summary monitoring of the behaviour (changes to size and number) of milk fat globules (MFG) can be used as an early indicator of the onset of mastitis.

In addition data collected during study produced strong evidence supporting the theory of the interdependence of the quarters within the udder. It was found that the

coefficient of correlation for size and number of particles for all four quarters within the udder was statistically significant. Particle counts and the VMD values behaviour were similar for the four quarters. This relationship was observed for all monitored animals. Moreover, the same relationship was also observed during both clinical and subclinical outbreaks of mastitis. Somatic cell count was affected only in an infected quarter while particulate content of milk “responded” to disease in all four quarters within the udder (even if only one was infected). These results were the most surprising and unexpected outcome, suggesting that four quarters within the udder work together as one organ not four separate units.

It was observed that the mean MFG cannot be used as a baseline to test individual animal deviations due to the unique particle profile of each observed animal. In all monitored animals particle counts obtained from PHA was found to be in the range of 10^{11} to 10^{13} with an average of 10^{12} particles per ml. The number of particles recorded in mastitis for one animal was at the healthy level for another. The particle pattern became a finger print for each animal and therefore MFG behaviour cannot be compared between animals.

Following the first phase of the study the monitoring period was set at 15 to 20 days. This protocol allowed for minimising the influence of any other parameters on particles, which may influence the outcome of an experiment e.g. the number of particles in single samples detected by the particle counter. It was also essential to understand how the age, nutrition and stage of lactation might influence the particles and affect the results. Therefore two animals were chosen to be examined during their lactation. The analysed data did not present enough evidence to establish the relationship between nutrition and milk fat globules size and number. However to better understand this association additional studies should be carried out.

Further work is required to optimise the monitoring device and build a fully automated system which will allow collecting and analysing data from the whole herd. This study proposed that particle pattern is unique for each animal – like a finger print. More research is needed to better understand the mechanism behind milk fat globules synthesis during inflammation.

The results obtained during this study provide new evidence with regard to physiological changes within the udder before and during mastitis outbreak and supported the theory of interdependence between quarters within udder. The particle count in milk can be used as an indicator of the health status of the single animal. Combined the PHA and microscope can be used as a new tool to determine and monitor the particle count in milk. The understanding of the particulate behaviour will help to minimise the chance of mastitis outbreak by early detection and also to reduce the chance of the cross-contamination between animals during the milking process. Milk fat globule size and number can be used as an efficient indicator of the onset of mastitis.

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Glossary of terms

AM	automatic milking
APP	Acute Phase Proteins
BHBA	β – hydroxybutyric acid
BL	back left quarter
BR	back right quarter
CLA	conjugated linoleic acid
CMT	California Mastitis Test
DCT	dry cow therapy
DM	dry matter
DNA	deoxyribonucleic acid
EC	electrical conductivity
ECR	endoplasmatic reticulum
FA	fatty acids
FFA	free fatty acids
FL	front left quarter
FMS	Facility Monitoring Systems Ltd., Malvern
FR	Front right quarter
GPL	glycophospholipids
HOCL	hypochlorous acid
mLPL	milk lipoprotein lipase
LDH	L-lactate dehydrogenase
LB	light blocking
LF	lactoferrin
LPC	Particle Counting Spectrometer for Liquids
LPD	lipopolysaccharide
LPL	lipoprotein lipase
LS	light scattering

MFD milk fat depression
MFG milk fat globule(s)
MFGM milk fat globule membrane
ML milk lipids
NMD number median diameter
VMD volume median diameter
NMR National Milk Record
PCT particle counting technology
PHA Peak Height Analyzer
PL phospholipids
PLP polystyrene latex particles
PMN polymorph nuclear neutrophil
PUFA polyunsaturated fatty acids
RNA ribonucleic acid
ROS reactive oxygen species
SC somatic cells
SCC somatic cell count
TAG triacylglycerols
WMT Wisconsin Mastitis Test

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1 Chapter I Introduction

1.1 Background to the global and UK dairy industry

The last century was characterised by fast population growth, rising incomes, and fast development of engineering and scientific knowledge. With greater awareness and understanding, the demands of 21st century's consumers are high quality and safe products from a global market. The food industry and its supply chains have made large steps in this direction over the last fifty years by giving consumers better quality products and increasing safety standards across the whole food chain. To be able to meet those demands the dairy industry has to provide a high quality basic milk product which can then be used to produce a wide range of dairy products. Good quality milk is associated with healthy dairy cows; hence improving the health status of a dairy herd is in the best interests of milk producer, processors and consumers.

The demand for dairy products derived from milk production, has been rising in recent years, and the milk production is increasing year on year [Dairy UK, 2009; White Paper, 2011]. The world milk production annual rate of growth has been 1.8% over the period 2000-2007. In the European Union milk production has been subject to a quota system. Introduced in 1984 it gives individual producers a limit for milk volume and butterfat. In 2008 the EU-27 produced over 133 000 million metric tonnes of milk and the forecast for 2009 was calculated to be 136 000Mt, which puts the EU as a leader in milk production. Currently about 30% of global milk output comes from small dairy farms, whose milk is mostly consumed by small local markets. However, the world dairy industry is indicating a trend towards larger herd sizes, closer collaboration between dairy farms and further processing [Dairy UK, 2009].

Currently the biggest challenge for the global dairy industry production is to decrease the incidence of animal health problems, particularly mastitis, due to its severe negative effect on milk production, animal welfare, and consequent reduction of the

farmer's income. Bradley (2002) defined this condition as inflammation of the udder with infectious or non-infectious aetiology. According to Hillerton (2000) and Berry (1998) the average clinical mastitis incidence rate is between 20-40 cases per hundred cows per year in the UK.

1.2 Animal welfare implications

Broom (1986) described welfare of individual as its state in regard to an attempt to cope with the environment. Broom and Johnson (1993) defined welfare as characteristics of animals that can be measured in a scientific way that is free from moral considerations. In recent years the concern about dairy cows welfare has increased which can be reflected in developments in housing and management practice on farms. The public pressure on animal welfare has increased in majority of European countries [Broom, 1992]. Animals experience pain in a comparable way to humans, as the neural pathways in all mammals are very similar. Sanford *et al.* (1986) described the behaviour of cattle when experiencing pain. Animals appear dull and depressed, lose weight and a reduction of milk yield can be observed. Severe pain is associated with a higher rate of respiration, a rigid posture, grunting and grinding of teeth, and laying down frequently. The condition of mastitis is highly associated with pain. Fitzpatrick (1998) described mastitis as a very painful condition and advised that pain relief should be considered as one of the fundamental therapies. The estimated pain of mastitis is illustrated by Huxley's scale (2007) in Figure 1-1. Mastitis is not the only aspect of cow welfare; Hillerton (1999) pointed out that dairy farmers should be able to claim high welfare status only if they obtain early and successful treatment of clinical mastitis. Results obtained by Kemp *et al.* (2008) indicated that cows diagnosed with moderate clinical mastitis had a significantly higher rectal temperature and heart and respiratory rates when compared with control animals. Moreover hock to hock distance was significantly smaller in control animals than in animals with moderate and mild clinical mastitis. Kemp *et al.* (2008) defines moderate mastitis as being characterised by changes in the appearance of milk, swollen and/or painful and hot udder(s) whilst mild mastitis is characterised by changes in the appearance of milk but udder condition remains normal.

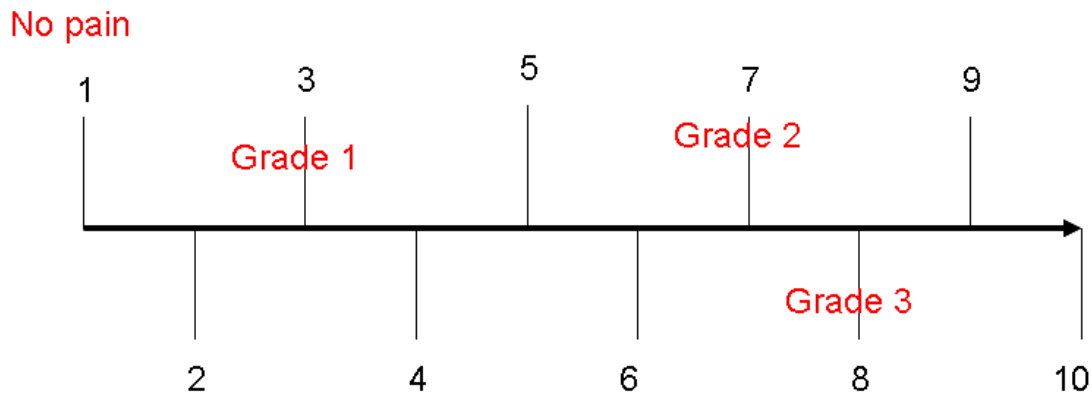


Figure 1-1 Pain of mastitis scale.

Grade 1 – clots in milk.

Grade 2 – changes in milk appearance and swollen quarter.

Grade 3 – changes in milk appearance, swollen quarter, increased temperature, reduction in feed intake [Huxley, 2007].

The animal welfare is closely related to stress to which dairy cows can be exposed too. According to Selye (1973) stress responses occur when the pressure of the environment becomes excessive or physiological threats and new defence mechanism are initiated. The Brambell Committee of the U.K. Parliament (1965) has formulated “five freedoms” which was later updated by the Farm Animal welfare Council (1992) in order to define obligation of humans and to ensure welfare of farm animals, this was to include psychological view of adaptation and stress.

- Freedom from hunger and thirst refers to the physiological requirements,
- Freedom from discomfort refers to the environmental needs,
- Freedom from pain, injury and disease refers to the pathological consequences of an adverse environment,
- Freedom to express normal behaviour refers to the importance of behavioural coping mechanisms for a successful adaptation,
- Freedom from fear and stress refers to the psychological component of adaptation.

Day to day factors and routines associated with the intense dairy management might have an impact on the stress level (e.g. transportation, handling, lameness, building design, prolong time in stall) and therefore affect mammary gland immunity and its ability to resist and/or overcome infection. More often dairy cows can be also exposed

to high temperatures and direct sunlight which can be linked to heat stress. Mammals maintain the body temperature by heat loss and heat production. Silanikove (2000) described in detail methods of measurement heat stress however there is no agreement or conclusion to the correct methodology. According to Harmon (1994) stress has minor impact on SSC if the udder and quarters are not infected. Waller (2002) pointed out that immune suppression can be caused by many factors and stress is one of them. However, further research needs to be undertaken in which to better understand the relationship between stress, immune responses and factors. The genetic factors should always be taken into consideration when assessing how an individual cow can handle stress in different situations. Stress associated with calving or adverse environmental factors can have a negative impact on the immune system of both adult and calf which could lead to higher susceptibility to infectious factors [Guidry, *et. al.*, 1976].

1.3 Economic impact

The last decade of the 20th century observed increased milk production as the result of a combination of improved management on dairy farms achieved through improvements in genetic selection, nutrition, veterinary care, increased knowledge of the host response to intramammary infections and also implementation of treatments which allow to adopt various control and prevention measures [Lucy, 2001; Shook, 2006; Oviedo-Boyso *et.al* 2007; Zadoks and Fitzpatrick, 2009; Atalla *et. al.*, 2010]. However, mastitis still has negative influence on the dairy industry.

The economic costs of mastitis can be associated with the following factors: milk production losses, cost of drugs, discarded milk, veterinary costs, labour, product quality, material investments, diagnostics and culling [Halasa *et al.*, 2007]. Houben *et al.* (1993) noted that a decrease in milk production is caused by both clinical and subclinical mastitis; however only losses attributed to clinical mastitis cases can be estimated. Milk payment can be based on unit weight of total milk or per kilogram of milk components such as fat and protein. Because subclinical mastitis does not indicate any abnormalities in milk appearance, many milk producers are unaware of low quality and losses in milk production. Results obtained by Hamman (2002) suggested that changes in milk composition occur when the somatic cell count (SCC) reaches 100 000 cells/ml and every additional 100 000 somatic cells (SC) results in up

to 5% less production. Discarded milk brings higher financial losses than decreased milk production. Many farmers use discarded milk to feed calves, which at the same time cuts the costs of milk replacement [Halasa *et al.*, 2007].

Antibiotics are often a necessary and frequent part of mastitis treatment. The cost of antibiotic treatment differs across countries. Antibiotic residues in milk and meat can be a source of human allergic reactions. Moreover use of antibiotics leads to development of resistant strains of bacteria that could be transferred to humans. The concern for public health may cause the presence of residues as one of the valuation metrics for milk price. [Bramley *et al.*, 1996; White and McDermott, 2001, Hillerton and Berry, 2005; Collins, *et al.*, 2010; Viridis *et al.*, 2010]. Probably the largest economic losses are costs associated with the premature culling and replacement of animals. The decision to cull is made by a dairy farmer only if replacement is the optimal option [Halasa *et al.*, 2007]. The production loss per lactation from one infected quarter has been calculated to be in the region of £1,600 [Bramley *et al.*, 1996], which in real terms is £2500 2011/2012 (with inflation value 2.9% per year). The average cost of mastitis in the UK was calculated at £87 per case per year [McInerney *et al.*, 1992] however summer mastitis costs can rise to £238 per case per year. Losses caused by summer mastitis were estimated at £7.7 billion to the UK industry [Hillerton *et al.*, 1992].

According to Blowey and Edmondson (1995) an average cost of one case of clinical mastitis is in the range of £100 to £200, with the average incidence of mastitis in UK farms between 40 to 50 cases per 100 cows. An increased number of somatic cells (SC) up to 310,000 with a penalty of 1.8 p/litre will cost the farmer £21,000 a year. However this calculation does not take into account veterinary treatment and milk wastage. In summary, mastitis is costing the UK dairy industry £200 million per year. The values above relate to the clinical cases of mastitis as they can be diagnosed and observed. However, for every case of clinical mastitis there are 20 to 40 times as many cases of subclinical outbreaks, therefore these costs cannot be precisely estimated.

1.4 Challenges for today

Research and knowledge about mastitis has been expanding for over fifty years. One of the biggest steps occurred in 1960s with the introduction to dairy farmers of a Five-Point Plan [Neave *et al.*, 1966; Smith *et al.*, 1967; Neave *et al.*, 1969] which cut the mastitis rate in dairy farming. This plan associated farmers with the management of mastitis by implementing the following; early detection and rapid treatment of clinical mastitis, dry cow therapy, post milking teat disinfection, culling chronically infected animals and maintaining milking process hygiene.

Currently SCC is an international indicator of udder health and its role in the control of mastitis has been discussed since the middle of the last century. In the 1970's milk with less than 500 000 cells/ml was considered healthy milk [International Dairy Federation, 1971]. Later research conducted by Dohoo and Leslie (1991) established data suggesting that a threshold of 200 000 cells/ml was an indicator of infection, a significant reduction from the previously held assumption that 500 000 cells / ml was healthy. The research is suggesting that the threshold of 100 000 cells/ml could be the established indicator between healthy and infected milk [Hillerton, 1999]. SSC methodology has a very high sensitivity and specificity, but prohibitive costs and impracticalities associated with the process including sample preparation, training, specialist equipment and timescales render it impractical as an in-line detection system. However, Rasmussen (2001) stated that SCC is the only parameter which can be used at the level of quarter, udder and herd as an efficient indicator of milk quality.

On the majority of dairy farms traditional methods remain in use to detect mastitis. Hillerton (2000) identified these traditional methods based on the examiner using:

- Experience – knowledge about changes to the cow's behaviour and attitude
- Smell – A purulent, unpleasant odour.
- Taste – A salty taste.
- Listening – Abnormal vocalisation that through experience have been established as being associated with pain and discomfort.
- Touch – Examination of udder swelling and elevated general temperature.
- Visual – Abnormal colouring including blood, also thickening and clots.

Early detection relies on the skills and experience of the herdsman. However, pressure for cutting costs, increased herd size and income maximisation may reduce the attention given to each animal. Unfortunately in the majority of dairies in the UK, only a short time is available for each cow. Hillerton (2000) pointed out that the visual observation of foremilk is still “the gold standard” for mastitis detection on the dairy farm. EU Hygiene Directive 89/362/EEC requires a diagnosis of foremilk appearance for every dairy cow before full milking is carried out. If there is any physical abnormality, milk has to be diverted from the bulk tank.

1.5 Automated milking systems and mastitis

Automated milking is a voluntary system which allows the cow to decide its own milking time / routine instead of being milked as a part of group/herd. Automated milking is a completely automated method using sensors and robotic arms for animal identification, teat-cup application and removal, cleaning and disinfection of teats and of milking units.

The first automated dairy farm was established in 1992 in Holland, since then the number of farms with an automated milking system has increased world-wide [Rasmussen, 2001]. An objective of this technology applied to milking is to increase milk production by increasing the number of milkings per day (Wiking, *et al.*, 2006). Data collected by Stelwagen (2001) showed that increasing milking up to three times a day can increase milk yield by up to 18%. Automated milking offers more frequent milking which allows flushing of the teat canal with greater frequency and so removing pathogens before they multiply. However automated milking with too short an interval (≥ 5 h) does not give enough time for udder tissue to recover and rebuild, and keeping the canal open for longer can prolong exposure to bacteria [Rasmussen, 2001].

In automated systems the requirement for foremilk inspection is not possible and needs to be replaced by an in-line mastitis detection system. Electrical conductivity (EC) is the main method used in automated milking but Whyte *et al.*, (2003) identified factors affecting EC such as: stage of lactation, changes of milk fat content,

frequency of milking, feed types, milk temperature and milk stone and/or milk fat build-up on the sensor electrode.

Automated systems are considered to have a future within dairy industry, therefore a new method/tool which can be easily incorporated into this automated system and also replace on-site foremilk sample checks (carried out by herdsman) need to be evaluated.

1.6 Particle sensing and monitoring

According to Kochevar (2006), in general particles can be divided in three types: inert organic, viable organic and inert inorganic. Inert organic particles are part of non-reactive organic material derived from living organisms. Viable organic particles are living organisms, capable of living, and germinating under certain conditions; including bacteria, spores and fungi. Inert inorganic particles are non reactive materials such as sand, minerals, iron and calcium salts. Particles can be produced in many ways and have a large variety of sources. Although organic particles are derived from carbon-based living matter it must be understood that particles may or may not be living. Inorganic particles are derived from matter that has never been alive.

Kochevar (2006) also divided particles into types according to their size, in increasing size order: subatomic particles, atoms, molecules, particles measured in microns (size range from under a micron to about 100 μ m) and large particles (over 100 μ m). Common particle sizes are presented in Table 1-1 which indicates a significant diversity in size. One important nature of particles is their tendency to group in more complex units. They can be classified as particle (single), aggregate, agglomerate or flocculate. Particles can present as a single particle or be held together by forces such as atomic or molecular (aggregation), adhesion or cohesion (agglomeration) and weakest forces (flocculation).

Table 1-1 Examples of particle sizes.

Adapted from Kochevar (2006); Morton, 1953; Walstra (1969); Mulder and Walstra, (1974).

Particle type	Particle size
Milk fat globules	<1 – 20 μm
Somatic cells	3 -15 μm
Bacteria	1.0 – 10 μm
Casein micelles	0.2 – 0.03 μm
Hair	50 -150 μm
Flu virus	0.07 μm
Pollen	7 – 100 μm
Dust	0.1 – 100 μm
Sneeze particles	10 – 300 μm
Visible	50 μm

Particle counters are broadly used as detectors of contamination in products and environments. Light particle counting technology was launched in the 1950s for military needs and then was developed in 1960s by High Accuracy Product [Hunt, 1993; Kochevar, 2006]. Particle counting technology (PCT) makes it possible to monitor and give results in real time, and thereby control level of particles and contamination. PCT has grown and expanded, and is now commonly used in many industries as a practical technique to monitor particle contaminations in a range of environments.

Currently particle counting instruments are generally too costly for application in the agricultural industry. At the early stage of this study it was established that milk consists of a high number of particles with different characteristics, which led to the assumption that the inflammation of the udder may affect intracellular synthesis of the particles in question. Therefore in this study a liquid particle counter, located at the

laboratory, was used to measure variation of milk particle numbers and size distribution during mastitis and the recovery period.

The main particulate component of milk in the current study was found to be milk fat globules (MFG). Milk fat is present in the form of droplets surrounded by a biological membrane. Size distribution of MFG has been observed between 0.2 -20 μm [Wooding, 1971; Mulder and Walstra, 1974; Heid and Keenen, 2005]. Research work with MFG started in the 1950s. Thanashi (1965) and King (1969) found an association between mastitis and MFG. Number and size distributions of MFG appeared to be affected by this disease. Diameter and number were measured by using direct microscopy but this technique was restricted to laboratory investigations and was very time consuming. However development of particle counting technology made it possible to measure these parameters on a laboratory scale in a rapid and accurate manner.

Liquid particle counters have been in use for many years. The major manufacturers of particle counters are Lighthouse, Met One, Climeq, IQAir, Kanomax and Fluke. Particles can be measured by the amount of light cut off or scattered by passing particle through a light beam. Liquid particle counters are used to count particles in variety of liquids e.g. water, pharmaceutical liquids, oils and hydraulic fluids. In the majority of cases they are used in systems designated to improve quality control by measuring filter efficiency. Particle counters use optical technology such as lasers, glancing light, white light, scanning optics or conventional microscopes. The method of use depends on particle size, shape, liquid flow rate and reflective index between a particle and its surroundings in addition to the environment and substance being evaluated.

Particles in liquid can be detected and measured by several methods such as Light Scattering (LS), Light Blocking (LB), coulter principle and direct imaging. According to Hill (unpublished) LB technology can be used for detecting particles greater than $1\mu\text{m}$ in diameter. The size of a particle is calculated from the amount of light the particle is able to block as it passes through a light beam. For this method it is crucial that the correct dilution is achieved as particles must pass through the light beam singularly if accurate results are to be obtained. LB counters are typically used for measuring particle contamination in hydraulic and lubricating fluids as well as

pharmaceutical preparations. It must also be noted that LS technology has the capability to detect particles from 0.05µm in diameter.

1.6.1 Facility Monitoring System

The current project was sponsored by Facility Monitoring System Ltd.

FMS based in Malvern was established in 2000. In 2008 FMS was bought by TSI Inc. TSI is a world leading supplier of contamination monitoring solutions including: airborne particle counters, liquid particle counters, and airborne molecular contamination.

FMS specialises in critical processes and storage monitoring systems and provides monitoring systems and services to market sectors including:

- Healthcare and Pharmaceutical (NHS and Private Hospitals, IVF Clinics)
- Environmental and Occupational Health,
- Industrial Processes,
- Air and Water Quality (Water Utilities)
- Artefacts and Data Storage,
- Automotive and Aerospace
- Biotechnology
- Industrial Clean Processes
- Microelectronic

FMS distributes equipment able to work in the range of operational temperature regimes such as cryogenic, freezers, fridges, ambient and incubators.

At the outset of this study, it was clearly demonstrated that the dairy industry urgently needed a new tool that could diagnose mastitis at the early stages. The Royal Agricultural College research team together with FMS engineers agreed that any new methods should have the following attributes: high sensitivity, low cost and able to operate in real time. However the decision to put into practice a particular control measurement (new method) on a farm is complex. It will also involve historical data

of disease among animals, expected change of improvement in the health, benefits and the ease with each it can be implemented.

FMS software and hardware are used across UK therefore the development team has been researching new areas of industry in which the usage of particle science may have found an application. The food industry is an area in which particle counting technology has found many purposes. Barrington Hill (FMS Commercial Director in 2007) approached the Royal Agricultural College with the vision of building a tool which will monitor particles in a milk stream in order to inform about the health status of lactating dairy cow. Currently available methods for mastitis detection do not provide sufficient and rapid results, therefore the desire to extent/introduce the particle technology to dairy industry was implemented. The idea was firstly to characterise milk particulates, secondly to understand their behaviour under different conditions and thirdly, to build a sensor which is suitable for farm operating conditions. This project was launched in May 2007

The objective of this project was to establish the scientific validity of using novel particle counting/sizing technology to monitor changes in the particulate content of milk and any associated link with health status of dairy cows. The establishment of a clear and predictable relationship between milk quality and animal health would allow the appropriate development of equipment and procedures to introduce a device for use in dairies to use as an integral part of animal management

The summary of the results obtained throughout this study indicates that frequent monitoring of the particular count in milk can be used as indicator of the health status of lactating cow. Moreover introduction of the particle counting technology into the dairy industry may lead to the development of technique able to detect mastitis at its early stage.

Null Hypothesis

The particulate content of milk is not affected by physiological condition of a dairy cow.

2 Chapter II Literature review

2.1 Milk components and their biosynthesis

Milk is a complex fluid secreted by the mammary gland of female mammals, designed specifically to nourish the young. Milk contains in excess of 250 components and from the commercial and nutritional perspective the most important are: fat, proteins, lactose and minerals (Table 2-1). The chemical composition of milk depends on many factors such as genotype, breed, nutrition, stage of lactation and health history [Jurczak, 2005].

Table 2-1 Approximate composition of milk

[adapted from Akers, 2002 and Jurczak, 2005]

Components	Gross composition [% w/v]				
	cow	human	sheep	rat	blue whale
Water	87.6	87.1	82.0	79	45.5
Total solids	12.4	12.9	18.0	21	54.5
Lactose	4.8	7.1	4.8	2.6	0.4
Fat	3.5	4.5	7.2	10.3	34.9
Casein	2.5	0.4	3.9	6.4	7.2
Whey	0.7	0.5	0.7	2.0	3.7
Ash	0.7	0.2	0.9	1.3	1.4

Milk components are synthesised in mammary epithelial cells. The main substrates imported from blood are glucose (precursor of lactose, ribose and glycerol), amino acids, fatty acids and minerals.

2.1.1 The carbohydrate fraction

Lactose is the main and the most important carbohydrate fraction in milk which determines its calorific value and sweet flavour. The main function of lactose is to maintain the osmolarity of the milk. Lactose is a disaccharide, one molecule of glucose and galactose are combined in a one to four carbon linkage as a β -galactoside. There are three enzymes associated with biosynthesis of lactose.

Lactose precursor is glucose; the latter is synthesised in the liver from propionate in the process of gluconeogenesis and transported to the udder. In the udder, a portion of

the glucose is converted into galactose. UDP galactase-4-epimerase appears just before parturition and its activity increases significantly during lactogenesis. Both monosugars are combined by lactose synthase inside the Golgi apparatus. Lactose synthase consists of two proteins galactocyl transferase and α -lactalbumin, which are required for maximal activity. The rate of lactation synthesis depends on the availability of α -lactalbumin [Ebner, 1970, 1971]. In milk there are two forms of lactose present identified as α -anomer and β -anomer which can inter-convert [Akers, 2002; Blowey and Edmonson, 1995; Jurczak, 2005].

2.1.2 The protein fraction

Milk proteins are specially designed for the ease of the digestion of a newborn calf. The casein family is highly digestible by enzymes of the calf's intestinal tract and provide a good source of calcium, phosphorus and various amino acids.

The majority of the milk protein is synthesised in the udder cells before transport into the ducts. They are defined as milk-specific proteins. Amino acids used for this synthesis are transported into the udder with the blood (essential amino acids) or synthesized by the secretory cells [Akers, 2002; Jurczak, 2005]. The milk-specific proteins include caseins (alpha_s-, beta-, kappa- and gamma-casein) and whey proteins (α -lactalbumin and β -lactoglobulin). The actual amino acid profile of milk depends on dietary factors and the genotype [Akers, 2002].

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Figure 2-1 Model of casein micelles.

The model B “coat-core” concept of Waugh (1967). Micellar core consists of α -s and β -casein and κ -casein is present at the peripheral layer forming coat. Model B structure of micelle built of casein fraction. S –calcium ions and colloidal calcium phosphate bonds [Moor 1967], (Adapted from Brunner, 1980).

The main protein in milk is casein, which constitutes between 78 to 86% of total proteins [Akers, 2002]. According to Jurczak (2005) the casein family of proteins belongs to phosphoproteins and consist of four fractions; casein- α_1 (55%), casein-beta (25%), casein-kappa (15%) and casein-gamma (5%), as illustrated on Figure 2-1. Each fraction has different amino acids and exhibits different properties. Casein fractions have high phosphate content which allows them to associate with calcium and calcium phosphate salts, as illustrated by Figure 2-2. When compared to other proteins, the casein family is richer in proline and glutamic acids and has less glycine and asparagines. Casein is present in milk in the form of micelles and is dispersed in the water phase of milk. The micelles are nearly spherical in a range of 0.03-0.2 μm with particle weight $10^7 - 10^9$ daltons and consist of all forms of casein held together by bridges made of calcium ion, colloidal-dispersed calcium-phosphate and citrate (Figure 2-2). Casein micelles have an open structure that allows water and other molecules to move freely in and out of the micelle. Ratio of α -s₁, α -s₂, β - and κ -casein appears as 3:1:3:1 [Brunner, 1980; Akers, 2002; Jurczak, 2005].

Figure 2-2 Structure of bridges of casein micelles [Jurczak, 2005].

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The other group of milk proteins constitutes the serum (whey) component. Whey comprises approximately 16-18% of total milk proteins and consists of albumin (α -lactalbumin, β -lactoglobulin, and blood serum albumin), immunoglobulins (IgG, IgA, IgM), lactoferrin, transferrin, nucleoprotein and free peptides. Some of the whey proteins are milk-specific proteins but some are derived from the blood stream. Serum proteins are free of phosphorus; however, they consist of large amounts of sulphur – containing amino acids [Jurczak, 2005]. Distribution of serum proteins varies between species, e.g. β -lactoglobulin in cow's milk constitutes more than 50% of whey proteins.

Milk enzymes are present in a variety of forms: as non-associated forms, as part of membranes delivered from the plasma membrane of the epithelial cells, connected with casein micelles, as part of microsomal particles and associated with somatic cells during inflammation (Swaisgood, 1995).

Lactoferrin and transferrin are counted as glycoproteins and their main function is to bind and transport iron. These proteins inhibit growth of bacteria and are a non-specific component in defence of the mammary gland [Akers, 2002]. Bacterial activity can be suppressed by lysosomes due to their ability to ingest bacterial polysaccharides. Moreover lactoferrin is able to bind iron and make it unavailable to the micro-organism.

Synthesis of milk proteins in epithelial cells follows the same mechanisms as other proteins synthesised in other tissues. During transcription the sense strand of DNA (after removing of non-coding fragments - introns) is transcribed to a complementary mRNA. The single RNA differs from DNA by having the sugar ribose instead of deoxyribose and the uracil instead of thymine. Activation starts when mRNA reaches the cytoplasm and binds with a small subunit of ribosome through pairing with ribosomal RNA. The translation takes place in the ribosome. Amino acids are

activated by ATP and controlled by synthetase activity. The transfer RNA (tRNA) transport amino acids to the ribosome, where tRNA is recognised by the anticodon which is complementary to the codon of mRNA. The mRNA contains codes for amino acids, which come in position appropriate for amino acid-tRNA complex. Growing peptide chains, design for secretion, are sent into Golgi apparatus for post-translational modification (Akers, 2002).

2.1.3 The lipid fraction

Milk is one of the most complex and the most variable sources of naturally produced fats. Milk lipids (ML) are present in the form of globules emulsified in the aqueous phase of milk. ML occur as triacylglycerols (TAG), phospholipids (PL), glycopospholipids (GPL), sterols and free fatty acids (FFA). Triacylglycerol is an ester divided from three individual fatty acids and glycerol. The composition of TAG depends on the kind and the amounts of FA available. However, distribution of FA in TAG is not random; according to MacGibbon and Taylor (2006) the esterification of FA is deliberate at the three positions of triacylglycerol. The short chain acids butyric (4:0) and caproic (6:0) are almost always esterified at sn-3, the medium chain acids from 8:0 - 16:0 at sn-1 and sn-2, when stearic (18:0) acid at sn-1, and oleic (18:1) at sn-1 and sn-3. Esterification of FA allows milk lipids to have melting point close to the body temperature of the cow [Timmen and Patton, 1989]. Phospholipids and glycopospholipids contain larger amount of polyunsaturated fatty acids (PUFA) than TAG. Phospholipids constitute about 0.5% of total lipids and they are present in milk fat globule membrane (MFGM). Cholesterol is the major steroid of ML, it constitutes 95% of the total amount and its majority is located in MFGM [Jensen and Newberg, 1995]. Approximately 10% of cholesterol is esterified [Jensen, 2000].

Milk fat contains approximately four hundred different fatty acids. According to Akers (2002) there are three sources of FFA in bovine milk; firstly, glucose via tricarboxylic acid cycle (in mitochondria conversion to pyruvate, citrate) and acetyl-CoA (in the cytoplasm); secondly, diet (hydrolysis); thirdly, *de novo* synthesis in the secretory cells from nonglucose sources. Fatty acids contain more than fourteen carbons with the half of the 16:0 carbons are generally dietary acids. In the rumen unsaturated fatty acids are biohydrogenated. Therefore the profile of FA present in

milk and tissue is more saturated than those in the diet. Short chain fatty acids are usually synthesised within the mammary gland [Akers, 2002]. Milk fatty acids are synthesised in the cytoplasm surface of the smooth endoplasmic reticulum. Precursors for *de novo* fatty acids are acetate and β -hydroxybutyric acid (BHBA). BHBA is present in the first four carbons of fatty acids. Acetate provides carbon for shorter fatty acids C4-C14 and some C16. NADPH is generated from the pentose phosphate or Krebs cycle. Malonyl CoA adds another two carbons to a fatty acid chain.

Most of the milk FA are present in trace quantities and only fifteen exceed the level of 1% of total saturated FA. Saturated FA represent 70% by weight of the total of all FA. Palmitic acid (16:0) comprises approximately 30% by weight, myristic acid (14:0) 11%, stearic acid (18:0) 12% [MacGibbon and Taylor, 2006]. The mono-saturated FA add up to 21% by weight with oleic acid (18:1) accounting for 24% by weight. Linoleic acid (18:2) and linolenic (18:3) comprise only 4% by weight of the total fatty acids [Mansbridge and Blake, 1997]. The majority of fatty acids in milk fat have a straight chain structure that is saturated and have 4 to 18 carbons (4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0), monounsaturated fatty acids (16:1, 18:1), and polyunsaturated fatty acids (18:2, 18:3) [Jensen, 2002; Jurczak, 2005; Fox and McSweeney, 2006].

The variation and the amount of FA in milk is associated not only with diet i.e. energy intake and dietary fats but with other factors such as season of the year, and other factors such as genetics (breed, selection), stage of lactation, rumen fermentation, and health status (mastitis) [Palmquist, *et al.*, 1993; Jensen, 2002].

The pathways of the process of the synthesis of the milk are very fragile and they depend on many factors, e.g. availability of micro elements and enzymes or pH. Greater understanding of these cycles will allow us to influence cellular activity in such ways as increasing or decreasing production of the component. However these processes also depend on health status of an animal, on the amount of stress they are put under, on the nutrition, the number on days in milk, age [Sanford *et al.*, 1986, Jenkins and McGuire, 2006; Huxley, 2007].

2.1.4 Particulate fraction of the milk and its relationship to mastitis

Milk is a biological fluid which has very high particle content. Milk coming from healthy cows contains at least three types of components: 1) “foreign” materials such as udder cells, leukocytes and bacteria, 2) milk fat globules, 3) colloidal particles of casein complex [Morton, 1953]. Durand *et al.* (2003) divided milk particles into two groups based on size, those greater than 3 μm (mostly milk fat globules) and those smaller than 3 μm such as proteins and other cellular material.

In respect of so-called foreign particles normal cow’s milk contains up to 10^5 somatic cell/ml and most viable cells are macrophages and lymphocytes. Mastitis increases this number to more than 10^6 cells/ml and changes the proportion of cells (95% of neutrophils) [Kehrli and Shuster, 1994]. Somatic cells are described in more detail in section 2.4.2.

Fat is present in milk in the form of triglyceride micro droplets – milk fat globules surrounded and stabilized by a biological membrane. Fat globules are the main particles of milk. The milk fat globule membrane (Figure 2-3) is composed of phospholipids, glycolipids, proteins, lipoproteins and enzymes. MFGM is a tripartite structure and originates from the apical membrane and endoplasmatic reticulum [Wooding, 1971; Heid and Keenen, 2005]. The function of MFGM is to protect milk fat globules from lipolysis and oxidation which causes surfacing of fat, decrease concentration of FFA and generation of off-flavours in dairy products [Mulder and Walstra *et al.*, 1974; Mehaia, 1995; Wiking, *et al.*, 2003].

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Figure 2-3 Structure of the milk fat globule membrane.

Adapted from Evers (2004).

Milk fat lipids are synthesized on the smooth endoplasmatic reticulum (ECR). Triacylglycerols may cumulate in the ECR causing the bulge of membrane which would drive the opposite monolayers apart. Eventually fat droplets surrounded by cytoplasmic reticulum derived phospholipids monolayer are pinched off into the cell's cytoplasm [Heid and Keenan, 2005]. During their transport through the cytoplasm these droplets grow in size by fusing with each other. Bargman and Konop (1959) first reported observation of lipid droplets approaching the apical plasma membrane until progressive envelopment. Completely enveloped fat droplets detach from the cell into the milk canal. This is the widely accepted mechanism of milk fat globule secretion [Heid and Keenan, 2005]. The mechanism controlling fusion of fat droplets and milk fat secretion is still unknown. The size distribution of fully formed MFG varies between 0.2 to 20 μm , with the average size diameter as 3 μm [Mulder and Walstra, 1974; Mehaia, 1995; Michalski *et al.*, 2002; Michalski, *et al.*, 2003]. Walstra (1969) suggested that MFG comprised three groups. The first group contains the small globules with diameter smaller than 1 μm which includes about 80% of the total number of fat globules. The second group contains a middle-size population (about

90% of total fat). The third group contains the largest globules – greater than 8 μm . The above segregation is arbitrary and has no biological or physiological meaning; however it has been referred to by other researchers. This study refers to volume median diameter which has better potential to characterise milk fat globules quality characteristics, e.g. to monitor stability, sensitivity to external and internal parameters, and prediction of change.

Milk fat globule size is linked to the composition of milk fat secreted and the concentration of long chain fatty acids in milk [Wiking *et al.*, 2003; Wiking *et al.*, 2004] as well as milk yield and protein content [Couvreur *et al.*, 2007]. The correlation between characteristics of MFG, their chemical structure and nutritional impact has not been studied in depth. Milk containing a higher number of small MFG requires more membrane material than large MFG, showing a positive correlation between quantity of membrane and composition of secreted milk. Research undertaken by Wiking *et al.*, (2004) indicated that an increased fat synthesis does not affect all subpopulations of MFG in the same way. This suggests that the mechanism of enveloping small and large droplets is not the same, explaining the different abilities to synthesise MFGM.

A number of studies have indicated that size and number of MFG are influenced by the environmental and physiological factors such as diet, stage of lactation, age and health status [Walstra, 1969; Mehaia *et al.*, 1995; Attia *et al.*, 2000; Cardak *et al.*, 2003; Wiking *et al.*, 2004]. The effect of mastitis on the milk yield and quality of milk has been widely studied and the conclusion is that the cow udder quarter most affected by mastitis yields less milk with the greatest changes in composition than the corresponding non-affected quarters. Research monitoring MFG dynamics during lactation cycle started in the 1960s. Thanashi (1965) and King (1969) determined the influence of mastitis on MFG size. They obtained results indicating decreased size and number of MFG during mastitis. It was suggested MFG are the main particles in milk, changes in their number and distribution during mastitis could become a new diagnostic tool for detecting and/or indicating this disease. The methodology available at the time of both researches was based on direct microscopy only, used technique was very time consuming and could not have been verified by any other methods.

Casein particles exist as a mix of proteins and salts combined in colloidal complex. According to Eigel *et al.*, (1984) caseins participated from milk by acidification to pH 4.6 at 20°C. The size range of casein particles extends from 200 nm diameter to less than 30 nm [Morton, 1953] and they cannot be seen by particle counter and light microscopy.

Technique used in this study allows to monitor and record changes in the distribution of milk particulates in foremilk samples. MFG are the main particles in milk, therefore any observed changes during the monitoring period can be linked to MFG and their synthesis. Since 1970's mastitis and its impact on milk composition was closely studied. However, its impact on the synthesis of MFG – main milk particles and their behaviour throughout the inflammation is not fully known. Particle counting and sizing technology gives an opportunity to bring new evidence regarding physiology of mammary gland at the cell level.

2.2 Morphology and physiology of mammary gland

2.2.1 Mammary gland anatomy

The udder is derived from a sweat gland and the inside lining of the teats and ducts consist of modified skin [Blowey and Edmondson, 1995]. The udder consists of two types of tissue: parenchyma which is involved in milk synthesis and secretion, and the surrounding connective (stromal) tissue responsible for providing passage for blood and lymph vessels. In ruminants the mammary gland is clustered together into groups of two e.g. sheep and goat, or into four e.g. cattle to create the udder. Each gland has its own teat and there is no milk flow between quarters. Anatomically the four quarters are separate and independent units. Therefore on many occasions herdsmen dried out one of quarters without any disturbance to the milk production by other quarters.

The teat has a single canal leading into the teat cisterns, which are separated from the gland by a fold of tissue. The purpose of this barrier is to increase defence against bacteria entry. The gland cisterns are connected to ducts draining milk from the secretory cells.

Support for the udder is provided by flat suspensory ligaments attached to the pelvic bone and tendons of the abdominal muscles in the pelvic area. Suspensory ligaments separate the udder vertically and provide most of the strength to hold the udder. The suspensory ligament consists of a superficial lateral ligament, a deep lateral ligament and the median ligaments. Fibres of the lateral ligaments surround both sides of the udder which is supported by connective tissue. Suspensory ligaments divide the udder into two halves left and right. However there are no anatomical barriers or direct connection between front and back quarters. The mass of udder tissue and milk stored within it, just before milking could weigh around fifty to seventy kilograms. The ageing process, over-engorgement and/or oedema of the udder can cause degradation of the fibres of the suspensory ligament which might reduce support and affect the shape of the udder.

Blood supplies the nutrition necessary for milk synthesis and carries away all metabolic waste. Average daily amount of the blood flowing through the udder is approximately 17,500 litres. Production of one litre of milk requires 400 litres of blood flowing through the udder. The udder is supplied by arterial blood; this continues to subdivide into internal and external iliac artery. The blood is drained from the udder by the external pubic vein (drains the blood from the cranial and caudal branches of the mammary vein) and milk vein (drains from the anterior basal border of the udder) [Adkinson, *et al.* 1993; Blowey and Edmondson, 1995].

Of the cow's four teats, the hind ones produce 60% of total milk yield. The teat length varies from 3 cm to 14 cm with a diameter from 2 cm to 4 cm. There are four layers forming the teat wall; the epidermis, the dermis, the muscles and the teat cistern lining. The external layer of the epidermis is built of dead keratinised cells; internal – deep epidermal pegs. The dermis has numerous blood vessels and nerve tissue. The teat consists of various muscles set in the dermis which maintaining tight closure of the teat opening and shortening the overall time of teat opening after milking which limits bacteria penetration. Double layers of cuboidal cells constrict the cistern lining. These cells have ability to move slightly during bacterial infection to allow white blood cells enter the gland [Blowey and Edmondson 1995, Nickerson and Akers, 2002].

Udder characteristics e.g. large teats, flat ends, low udder could be associated with mastitis rate as they can influence the chance of the injury followed by exposure to mastitic pathogens (Rogers and Spencer, 1991). According to Seykora and McDaniel (1985) animals with firmly attached udders are less susceptible to mastitis than those with pendulous udders. The shape of the teat's end is also associated with mastitis frequency. However no agreement was found about the correlation between the other teats traits and mastitis prevalence.

2.2.2 Morphology of the mammary gland and milk secretion

Secretory tissue in the mammary gland is arranged in lobes, each is composed of lobules. Each lobule is built of groups of alveoli surrounded by blood vessels. Every alveoli consists of a single layer of epithelial cells (Figure 2-4).

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Figure 2-4 Mammary alveolus.

Adapted from McManaman and Neville (2003).

The alveolus are surrounded by myoepithelial cells, which when stimulated by the hormone oxytocin produce contractions leading to milk ejection. Myoepithelial cells

are attached to the connective tissue basement membrane [<http://animsci.agrenv.mcgill.ca/courses/460/topics/3/text.pdf>, McManaman and Neville, 2003]. Epithelial cells show high activity during lactation and have cytoplasm filled with numerous mitochondria and endoplasmic reticulum. The precursors of milk components are taken from the blood stream into the cell through a basal and lateral membrane. Cells are connected to each other via a tight junction structure, which does not allow the passage of precursors and/or components of milk under normal conditions. The mammary gland has five pathways for milk secretion as illustrated in Figure 2-5 [McManaman and Neville, 2003]. The exocytotic pathway (I) involves the major milk proteins, water, citrate, lactose, and calcium. Those substances are packaged into Golgi apparatus and transported to the apical region of the cells [McManaman and Neville, 2003]. During the lipid secretory pathway (II) milk fat precursors are acquired at the basolateral membrane. Milk fat lipids are synthesized in the smooth endoplasmic reticulum and form into a structure called small droplet and/or lipids body [Wooding, 1971; Mather and Keenan, 1998; McManaman and Neville, 2003]. It has been reported that small droplets fuse with each other on the way towards the apical membrane. However, Wooding (1971) did not observe this process. The apical membrane envelops droplets and form milk fat globules (MFG) which are then pinched off into the lumen [Wooding, 1971; Mather and Keen, 1998; McManaman and Neville, 2003]. The transcytotic pathway (III) depicts vesicular transcytosis of proteins such as immunoglobulin. Pathway (IV) includes transport monovalent and polyvalent ions of small molecular size (e.g. glucose, amino acids). Cellular transporters for this pathway are the Golgi apparatus and secretory vesicle membrane. [McMananan and Neville, 2005]. The paracellular transport pathway (V) is the passage between cells through tight junctions. During mastitis tight junctions are more “open” causing leaking across the blood-milk barrier [McMananan and Neville, 2005].

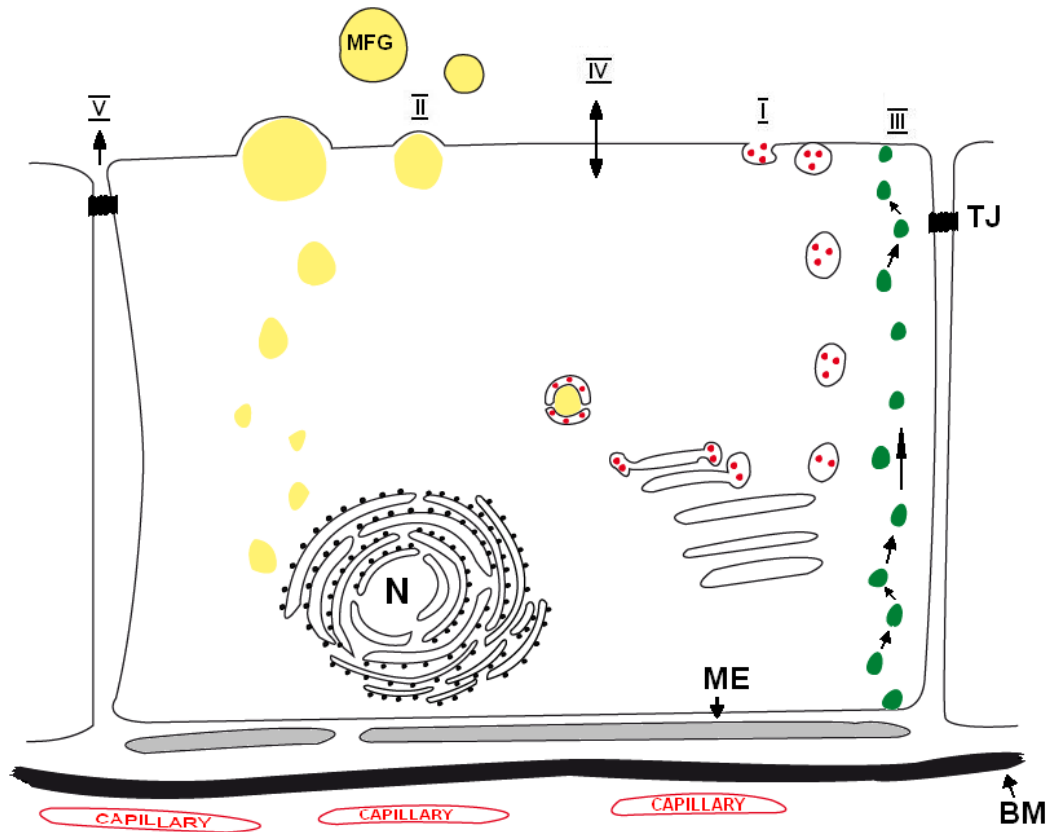


Figure 2-5 Alveoli epithelia cell and pathways for milk secretion.

Abbreviations: TJ, tight junction, ME, myoepithelial cell, N, nucleus, BM, basement membrane. Pathway I secretion of milk proteins, lactose, calcium, and other components of aqueous phase of milk (from Golgi Apparatus); Pathway II secretion of MFG by envelopment in apical plasma membrane; Pathway III transcytosis of proteins such as immunoglobulins from the interstitial space; Pathway IV transport of monovalent ions, water, glucose; Pathway V is active only during pregnancy, involution and inflammations such as that caused by mastitis. Adapted from Mather and Keenan (1998), McManaman and Neville (2003), Heid and Keenan (2005)

The milk produced by epithelia cells is accumulated in alveoli and cisterns. Milk ejection or milk let-down occurs when an active pressure is put on the cisterns. The total volume of milk stored in cistern does not exceed 20%; this is for 12 h milking interval [Pfeilsticker et al., 1996]. Milk is increasingly transferred to cisterns several hours before milking [Knight et al., 1994] and shortly after milking almost all

cisternal fraction is removed [Knight et al., 1994; Bruckmaier and Hilger, 2001; Ayadi et al., 2003]. According to Bruckmaier et al., (1994a, 1994b) there is a positive correlation between the size of cisterns and the number of lactation; the oldest cows were found to have the largest cisternal fractions. Milk ejection is induced by the release of sufficient concentration of neuropeptide oxytocin which causes contraction of the myoepithelial cells surrounding epithelia cells. The secretion of this hormone is stimulated by the sucking of the calf and hand or mechanical milking. The stimulation process may take from forty seconds to two minutes, therefore it is very important that sufficient time has been given before the milking process start (especially in the late stage of lactation) to avoid milking being performed on empty teats. Bruckmaier and Blum (1992) pointed out this may cause cavities to collapse and reduce milk-ability during the remainder of milking. Moreover according to Johansson *et al.*, (1952) milk fat content continuously increases in the milk fraction during the milking. Hence as suggested by Tancin *et al.*, (2007) the optimization of milk flow will positively influence the distribution of milk constituents in milk fractions during milking. In summary, the inefficient release of oxytocin and/or too early milking may affect the milk composition.

2.3 Mastitis in dairy farming

2.3.1 Definition of mastitis

Mastitis is defined as the inflammation of the parenchyma of the mammary gland caused by microorganisms that invade the udder, multiply, and produce toxins that are harmful to the gland. According to Bramley, *et al.*, (1996) the term mastitis is derived from the Greek word *mastos*, meaning “breast” and *itis*, meaning “inflammation of”. Despite considerable knowledge about mastitis and its aetiology, this disease is still prevalent in many dairy herds, it remains most difficult to eradicate or control, and it has a great negative financial impact on the dairy industry worldwide [Halasa *et al.*, 2007]. These losses are caused by several factors such as a decrease in the total milk

output with marked compositional changes in the milk (a reduction in the quality and industrial usability), cost of treatments, labour costs, and increasing the chance of premature culling of cows [Halasa *et al.*, 2007]. Sub-clinical mastitis is a condition with no visible, external changes in the appearance of udder and milk. This condition causes the milk yield to decrease, bacteria and somatic cells population to increase in the secreted milk and its composition to change. The symptoms of clinical mastitis are swelling of the gland, udder pain, hardness, elevated temperature, lethargy and inappetence or anorexia. Clinical disease also may affect the quality and the quantity of milk [Eberhart *et al.*, 1987; Blowey and Edmondson, 1995; Bramley *et al.*, 1996].

The sudden onset of disease and severe signs such as swelling, pain and abnormal milk characterise acute mastitis where the condition is persistence, but with no severe symptoms it is termed chronic mastitis. [Blowey and Edmondson, 1995; Bramley *et al.*, 1996].

2.3.2 Characterisation of pathogenic microflora

There are many causes of mastitis including micro-organisms and associated toxins, physiological trauma and chemical irritants. However pathogenic microflora is the main causative agent [Bramley *et al.*, 1996]. There are approximately 200 pathogenic microorganisms that have been implicated. These can be divided into two distinct classes: firstly, contagious e.g. *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and secondly environmental e.g. *Streptococcus uberis*, coliforms (*E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*), *Bacillus cereus*, *Bacillus licheniformis*, fungi and yeasts [Harmon and Langlois, 1986; Blowley and Edmondson, 1995]. For contagious pathogens an infected udder is the major reservoir. Some of those bacteria possess very strong adhesive properties, enabling growth into the gland wall. The source of environmental pathogens and contamination is the environment of the dairy cattle e.g. digestive tract of cattle or their surroundings such as faeces, soil, bedding material, manure and soil [White and McDermott, 2001; Collins *et al.*, 2010; Viridis *et al.*, 2010]. The first group of micro-organisms tend to cause periodic clinical mastitis. Between 70% - 80% of the individual cases of infections in UK are caused by coliforms, 50% of environmental streptococci may produce cases of clinical mastitis. While contagious infections occur

during the milking procedure, environmental infections develop between milking. The reservoir for *S.aureus* is the mammary gland. Infections caused by staphylococci are difficult to treat and once established very hard to eliminate. *S.aureus* is able to exist and survive within macrophages such as polymorph nuclear neutrophil (PMN) which protects bacteria from circulating antibiotics. However PMN cannot survive for long periods in the outside environment and they are transmitted from one cow to another during the milking process and/or the hands of milkers [Harmon, 1994]. Many strains of *S.aureus* can produce beta-lactamase which may make them resistant to some types of penicillin. *Escherichia coli* is the most common pathogen causing mastitis [Blowey and Edmondson, 1995]. The most common symptoms of *E. coli* mastitis are hard, hot swollen quarters and watery discharge. *E. coli*, unlike *S. aureus*, does not adhere to the epithelial cells in the teat and gland cisterns. These bacteria release an endotoxin – lipopolysaccharide (LPD) [Bramley, 1981; Nickerson and Heald, 1981; Blowey and Edmondson, 1995; Sol, *et. al.*, 2000; Olviedo Boyso *et.al.*, 2007, Burvenich, *et. al.*, 2003].

2.3.3 Methods of detection

Effective mastitis control strategies ideally need to be based on early detection. This allows a better cure rate and facilitates proactive instead of reactive management strategies to minimise the negative effects of disease [Fricke, 2002; Deluyker *et al.*, 2005]. Unfortunately new trends of herd management, such as an increase in the number of cows per labour unit results often in decreased time and attention given to each cow [Lucy, 2001]. Enumeration of somatic cells in milk is the most popular technique to monitor and diagnose mastitis. SCC indicates the presence of infection, its severity and stage. SCC in milk became the world standard for mastitis diagnosis [Dohoo and Leslie, 1991]. Currently the counting of somatic cells by microscope using methylene blue staining is the reference method for direct estimation of these cells. Unfortunately this method is time-consuming and requires trained staff [International Dairy Federation, 1995]. Somatic cell levels can also be measured by using indirect methods: California Mastitis Test (CMT) or Wisconsin Mastitis Test (WMT); and direct methods: Coulter Milk Cell Counter (which counts particles as they flow through electric field), Fossomatic (counts fluorescent dyed cells) and

Somascop (Delta Instruments Drachten, the Netherlands, based on flow cytometry) [Miller *et al.*, 1986; Moon *et al.*, 2007].

CMT and WMT are relatively simple tests detecting mastitis. They do not give a numerical result, just an indication of whether the count is high or low. These tests are cheap and give results immediately for a single quarter. Negative results are obtained when milk, after mixing with reagent, remain watery and highly positive when milk remains in gel form [Blowley and Edmondson, 1995; Bramley *et al.*, 1996, Viguier *et al.*, 2009]. However these methods cannot be used in automatic milking systems, which require fast and automated measurement techniques providing correct data for mastitis control.

Clinical and subclinical mastitis affects milk composition. Changes in milk's physical properties can be used as indicators of mastitis. EC depends on the concentration of ions such as sodium, potassium, calcium, magnesium, chloride and others. Ionic balance is changed during mastitis due to the tissue damage – leaking of components across blood-milk barrier [Wheelock *et al.*, 1966]. Increase of EC to 6.0 milisiemens (mS)/cm indicates pathogenic changes in the udder [Linzel *et al.*, 1974]. However according to Spakauskas *et al.*, (2006), EC does not always correspond to SCC. Prentice (1961) observed that fat content is an important factor affecting EC. According to Fernando *et al.* (1981) EC changes through different fraction of milk, with the highest levels in stripping fraction for healthy and mastitic milk. Results obtained by Woolford *et al.*, (1998) suggest that the first foremilk samples give the highest diagnostic sensitivity for EC. Claycomb *et al.* (2009) reported that the measurement of EC from foremilk in individual quarters has greatest sensitivity of detection of clinical mastitis than convention EC measurement in clusters. Hamann and Zecconi (1998) pointed out that foremilk samples from healthy quarters have mean value for EC in the range 4.9-6.4 mS/cm, when the range for quarters diagnosed with clinical mastitis is over 8 mS /cm.

The presence of mastitis may be correlated to amount of L-lactate dehydrogenase (LDH). A model based on the measurement of concentration of LDH was able to detect mastitis on average 3.5 days earlier than when clinical mastitis occurred. Sensitivity of LDH was greater than 80% and specificity greater than 99% [Chagunda *et al.*, 2006].

Karp and Petersson-Wolfe (2010) described the AfiLab system which measures the concentration of fat, protein and lactose for Holstein, Jersey and crossbred breeds. Results indicated that milk component data may be an effective way of identifying disease on its early stage. Centrifugal micro-fluidic devices for determination of protein content (Barry *et al.*, 2010) and SCC and fat percentage (Gracia – Cordero *et al.*, 2010) have been introduced as a rapid, low cost, on side method for farmers. Both methods use CD-based centrifugal microfluidic device containing microfluidic chambers with the capacity to perform a single test. The Orange G dye solution was used to determine protein content when sedimentation procedure and microscopes were used to estimate SCC and fat percentage. Both methods required a low volume of sample and allowed analysis of eleven to twelve samples at once.

Tsenkova *et al.* (2001) reported using near-infrared spectroscopy as a new tool to detect mastitis where SCC determination is based on the related changes in milk composition.

Early detection of mastitis and treatment is crucial to minimise damage caused to the epithelial cells inside the udder and reduce the possibility of spreading contagious pathogens during the milking process. The response to antibiotic treatment depends on the species of bacterial pathogen involved with the mastitis outbreak. An example can be Coliform infection, as pointed out by Wilson *et al.*, (1999), which have a high self cure rate when antibiotics have not been used. However, conventional bacterial culturing methods (e.g. Petrifilms) are slow (22-24 hours) and cannot be used as onside test on dairy farms. Koskinen *et al.*, (2010) indicated use of real time Polymerase Chain Reaction (PCR) as a diagnostic tool for identification of mastitis pathogens and helping to choose the right antibiotic therapy. Non-coding RNA e.g. ribosomal RNA, messenger RNA can be used for bacterial strain typing. Clancy *et al.*, (2010) reported use of messenger RNA in post-labelling sandwich assay from mastitic milk to detect *E.coli*. This method allowed detecting *E.coli* after two hours of incubation, with limit of detection 5×10^6 bacterial cell/ml. However there is a concern that the very high sensitivity of PCR test might produce false positive results for samples from healthy animals.

Whyte et al., (2003) suggested that changes in the concentration of lactate, acute phase proteins (APP) can be used by on-line sensors for mastitis detection. Lactate is a metabolite of growing bacteria. Milk lactate concentration is increased during mastitis and its one of the very earliest indicators of the presence of bacteria [Davis, *et al.* 2004]. APP are produced in an acute phase response to inflammation, trauma and disease. They are an extensive group of proteins regulating immune response to inflammation by functioning as mediators and inhibitors of inflammation, transporting proteins, and take part in tissue repair. According to Whyte *et al.*, (2003) in veterinary medicine APP used to monitor the physiology of animals include haptoglobin, serum amyloid A and C-reactive protein. Milk amyloid A is synthesised in the udder and its levels increase before SCC reach critical levels [Eckersall *et al.*, 2001].

The Coulter Counter is an instrument designed for counting and sizing particles and cells by measurement of changes in electrical conductance of a small aperture during the fluid flow. This method has a variety of applications in microbiology, (counting cells and microorganisms), food manufacture and quality control. Also the Coulter Counter has been widely used to determine SSC in foremilk samples from healthy and inflamed udder quarters (IDF Standard 148, 1991). This technology allows continuous counting and sizing of particles. Coulter consists of a number of microchannels which are divided into two separate chambers containing electrolyte solution. Particles flowing through change the electrical resistance of the liquid. These changes are recorded and translated into electric current or voltage pulses which correlate to size, mobility or number of particles. Successful use of coulter counter in detecting mastitis has been described by Frohling *et al.* (2010). An increase of somatic cells was recorded during inflammation with a peak of particle size between 8 and 12 μm .

Above review indicates that the dairy industry urgently needs new tool which will allow early detection of pathogenic changes within udder in timely manner.

2.3.4 Influence of mastitis on milk composition

There are a large number of factors affecting the composition of milk in a commercial dairy farming operation such as: age, breed, nutrition, stage of lactation, genes, stress and other environmental variables [Bansal *et al.*, 2005, Sheldrake *et al.*, 1983], but one of the biggest impact on milk composition is mastitis [Wielgosz-Groth *et al.*, 2003]. Major changes in milk composition and its bacterial properties were observed during the early stages of inflammation [Sordillo *et al.*, 1987]. These changes reflected degree of damage of cellular permeability and stability of milk – blood barrier as well as severity and type of infection [Kitchen, 1981]. Figure 2-6 illustrates changes to milk composition caused by mastitis.

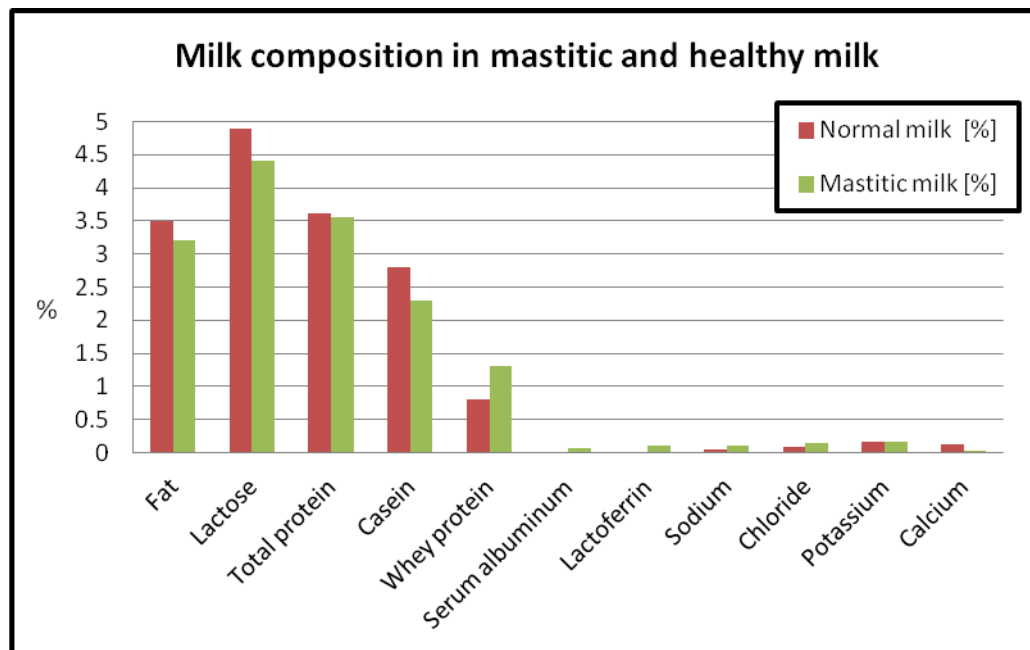


Figure 2-6 Changes in milk composition associated with mastitis. (adapted from Bramley *et al.*, 1996)

Lactose is the main sugar and the osmotic regulator in milk. Reduced concentrations of this sugar in mastitic milk is connected to its leaking from the milk into the blood stream [Kitchen, 1981; Shuster *et al.*, 1991; Auldism *et al.*, 1995; Auldism and Hubble, 1998; Fernandes *et al.*, 2004]. This sugar can be synthesised only in the mammary gland tissue, however, concentration of lactose is detected in the blood and urine of animals diagnosed with mastitis.

In the literature there are different opinions about the influence of mastitis on the total fat content [Shuster, *et al.*, 1991; Auldism *et al.*, 1995; Auldism and Hubble, 1998]. Kitchen *et al.*, (1978), Schultz (1977), Auldism and Hubble (1998), Wielgoszcz-Groth (2003) have all reported decreases in concentration of fat when this inflammatory condition exists. Lower concentration of fat in milk could be linked to the reduced synthetic and secretory capacity of the mammary gland. Changes in milk quality may enhance the activity of milk lipoprotein lipase (mLPL) causing damage to MFGM. This enzyme is present in raw milk but is able to hydrolyse only under certain conditions. The activation process of this enzyme- redistribution mLPL from casein fraction and bound with fat - may be switched on by changes in pH and temperature according to results obtained by Deeth and Fitzgerald (1976), Azzara and Dimick (1985b). Moreover, leukocytes invading the mammary gland are able to engulf MFG what could decrease numbers of MFG and increase concentrations of FFA [Harmon and Heald, 1982; Azzara and Dimick, 1985a]. Milk containing a high level of SC is more susceptible to lipolysis, oxidation of fatty acids and breaking down of triglyceride structures in MFG [Auldism and Hubble, 1998]. However Shuster *et al.* (1991) and Pyoralo (2003) recorded contradictory results. There is a suggestion that fat synthesis is only slightly affected by inflammation, but the high decrease of the total volume of milk increases the fat proportion. Moreover Murphy *et al.*, (1988) found no effect of mastitis on milk lipase activity. They suggested that elevated lipolysis is linked to increased susceptibility of the milk fat to this activity.

The amount and proportion of milk proteins change during mastitis as illustrated in above figure. Inflammation, by influencing the vascular permeability, causes leaks of serum proteins into milk. Concentration of serum albumin, immunoglobulins and transferrins is elevated [Auldism *et al.*, 1995; Auldism and Hubble, 1998; Jones and Bailey, 1998; Shuster, *et al.*, 1991]. Decrease in the level of casein is due to post – secretory degradation by exogenic enzymes and the reduction in its production as a result of damage to gland tissue [Auldism *et al.*, 1995; Auldism and Hubble, 1998].

The balance of type and concentration of enzymes is changed. Increased levels of lactoferrin during mastitis might be connected with the antibacterial properties of this protein. Lactoferrin concentration in normal milk can be increased up to thirty times during mastitis [Harmon *et al.*, 1976]. Damage to gland tissue and the blood-milk barrier result in elevated concentration of plasmin. This enzyme is derived from

plasminogen which originates in blood [Auldism and Hubble, 1998; Shuster, *et al.*, 1991].

Potassium is the predominant mineral in milk and its concentration decreases during mastitis. In contrast sodium and chloride levels increase during mastitis. This process is associated with tissue damage to the blood and milk interface. Milk with high SCC contains a lower level of calcium due to disruption of casein synthesis. The abnormal concentrations of minerals increase conductivity and pH of milk [Bramley *et al.*, 1996; Auldism and Hubble, 1998; Wielgoszcz-Groth, *et al.*, 2003].

The number and secretory activity of mammary gland epithelial cells determine productivity during lactation. Increased milk production during early lactation is an effect of the high activity of secretory cells. During late lactation low milk yield is a result of declining cell number and activity [Capuco *et al.*, 2003]. However inflammation of the mammary gland changes milk production significantly [Kitchen, 1981]. Lower milk production during mastitis is the result of the physical damage to gland tissue caused by pathogens, their toxins or inflammation mediators. These factors disturb milk synthesis by damaging secretory cells, interaction with substrates for milk synthesis or by hormonal changes altering the metabolic activity of milk producing cells. Lower milk yield can be a result of increases in permeability of the blood – milk barrier and leaking of milk components from the udder [Shuster *et al.*, 1993, Bansal *et al.*, 2005]. Sordillo (*et al.*, 1989) reported that tissue from quarters infected with *Staphylococcus aureus* had less alveolar area and the cells contained lower number of organelles associated with milk production when compared with tissue from uninfected quarters.

2.4 Defence mechanisms against mastitis

The mammary gland is an organ designed to provide nutrition and its tissue plays a central role in the immune system by protecting from and responding to infections like those of pathogenic microbes. Pathogen attack is defended by combined anatomical, cellular and soluble factors. This triple mechanism works in coordination to increase resistance and susceptibility to inflammation. To support this natural system the current practice of mastitis control and prevention is based on milking hygiene, decreasing time of exposure to environmental pathogens, controlling the

spread by vectors and reducing reservoirs e.g. dry cow antibiotics therapy, post-milking teat disinfection and culling [Sordillo *et al.*, 1987; Oviedo-Boyso *et al.*, 2007].

The immunology defence strategy in gland tissue can be divided into two groups: innate immunity (non-specific responsiveness), and specific immunity, the balanced coordination of which helps to provide optimal protection from disease.

2.4.1 The anatomical defence of mammary glands

Mastitis occurs when bacteria enter the mammary gland through the teat canal and multiply in milk. [Eberhart *et al.*, 1987]. The teat is the first line of defence. Its end is built of sphincter muscles which keep it closed during the interval between milking [Murphy and Stuart, 1953]. The teat canal is lined with keratin and the structure and chemical content of this protein provides anti-microbiological activity. Keratin can entrap pathogens before they enter the gland and it contains bacteriostatic proteins and fatty acids [Treece *et al.*, 1966; Hibbitt *et al.*, 1969]. Capuco *et al.*, (1992) removed keratin from the teats of dairy cows and exposed them to bacterial challenge using *Staphylococcus agalactiae*. The rate of infection in quarters when keratin was removed was greater when compared with control animals with normal levels of keratin. This indicates the critical role of this protein as a barrier against infection.

2.4.2 The cellular defence of mammary gland

When the anatomical defence fails and the bacterial pathogens penetrate the teat sphincter, cellular defence is the next necessary step to overcome infection. Somatic cells are those containing several types of leukocytes or white blood cells which include neutrophils, macrophages, lymphocytes and epithelial cells. During mastitis the number of somatic cells/ml of milk may increase from $< 10^5$ / ml to over 10^6 /ml within ten hours [Pappe and Weinland, 1988; Persson *et al.*, 1992]. The main peak of SC concentration appears in early inflammation [Shuster and Harmon, 1991; Lauzon *et al.*, 2006]. When the infection appears, somatic cells move rapidly from the blood stream to the mammary gland as the immune system responds. Bacterial enzymes, toxins and cell-wall components may have a direct effect on the function of mammary glands – attracting somatic cells - as well as stimulating the production of numerous

mediators [Sordillo *et al.*, 1997]. Pathogen toxins activate white blood cells and epithelial cells to secrete cytokines that recruit PMN whose main function is phagocytosis [Pappe *et al.*, 2003]. After the elimination of bacteria the SSC return to normal levels. These two migration ways of leucocytes and release of enzymes are correlated to the morphologic destruction of parenchyma tissue resulting in decrease of milk production. Firstly the duration and secondly the frequency of somatic cells migrations have a major impact on the quality and the quantity of milk [Harmon and Heald, 1982; Sordillo *et al.*, 1987; Sordillo and Nickerson 1988; Sordillo *et al.*, 1989].

The major line of defence for the mammary gland is the influx of polymorphonuclear neutrophil (PMN) leucocytes to the udder tissue. Neutrophils are the non-specific, predominant cell type during early lactation constituting over 90% of leucocytes, uniform in size with diameter between 14 μm to 15 μm [Sordillo *et al.*, 1987, Sordillo *et al.*, 1989, Sordillo *et al.*, 1997]. Single PMN contain polymorphic segmented nucleus, numerous cytoplasmic granules (providing constituents for killing bacteria), glycogen (energy source) and highly convolute surface and formation of intracellular phagocytic vacuoles [Pappe *et al.*, 2003]. On the outside surface of PMN receptors are present that function by detecting chemoattractants (which lead towards infected areas), adhesion to provide movement, binding immunoglobulin, components, bacteria and toxins [Pappe *et al.*, 2003]. During infection PMN adhesion to vessel walls increases significantly. Chemical messengers or agents released from leucocytes or from damaged tissue attract PMN into milk in an abnormally large number [Craven and Williams, 1985; Dosogne *et al.*, 2003;]. According to Bramley *et al.* (1996) rapid response of PMN can be observed in milk after a 12 to 24 hour lag after initial infection. Neutrophils can take part in immune-response against infection as they phagocytise and kill bacteria by setting off a series of immune responses – production of reactive oxygen species (superoxidase ions, hypochlorite ions, hypochlorous acid and hydrogen peroxides), low molecular weight antibacterial peptides [Sordillo *et al.*, 1997; Sordillo, *et al.*, 2002; Mehrzad *et al.*, 2002; Pappe *et al.*, 2003]. At the same time PMN attack other particles of milk such as milk fat globules and casein causing decrease of their density in milk during infection. Bacterial toxins, enzymes and other mediators of inflammation damage gland tissue and this results in reduced synthesis of milk and a leakage of blood components into the milk. Very often clots formed from leucocytes block small ducts and stop milk

removal causing the formation of scar tissue or even permanent loss of function of that part of the gland [Craven and Williams, 1985; Eberhart *et al.*, 1987; Shuster and Harmon, 1991; Shuster *et al.*, 1993; Burvenisch, *et al.*, 2003]. However infection may subside and tissue repair may occur bringing function back in the same lactation or the subsequent one [Harmon, 1994]. Activity of PMN during infection can be measured by chemical luminescence reaction based on oxidation of the H₂O₂ that is released during PMN activity [Allen, *et al.*, 1972].

The milk PMN has much less activity than blood PMN, which could be result of coating by milk casein, engulfing MFG and casein instead of bacteria, and/or low oxygen level in milk [Blowey and Edmondson, 1995]. When neutrophil activity is not sufficient and pathogens survive their infiltration, they are replaced with T and B lymphocytes and monocytes. However, neutrophil defence is highly effective and PMN are still the most important type of cell in immune response of the mammary gland [Rainard and Riollot, 2003].

Lymphocytes are able to recognise antigens through membrane receptors – specific for pathogens. Two types of lymphocytes T and B differ in function. Lymphocytes type T recognize invading pathogens while lymphocytes type B produce antibodies against them. Ttype T are classified in two groups T $\alpha\beta$ (Cd4T-helpers; CD8-suppressors) and T $\gamma\delta$ [Oviedo-Boyso, *et al.*, 2007, Sordillo, *et al.*, 2002].

Macrophages are the predominant cell type in milk from non-infected udders [Lee *et al.*, 1980 from Craven and Williams, 1985]. Tissue and blood macrophages recognize and phagocytise pathogen [Boulanger *et al.*, 2003; Stein *et al.*, 2003]. Macrophages release cytokines and leukotrienes which stimulate migration of PMN into the milk during infection.

2.4.3 The soluble defence mechanisms of the mammary gland

The mammary gland soluble defence combines three isotypes of immunoglobulin IgG (IgG1 and IgG2), IgA and IgM [Butler, 1969; Devery-Pocius and Larson, 1983] and cellular factors include: lactoferrin (LF), complement, lysozyme and the lactoperoxidase-thiocyanate-hydrogen peroxide system.

Four classes of immunoglobulins (IgG, IgG₂, IgA, IgM) are produced by B lymphocytes. Their concentration depends on physiological condition of udder, stage

of lactation, permeability of secretory tissue and level of activity of B lymphocytes [Sordillo *et al.*, 1997, Sordillo *et al.*, 1987].

LF is an iron-chelating protein synthesised by granules on the surface of PMN and glandular epithelial cells [Baggiolini *et al.*, 1970; Masson *et al.*, 1966] and LF can inhibit the growth of pathogens in the presence of bicarbonate. According to Harmon *et al.*, (1975) concentration of LF is correlated to SCC and stage of lactation.

Lysozyme (EC 3.2.1.17) is a protein removing peptidoglycans from cell-wall Gram-positive and membrane Gram-negative bacteria. Milk lysozyme consists of 154 amino acids and is significantly different to human and egg lysozyme [Eitenmiller *et al.*, 1976].

Lactoperoxidase is an enzyme showing antimicrobiological activity against Gram-positive and Gram-negative bacteria in the presence of thiocyanate and hydrogen peroxide (International Dairy Federation, 1988; Kussendrager and Hooijdkamp, 2000).

Complement is the term describing a group of proteins present in bovine milk and serum, its level increases during inflammation. These proteins are produced by hepatocytes, some monocytes and macrophages in different tissue. Their function is firstly to promote recruitment of neutrophils, and secondly to kill bacteria. Some types of complement are specified against only Gram-positive or Gram-negative bacteria. However there are examples such like C3b and C3bi showing antibacterial activity against both groups [Oviedo-Boyso *et al.*, 2007].

Cytokines belong to heterogeneous group of hormone-like proteins, that are produced locally in the udder in very small concentrations. Cytokines take part in the regulation of activity of immune cells by increasing the bacterial capacity of macrophages and neutrophils, stimulating recruitment of neutrophils to infected tissue and inducing the maturation of dendritic cells [Sordillo *et al.*, 1991; Alluwaimi and Cullor; 2002; Horne *et al.*, 2002; Alluwaimi 2004]. Cytokines are also involved in initiating the development of plasma cells producing antibodies. Some documented evidence of such association and more of activity are shown below in Table 2-2.

Table 2-2 Examples of cytokines, their functions and source.

[Adopted from Oviedo-Boyso *et al.*, 2007]. About thirty kinds of cytokines are identified and described, each can interact with others.

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The udder defence system against mastitis is complex. It combines three pathway protections systems which are very carefully integrated to minimise disease outbreak. However the natural triple guards have not always being able to prevent and/or self cure an inflammation and therefore herdsman management is required.

2.5 Tissue damage during mastitis

Damage to mammary gland tissue during mastitis is a result of the activity of pathogenic bacteria and their enzymes, PMN, animal's proteases, and cytokines. However pathogenesis of the mammary tissue damage during mastitis is multifactorial and the mechanism of tissue damage is not fully understood.

Zhao and Lacasse, (2008) described two types of cell death; apoptosis and necrosis. Apoptosis is the controlled, programmed process of cell death in multicellular organisms in response to various inducers. Apoptosis is a necessary way for removing epithelial cells during lactation and post lactation period and to ensure the correct development and function of mammary gland [Cappucio *et al.*, 2003]. However Long *et al.* (2001) provided results that *E.coli* infection induces epithelial secretory cells death via apoptosis. Necrosis is a consequence of wide injuries and follows a characteristic pattern of morphological changes. Necrosis causes uncontrolled death of cells. According to results obtained by Sordillo and Nickerson (1988), Trinidad *et al.*, (1990) and Heald (1979) *Staphylococcus aureus* infection results in necrosis of the mammary gland cells. Histopathological study of the tissue indicates a greater degree of replacement alveolar lumen with alveolar stroma compared to uninfected quarters.

The detection of pathogens by microphages and epithelial cells results in the release of chemoattractants which attract leukocytes, mainly PMN, from the blood system towards the mammary gland. PMN kill pathogens by oxygen-dependent and oxygen-independent systems. Harmon and Heald (1982) observed that an elevated number of PMN during experimentally induced *Staphylococcus aureus* mastitis is correlated with great damage to mammary gland tissue. Tissue samples taken from quarters induced with *S.aureus* mastitis showed replacement of secretory parenchyma by epithelium without secretory ability [Heald, 1979]. Microscopic examinations of gland tissue infected with *E.coli* show damage of epithelial cells by lysing or phagocytosing PMN. Activated PMN are cytotoxic for epithelial cells. [Capuco *et al.*, 1986; Laruzon *et al.*, 2006]. Moreover infusion of LPS cause acute mastitis and

stimulates PMN to damage mammary gland epithelial cells via superoxidase production [Boulanger *et al.*, 2002; Lauzon *et al.*, 2006]. However endotoxin has no direct damaging impact on epithelial cells [Frost *et al.*, 1984].

Oxidative stress is caused via release of extracellular reactive oxygen species (ROS) such as hydrogen peroxide, radical OH⁻, hypochlorous acid (HOCl), [Boulanger *et al.*, 2002, Lauzon *et al.*, 2006]. ROS destroy bacteria by an oxygen-dependent mechanism but at the same time they are associated with tissue damage during mastitis. ROS can overwhelm the animal oxidant system leading firstly, to decrease in milk production; and secondly, to potentially the complete loss of an infected quarter. It was found that catalase, melatonin and some antioxidants (catechin, deferoxamine, and glutathione ethyl ester) have protective effects against ROS [Boulanger *et al.*, 2002; Lauzon *et al.*, 2006; Lauzon *et al.*, 2005; Mehrzad *et al.*, 2005].

Mastitic milk shows higher than normal phospholytic activity, leading to an imbalance of proteinase-antiproteinase mechanism [Nickerson and Heald, 1981]. During mastitis proteases associated with PMN are highly involved in opening tight junctions and this will lead to tissue damage [Mehrzad *et al.*, 2005].

Benites *et al.*, (2002) examined the mammary parenchyma collected from 46 slaughtered dairy cows naturally infected. Firstly 12.5% of samples did not show histopathological changes. Secondly 63.3% showed loss of parenchyma, which was replaced by connective tissue, due to the repair process. These results suggest a high occurrence of histopathological changes during infection leading to reduction in milk production. Apoptosis regulates the life span of PMN. Prolonged activity of PMN can cause damage to mammary gland tissue. Elimination of apoptotic PMN by macrophages following bacteria neutralisation is essential to reduce injury to secretory cells [Pappe *et al.*, 2003].

2.6 The interaction between herd management factors and mastitis incidence

2.6.1 Nutritional impact on milk composition

During the last thirty years nutrition and its influence on milk production and composition has been of great interest to scientists. The ability to control and modify milk quality could lead to improved processing of milk and the manufacturing of

dairy products. Milk is 96% fat free and its dry matter consists of a high content of saturated fat. Moreover the manipulation of milk composition might help to enhance human health (trans-acids e.g. conjugated linoleic acid CLA) and overcoming diseases i.e. lactose intolerance and osteoporosis.

Milk fat is the most responsive to an animal diet component of the milk and therefore, the easiest to manipulate [Palmquist *et al.*, 1993]. Bovine milk fat comprises about 26% of milk dry matter and majority of it is saturated fatty acids.

Mansbridge and Blake (1997) identified four main sources of fatty acids in the ruminant diet: forages, oils and oilseeds, fish oil and fat supplements. Forages affect the FA content in milk due to cellulose and hemicellulose content, both fermentable by rumen microorganism to acetate and butyrate (precursors of *de novo* FA), and oil (up to 50g per 1kg of DM). Oilseeds or seed oil are a good source of polyunsaturated FA such as oleic (18:1) acid, linoleic (18:2) acid and α - or γ -linolenic (18:3) acid. Higher intake of oilseeds and/or oils by a lactating animal can result in decreased concentration of C4-C16 milk FA and elevated levels of long-chain (18:0, 18:1, 18:2, 18:3) FA. However, untreated or extruded oilseeds have poor protection from ruminal metabolism. Marine fish oil contains long chain unsaturated FA (n-3). An *in vitro* study was described by Ashes *et al.* (1992), in which fish oil and a casein mixture (1:1 w/w) was incubated with sheep rumen content. Results showed a reduction in concentration of 18:1 but no influence on 20:5 and 20:6 was observed. Further research is required for the use of marine fish-oil as a regulator of FA profile in milk. Fat supplements can affect the content and the composition of milk FA. However the scale of changes depends on the degree of protection from microbial and enzymatic activity. Ashes *et al.*, (1997) listed the supplemental fats: whole, extruded, or exploded oilseeds, calcium salts of long-chain FA, pelleted or prilled fats containing TAG and starch, animal and vegetable blended fats, yellow grease, formaldehyde-treated oil, protein supplements and butylsoyamides. The important characteristics of fat supplements are not only the quality and quantity of FA but also digestibility, transfer of absorbed FA, the degree of inertness or protection in the rumen, and the effect on mammary gland metabolism.

Some diets can facilitate reduction in milk fat production, defined as milk fat depression (MFD). Diet-induced MFD decreased yield of all fatty acids but the greatest reduction is observed for short- and medium-chain FA, which are synthesised

de novo. According to Bauman and Griinari (2003) induced MFD is related to the interrelationship between rumen microbial processes and tissue metabolism. Jenkins and McGuire (2006) pointed out, that a higher intake of grain (fermentable starch) > 50% of DMI stimulates milk yield and also induces milk fat depression. MFD is associated with changes in FA profile. Grain feeding stimulates synthesis of the *trans* FA by rumen microorganisms which associated with MFD [Palmquist and Schanbacher, 1991; Jenkins and McGuire, 2006,] and might decrease concentration of short-chain FA [Palmquist *et al.*, 1993]. Peterson *et al.*, (2003) induced MFD with a high concentrate and low forage diet. Obtained results suggested that diet-induced MFD resulted in a corresponding reduction in mRNA quantity for genes associated with mammary lipid synthesis. Moreover those changes were correlated with the appearance of *trans*-10, *cis*-12 CLA. Davis and Brown (1970) pointed out that increased concentration of *trans* 18:1 fatty acids were usually associated with MFD. Bauman and Griinari (2003) proposed a hypothesis of MFD “under certain dietary conditions, the pathways of rumen biohydrogenation are alerted to produce unique fatty acids intermediates, which are potent inhibitors of milk fat synthesis”. In brief MFD is perceived when the diet alerts the rumen environment and the diet contains polyunsaturated fatty acids [Bauman *et al.*, 2006].

Increased concentration of unsaturated FA in animal's diet may not allow the manipulation of the FA profile in milk. Transfer of dietary FA into milk fat depends on ruminal biohydrogenation, digestibility and deposition in adipose tissue [Palmquist *et al.*, 1993]. Biohydrogenation is microbiological process which takes place in rumen. Microorganisms present in the rumen transform and convert unsaturated FA through enzymatic reaction to saturated acids. However, there are currently available “rumen-protected” fats which elevate concentrations of unsaturated FA in milk. In summary, introduction of the moderate levels of saturated fats may increased total milk fat by a small amount. However a similar amount of unsaturated FA and large amounts of most sources of fat will have the effect of decreasing total milk fat concentration

Fat supplementation is correlated with reduction of protein content, particularly casein. An addition of 100g of supplement fat into diet decreases milk protein about 0.03 per cent and increased net protein nitrogen [Jenkins and McGuire, 2006]. The reasons for these observations are complex e.g. the adverse effect of free fat of dry

matter intake, activity of micro-organisms and their protein synthesis, carbohydrate fermentation [DePeters and Cant, 1992; Stern *et al.*, 1994].

Protein quality and quantity plays an important role in cheese manufacture. Cheese curd structure, firmness and yield depend on casein. The latter comprises over three quarters of all milk proteins. Correct fat supplementation and/or dietary influence should not affect protein content and have a positive effect on its profile. Emery (1978) in his review article, explained that diet influences of the protein composition is small if compared with environment and/or genetic impact. Transmission of dietary nitrogen onto milk protein output has as low efficiency, 25-30% [Bequette *et al.*, 1998]. However, the nutritional changes will provide a quicker response than any other factor [Emery, 1978]. According to Jenkins and McGuire (2006) factors affecting the most protein content are forage-to-concentrate ratio, the volume and source of dietary protein and dietary fat. Decreased forage proportion in animals diet results in increased protein content. Reduction of forage proportion in a diet to 10% could elevate protein content by 0.4 per cent. However, forage is an essential part of a well balanced diet and it also improves the digestive process. Emery (1978) pointed out the low transfer of dietary protein into milk. An increase of 1 per cent dietary protein will result in an increase of only 0.02 per cent in milk protein content. Dietary protein has greater impact on total milk production (increased) than on milk protein concentration [Kirchgessner *et al.*, 1967]. Emery (1978) in his review also highlighted that a change in diet such as feeding more energy, more protein or less fibre will significantly affect milk protein concentration. The same changes may increase milk yield but may or may not affect fat composition. Moreover addition of 4-12% of fat (hydrogenated marine fat, lauric acid, protected or unprotected soybean oil, or coconut oil) declined milk protein concentration by 0.1 to 0.3 %. DePeters and Cant (1992) confirmed a negative influence of fat feeding on the milk protein content. Illg *et al.*, (1987) reported that feeding protected amino acids e.g. methionine increased milk proteins. Emery (1987) agreed, however, protection from ruminal influence cannot be fully achieved; firstly ammonia concentrations in rumen must support microbial protein synthesis; secondly high photolytic activity and amino acid absorption occurs in the lower digestive tracks.

The total mixed ration (TMR) is used widely in dairy industry, particularly for the winter period, to help dairy cow achieving their maximum performance through consistent blend and nutrient balance. TMR fed regularly allow cows to consume as close to energy requirements as possible and which maintains physical or roughage characteristics required for good rumen function. Ideally different TMR should be fed to the different group of milk producers based on stage of lactation and yield (low, medium and high) or different days in milk, reproductive status, age and/or health too ensure that required nutrients are delivered. However, this has been found to be difficult to achieve or inappropriate for small herds (50-70 animals). Also mixing equipment is costly and it must be capable of thoroughly blending all ingredients [Lammers, www.das.psu.edu/teamdairy/]. Table 2-3 below illustrates the level of nutrient required for each yield level group.

Table 2-3 Nutrient specification for three group system (100% dry matter basis).
<http://www.ag.ndsu.edu/pubs/ansci/dairy/as769.pdf>

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NE – netto energy calculations for lactation; ADF - acid detergent fibre; NDF – neutral detergent fibre.

Certain diets for dairy cows may be more conducive to milk productivity and desired quality, when correctly balanced ingredients and supplements are fed, therefore care should be taken with formulating to achieve such objections.

2.6.2 Nutritional impact on mastitis

The dairy cow's diet is designed to enhance the milk production and its quality by regulating the energy status which in turn informs milk composition. In addition a well balanced diet will improve the health status of a cow by modulation and support of the immune system.

A number of studies have looked at an influence of the supplementation of dairy herd diet on intra-mammary infections. In summary it has been agreed that an animal's resistance to mastitis can be strengthened by supporting the anti-oxidant system. The correct supplementation of vitamins and minerals can increase the resistance against the disease.

Vitamin E is a fat-soluble anti-oxidant. It enhances the functional efficiency of neutrophils through protection from oxidative damage of toxic oxygen molecules necessary for intracellular killing of ingested bacteria (Herdt and Stowe, 1991) as well as maintaining cellular membrane fluidity (Bendich, 1993). Vitamin E supplementation to dairy cows in early lactation results in increased antibacterial activity of bovine blood neutrophils. Vitamin E and selenium (Se) are integral components of the antioxidant defence of cells [Smith *et al.*, 1997]. Adding supplemental vitamin E and Se into an animal diet can enhance immune function by the influence on phagocytic activity. According to Smith *et al.* (1984) dry cows fed with 1000 IU of supplemental vitamin E and/or injection of 50 mg Se showed a 37% lower number of outbreaks of clinical mastitis when compare with control animals. Hogan *et al.* (1990) reported that adding a supplement of vitamin E to the diet increased intracellular kill of *S.aureus* and *E.coli*. These results were confirmed by Weiss *et al.* (1997) and Ashutosh and Singh (2008), who pointed out that vitamin E and Se added to an animal's diet could increase defence against mastitis [Smith *et al.*, 1997; Weis, 2002,]. According to Chew (1987), Hemingway (1999) vitamin E and selenium stimulate function of bovine PMN which results in lower duration time of clinical mastitis and its frequency.

Beta-carotene is the dietary precursor of vitamin A, is metabolised in the intestinal mucosa to retinol and then transported with fat to the liver. Green forages are rich sources of vitamin A therefore an addition of this vitamin should be specially considered for diets lacking in this component. Vitamin A has numerous functions

unfortunately not all are fully understood. It has been noted as having a role in the resistance to infection e.g. mastitis in prepartum dairy cows (NRC, 2001). Results obtained by LeBlanc *et al.*, (2004) confirm the protective role of α -tocopherol and retinol in prepartum mastitis. However many factors such as the metabolic status of an animal might affected the necessary volume of the supplement in the order to minimise chance of disease outbreak. Results obtained by Dahlquist and Chew (1985) pointed out that an addition of 53,000 IU/ day of vitamin A and 300mg of β -carotene / day from three weeks prior drying off will reduce the chance of mastitis outbreak by up to 50%. Vitamin A and β -carotene stimulate immune cells and prevent initiation of fatty acid peroxidation chain reaction [Sordillo *et al.*, 1997; Weiss, 2002]. According to Vecqueray *et al.* (2009) animals on a diet based on high levels of concentrates and poor quality forages, low in green forages, and during the prepartum period should be given supplements of vitamin A.

Minerals are not antioxidants themselves; however, they are essential for antioxidant enzyme activity. Zn, Cu, Mn play a crucial role for the activity of dismutase, Se is essential for glutathione peroxidase activity, Fe is required for catalase activity (Bendich, 1993).

Results obtained by Scalettie *et al.* (2003) indicated a relationship between copper supplementation and SCC in milk. Cows which were supplemented with Cu have a lower SCC as well as a lower clinical udder score. Moreover it was observed that Cu addition decreased severity of *E.coli* infection (duration was unchanged).

According to Miles and Calder (1998) intake of dietary fatty acids can have an influence on immunity by effect on liver acid oxidation and the production of cytokines and molecules essential for immune reactions and response.

The knowledge of the relationship between SCC/mastitis and nutrition is not yet well known when compared with associations with hygienic or environmental factors. Manipulation of the diet by an addition of vitamins and minerals will improve a dairy cow's resistance against mastitis. However supplementation can be dangerous for the animal and therefore only recommended doses should be introduced since overdose might be toxic and lead to death.

2.6.3 Mastitis treatments: lactating cow therapy and dry cow therapy

The main goal of the treatment of a cow is to reduce existing infection or eliminate the threat of mastitis. The animals can be treated at two levels: while cows are in milk or during their dry period. The purpose of the dry cow therapy is to remove infection still present in the udder at the end of lactation cycle and prevent infection from being passed into the next lactation. The aim of lactating cow therapy is to cure diagnosed infection. There are three main methods of treatment: parenteral therapy (injection), intramammary therapy (infusion into the udder through the teat) and oral treatment.

When the herdsman diagnoses a cow with an infection it should firstly be kept back to be milked after all healthy animals leave the milking parlour; secondly, it should be treated with antibiotics and a milk sample should be sent for microbiological testing. Removing the animal from the milking process can decrease the likelihood of the spread of bacteria. However, this procedure is not consistent across all farms. Usually infected cows are milked with a separate cluster connected to a dump bucket. Antibiotics available on the market offer a wide spectrum of activity. The following criteria when choosing an antibiotic treatment: the kind of bacteria involved and its sensitivity, the ability to penetrate the udder and persist in it, effectiveness, properties (e.g. fat or water-soluble) and costs [Mackellar, 1991]. Penicillins (gram-positive spectrum e.g. penicillin G, penethamate, nafcilin) and synthetically modified penicillins (gram-minus spectrum e.g. ampicilin, amoxicillin), erythromycin (gram-positive spectrum), tetracyclines (broad-spectrum), aminoglycosides (activity against coliform and beta-lactamase, e.g. streptomycin, neomycin) are included as the most commonly used antibiotics.

Some cases of mastitis e.g. *S.aureus* infection (Table 2-4) have a very low self-cure rate, an antibiotic treatment is a necessary therapy to overcome the disease. Currently there is more awareness regarding antibiotic residues and its effect on environment, and human health. Use of mastitic milk, which contains an elevated number of pathogens and antibiotic residues as, feed for calves leads to selection of the resistance bacteria. EC Regulation No 852/2004 on Hygiene of Foodstuffs and No 853/2004 required that milk containing antibiotics is not to be placed on the market.

Table 2-4 Mastitis pathogens: source and control (adapted from Bramley *et al.*, 1996)

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Dry cow therapy (DCT) is an intramammary administration of long acting antibiotics into each quarter following last milking. DCT is extremely important for reducing contagious pathogens still present in the udder and decreasing number of infection during off milk period when the udder is extremely susceptible to new infections [Dingwell, et. al., 2004]. This treatment does not require milk discharge and an animal body response to DCT is more effective if compare with lactating cow therapy. DCT helps reduce contagious and environmental streptococci infections however it does not protect from coliform mastitis.

Results obtained by Smith *et al.*, (1985) suggested that coliforms are able to invade the teat during the end of lactation and survive within the udder until the periparturient period.

Resistance to mastitis is complex and consists of genetic components, physiological and environmental factors. Rupp and Biochard (2003) defined resistance as the ability to avoid infection or the quick recovery from it. Research

work to enhance animal resistance to mastitis is ongoing and currently there are no commonly used vaccines available to protect against it. Vaccination stimulates natural antibodies to fight against pathogenic microorganisms and results in increased body immunity against these specific bacterial factors. The number of pathogens causing mastitis is considerable, many different species and immunologically different strains make it very difficult to construct effective vaccination. In the United States of America vaccination based on *E.coli* (O111:B4) J5 bacteria was injected into dairy cows to boost their immunity. Results obtained indicated a reduction of clinical signs and the duration of mastitis. *E.coli* J5 is naturally occurring structurally modified bacteria strain which enhances immunity to itself as well as other strains of Gram-negative bacteria [Hogan *et al.*, 1995; 1995; Bramley *et al.*, 1996]. In addition the core-antigen technology has been implemented to format bacteria on J5 mutant *Escherichia coli* in order to reduce incidence and severity of coliforms mastitis. The aim will be to reduce the incidence of clinical coliform mastitis associated with early lactation through vaccination regimes which will include multiple administrations during the dry period. According to Bramley *et al.* (1996) the cow's genetic susceptibility to mastitis is associated with milk yield. The animals with high milk producing ability are more susceptible to mastitis. However only 10% of mastitis can be correlated with genetics and 90% is attributed to effective management. Moreover antibiotics still remain the most effective way to treat established disease.

2.6.4 Herd management and hygiene

Continuous and careful farm management and animal monitoring is essential for healthy and high productive dairy cattle. Effective management should combine and include the following factors: clean bedding, quality housing, good ventilation, high standard of hygiene, good monitoring of animals and accurate records kept of associated with health, disease issues and history. Table 2-5 bellow illustrates key points of good management practice.

Table 2-5 Herd management key points [Schukken *et al.*, 1990; Peeler *et al.*, 2000]

Category	Issue	Proposed action plan
General hygiene	Increased exposure to environmental pathogens (straw yard poor freestall cleaning, milk leaking outside the parlor)	<ul style="list-style-type: none"> ➤ Frequent cleaning e.g. calving areas should be cleaned at least once a month, collecting yard twice a day ➤ sufficient ventilation
Microorganisms	High bacterial challenge and/or issues with resistance	<ul style="list-style-type: none"> ➤ Sufficient post-milking disinfection of teats and milking clusters ➤ Minimising milk leakage
General management	Recurring mastitis events	<ul style="list-style-type: none"> ➤ Implement longer dry period and high replacement rate. ➤ Mastitis therapy should be involved when single clots are seen in foremilk. ➤ Good accuracy of clinical mastitis evaluation and SCC measurement ➤ Close monitoring of animals with non-functional quarter/quarters ➤ Stable dairy management
Dietary components	Different diet groups	<ul style="list-style-type: none"> ➤ Balanced concentrate in cow basal diet.

Management practice as presented in the table above has an impact on the rate of disease incidence and the cure rate [Barkema *et al.*, 1999]. In addition, it is very important to understand which pathogen is the cause of mastitis; however the micro-

testing results take too long to agree on an appropriate treatment. Therefore development of process should take place on each farm as without the understanding of the cause a prevention program cannot be designed and implemented. Moreover all animals with an average SCC being close or just over the threshold (250.000 cell/ml) should be tested - bacteriological culturing, to detect or exclude mastitis.

Menzies and Mackie (2001) suggested that frequently changed bedding, sufficiently ventilated housing and encouraging animals to remain standing after milking would help minimise clinical and sub-clinical mastitis outbreak rate. They suggested that dairy cows should be provided with a dry and clean environment. Undersized cubicles increase faecal contamination of the udder. Moreover a poor ventilation system and drainage provide an excellent environmental for pathogens. Dairy farm design should be improved to reduce mastitis outbreaks. However economics force dairy farmers to cut costs by increasing stock density of animals [Johnes, 1990; Smith and Hogan, 1993].

Bedding, housing and ventilation

The bedding layer should absorb moisture, minimise multiplication of micro-organism but also provide cushioning for resting animal. Bedding materials can be divided into two categories: organic e.g. straw, wood shavings, sunflower hulls, paper products, recycled manure solids, and inorganic e.g. washed sand and limestone. They all have their advantages and disadvantages. Organic bedding is used because it can absorb moisture but being biodegradable also provides nutrients for micro-organisms. It was suggested that inorganic materials, if compared to organic materials, have lower bacteria count. The inorganic materials offer no nutrition for bacteria and moreover allow for water, milk and urine to drain through, which will decrease retained moisture. According to Smith and Hogan (2000) sand could have all the properties of a “gold standard” of bedding materials. Properly maintained, sand provides a comfortable cushion for an animal and does not support the growth of pathogenic bacteria. However Smith and Hogan (2000) pointed out that particle size of bedding materials has an important impact on pathogen population. Small particles usually cover teat skin giving excellent opportunity for bacteria to multiply and increasing number of mastitis outbreaks.

According to Zadanowicz (2002) there is significant correlation between bacterial counts on the teat and sawdust or sand bedding. Bedding is a source of micro-organisms involved in the udder inflammation. Environmental bacteria live and multiply in bedding material and the teat end is in contact with the bedding. The major objective is to frequently replenish bedding material which will then reduce growth of bacteria implicated in mastitis infection [Hogan and Smith, 1992]. Maximum bacteria population in organic bedding is reached 24 hours after it is laid down. Dodd *et al.*, (1984) specified that the rear end of cubicles should be changed once a day to decrease contamination. Keeping solid floors clean is also very important; they should be cleaned twice a day by using a tractor and scraper.

McFarland and Gamroth (1994) found that chopped newspaper can be used as a bedding material. It is cheap, moisture adsorbing and reduces barn odours, and growth of bacteria. Mixtures of paper and saw dust or straw bring similar benefits. Visser (1994) described an experiment which monitored dairy cows' preference for specific bedding materials. The following order was established 1) canvas with a coarse river sand base 2) canvas with corn stalk base 3) river sand 4) calcrete 5) fine sand.

Lehenbauer *et al.* (1994) pointed out that housing type has a significant influence on SCC. According to Britten (1994) there should be 10 per cent more cubicles available than the number of animals to avoid cows lying in the walk way, which increases exposure of the udder to contamination. Increased cow-to-cow contact leads to spreading mastitis pathogens across animals.

The grids and over-slurry channels should allow animals to enter and exit the stall easily and comfortably. McFarland and Gamroth (1994) proposed dimensions for dairy cows based on their weight. Design of the free-stall must encourage cow to enter it. An animal will not enter a free-stall if it is too small, not comfortable or poorly bedded. Cows lying in alleyways became extremely dirty and more susceptible to stress (Bramley *et al.*, 1996). Hospido and Souesson (2005) indicated the importance of housing condition as it has a great influence on udder health. However there is no overall conclusion and/or agreement about the most suitable housing system.

Natural ventilation is preferable. Air exchange is achieved by wind and thermal buoyancy. House and Rodenburg (1994) pointed out that natural ventilation provides more daylight, is noise-free and has no costs. Continuous and/or near continuous wall

openings allow maximum air and moisture exchange with outside environment. Natural ventilation has been successfully used in bedded housing barns, free stall barns, tie stall barns [Holmes and Graves, 1994]. However usage of only natural ventilation cannot solve the following problems: water condensation and overheat in summer, and draft in winter.

Udder hygiene

An improved hygiene regime around the milking routine will contribute to reduction of the mastitis rate. The udder is constantly exposed to pathogens and poor cow hygiene is a common problem in dairy herds. Udder and leg hygiene was found to be associated to incidence of intramammary infection due to environmental pathogens [Ellis *et al.*, 2006]. According to Schreiner and Ruegg (2003) milk from cows with dirty udders contain 1.5 times more likely major environmental pathogens. Results obtained by Breen *et al.* (2009), based on observation of eight dairy cattle in the UK, shows a strong correlation between udder hygiene and the increased risk of *E.coli* infection. Menzes and Macke (2001) indicated that milk leaking between milking or incomplete milking, decreased distances between the teat and the floor and asymmetric udder could increase risk of development of the infection.

An effective milking routine should remove all milk from a cow with minimal risk of bacterial infection. Zecconi *et al.*, (1994) suggested that there is significant relationship between prevalence of mastitis and pathogenic micro-flora isolated from milking parlour. Good milking practice including pre- and post-milking disinfection of the udder, proper disinfection of milking equipment and milking parlours can limit the spread of bacteria in the parlour and reduce the incidence of mastitis.

Pre-milking teat preparation can reduce incidence of clinical mastitis by 50% according to Bramley *et al.*, (1996). Good milking routines will provide clean and dry teats and enable detection of mastitis without it spreading from cow to cow. Post-milking routines will apply disinfectant on the whole length of the teat. According to Cox (2009) post-dip should be applied within four seconds after cluster removal. Post milking disinfection was found to be correlated with the incidence rate of mastitis outbreak [Eberhart *et al.*, 1983; Schukken *et al.*, 1999].

Injuries in dairy cattle are usually linked to bad management e.g. poor housing, slippery walkways, and wide gutter grids. An injury of the teat or udder is associated with a greater chance of developing mastitis as it exposes the udder to pathogens and makes the milking process more difficult.

Herd management plays a key role in the process of reducing the rate of mastitis. The competent herdsman should be fully aware of normal animal behaviour and able to notice a difference in their actions. Moreover adequate and proactive milking procedures must be put in place and be followed by all members of team; especially pre- and post-milking disinfections. Providing good and clean accommodation will reduce the stress and the likelihood of the disease development. A management plan should be based on three risk factors a) resistance of an animal (or group of animals), b) exposure to pathogens (and understanding which pathogen is involved in order to plan an accurate treatment), c) record and recognise the cure rate (be able to identify the main aspects affecting it).

2.7 Potential of particle counting technology on mastitis

The particulate size distribution and number give a good characterisation of the properties of disperse liquid system. There are many methods and instruments used to characterise particles, the most common are centrifugation, ultrasonic spectroscopy, turbidimetry, image analysis, light blocking and light scattering [Nogowski *et al.*, 2007]. Particle counting technology (PCT) has been established in medical, manufacturing and electronics fields. Particle counters are used in medical practice to monitor and test particle contamination in blood streams. Free alien particles can transfer into blood streams causing blockage. Drinking water also requires for particles to be monitored in order to detect *Cryptosporidium* and *Giardia*. Particle counters are used as well in semiconductor industries to control quality of fluids used in manufacture. High purity of liquids such as ultra pure water, chemicals and reagents is very important for nano-structure on the microelectronics devices, therefore PCT has found wide use in this industry [Nogowski, *et al.*, 2007; Broadwell, 2000].

Optical techniques for particle characterisation are very common in liquid monitoring. Particles in liquid respond to light in many ways such as reflection, absorption, refraction, diffraction, scatter and transmission. Hunt (1993) defined the majority of the optical detection methods, as follows:

- Forward Detection –providing size counting – light is reflected in narrow angle (6.34-18.95°). The angle depends on the surface area which indicates size of particles.
- Fraunhofer Diffraction – providing size distribution
- Light Obstruction (Light Blockage)- described below
- Light Scatter – described below
- Turbidity – measuring of the cloud appearance within liquid because of particles. The means of detecting this varies considerably from straight through 90°scatter, 180° multireflection, multiangle scatter, etc.°
- Phase/Doppler scatter – two detectors record the Doppler shift caused by moving particles
- Photometric Dispersion – based on detection of the fluctuation of the light level around the main level
- Photon Correlation Spectrometry detection (by highlighting by a light beam) of the motion (Brownian Motion) of the particle due to molecular bombardment
- Time of Transition use blockage or back scattering of a rotating laser beam

Particle counters are divided into three main categories based on their application; aerosol particles counters, liquid particles counters and high-energy particles counters. The first one is used to measure particle density in atmospheric air. Mostly used to determine air pollution and monitoring the cleanliness level in a controlled environment e.g. in the pharmaceutical industry, hospitals and laboratories. Liquid particle counters measure number and size of particles flowing through the fluid. Phase liquid particle counters are widely used to determine quality of drinking water or cleaning solution as well as to determine purity of hydraulic liquids.

Light Scattering measurement is based on detecting the amount of light reflected and adsorbed by a particle, referred to as scattered light. When liquid flows through a sensor, particles present in the sample scatter light. A detector records a scattered

burst of light which is converted to electrical pulses. The size of the particle is obtained by comparing height of pulse with a standard calibration curve. [Nogowski *et al.*, 2007; Broadwell, 2000; Hunt, 1993]. Light scattering counters with white light detect particles in size ranges of 1 μ m to 300 μ m, or 0.2 μ m to 10 μ m with laser light; this method requires clear or semi-clear liquids [Hunt, 1993].

Particle counting technology used in this study was based on a light blocking measurement. This method was launched in 1962 in California, United States when High Accuracy Products was founded by Leon Carver. High Accuracy Products was a pioneer in the optical counting industry by developing effective automatic particle counters for aerospace hydraulic applications. Airborne counters were developed by Royco Apparatus Ltd. incorporated in 1970. Those two companies were joined by Pacific Scientific, purchasing High Accuracy Products in early 1970's and ROYOCO (early leader in semiconductor clean room market) in 1980. At this level sensors were able to detect particles at size 1 μ m to 9 mm with concentration as high as 20 000 particles/ml. Light blocking (known also as light extinction, absorption) measurement, is based on how much light is absorbed or reflected away from the detector by a particle during liquid flow. Every single particle blocks the light beam. A photo-detector records changes in the light when particles cross the beam and translates them into electrical pulse. The height of pulse is proportional to the square of the particle radius. Calibration of light blocking sensor is valid only for a specific type of particles and flow rate. [Hunt, 1993; Broadwell, 2000; Nogowski *et al.*, 2007]. This method required a low particle count as its intention is to have one particle in the beam of light at one time. A confidence of 95% can be achieved by ensuring the flow rate is as per specification and concentration of particles is below to confidence limit. A typical size range is between 2 μ m to 100 μ m.

In this study PHA was integrated into a light blocking particle counter as a module to calibrate the light blocking sensor. PHA are commonly used in nuclear and elementary physics particles research for the determination of energy spectra of nuclear radiations. This device allows the recording of electronic pulses, of different heights coming from particles, if their amplitude falls within specified limit. The

pulses are separated according to their height (equivalent of sorting particles to their energy) into channel/channels for later spectral analysis. The pulse leaving the detector has amplitudes proportional to the energy which was deposited by particles in the detector. Measurement is made with either multi or single channel analysers.

Both particle counter and PHA are used to constantly monitor of particle numbers, patterns and behaviour in both liquid and air environment. The network of sensors is typically connected to a facility monitoring system which is using logic systems to track dynamic performance of particles in real time. Combined particle counter and PHA were designed to monitor physiology and health status of the udder based on the particle behaviors. Research was required to evaluate the feasibility of introducing this particle monitoring device into dairy industry and its application at commercial farm level.

The sections above have introduced the problem of mastitis and its impact on the dairy industry and animal welfare.

Available research points out that many parameters of milk such as electric conductivity, pH, SCC are a good indicator of mastitis. However, it has been highlighted that the dairy industry urgently needs to improve the diagnostics and treatment of mastitis in order to make dairy farming more profitable and improve the welfare of the dairy cows. Milk particulate dynamics have been linked with mastitis by King in last century (1957, 1969), however there was no recent evidence illustrating an impact of this disease on MFG. As mentioned earlier, at the beginning of this study it was assumed that SC, their count and behavior will be the main domain of this thesis. Nevertheless following the first results it was clear that MFG are main particulate in milk - therefore there was a possibility that this group of particles might be affected by the mastitis. Hence an experimental protocol has been put in place. In phase one - experimental conditions and methodology, by using latex particles, samples of milking from healthy and infected animal, were established. In phase two, monitoring of animals diagnosed with mastitis was undertaken.

The following chapter will present the step by step sequence and experimental progression from the design through to operational protocol. The particle counting system designed for this research work combined all the characteristics (high

sensitivity, small volume of sample required, speed, able to work on-line, low cost, no chemical reagent) which facilitates the construction of a sufficient tool to monitor changes in particulate content of the milk. The system has been tested at the laboratory scale only, this research represent an important step and direction towards building in-line sensors for milking parlours.

3 Chapter III Materials and Methods

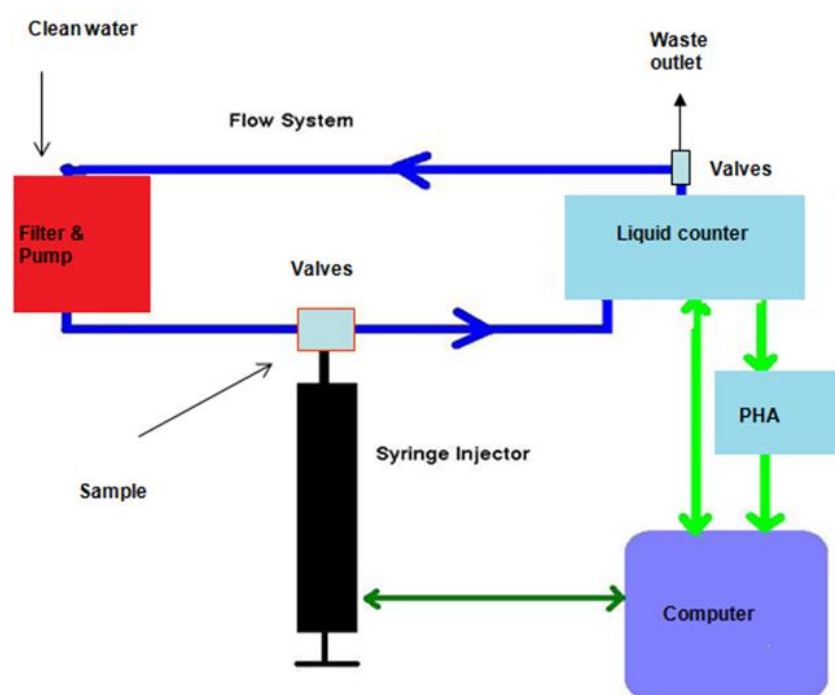
3.1 Establishment of particulate monitoring system

3.1.1 The design and development of particulate monitoring system

The purpose of the apparatus was to process milk samples to count and measure the size distribution of particles. This required a stable flow rate and particle-free water, the optimum dilution of the sample, accurate presentation of the sample to the particle counter for measurement, the measurement of particle counts at given size thresholds and the measurement of particle count distributions using PHA.

Apparatus used to determine particles in milk consists of injector – diluter, filter - pump, liquid particles counter, PHA and computer (Figure 3-1). Additionally some valves were included to enable the system to self-clean and dispose water with sample material to the waste outlet. The software was written by MicroResearch Ltd. The apparatus was built in the College. The following commentary provides more specific information on the function of individual components.

Figure 3-1 The particulate monitoring Apparatus



3.1.1.1 Filter and pump

The filter used is a 0.2 μm , polypropylene Submicronic Filter Cartridge RZ-06479-60. Particle free water used in this project is defined as deionised water which contains no particles greater than 0.1 μm in diameter. The water circulates in a closed system. Between every sample the water was checked for the number of particles (blank check).

3.1.1.2 Syringe Injector/Diluter

The syringe injector was a Hamilton Microlab 500B/C with 10ml single Drive Unit (Appendix 1). The drive unit contains a drive motor, the syringe drive arms, valve assembly, input and output arm. The syringe module has a two way rotary valve. To facilitate washing and dilution an additional two way valve is fitted to the output of the module. One leads to the waste outlet, the other to the particle counter. The syringe injector and attached valves are controlled by a computer using an RS232 interface. Digital outputs were used to control the valves in the rig.

3.1.1.3 Particle Counter

The liquid particle counter is a Particle Measuring Systems Inc. LiQuilaz E20 – Particle Counting Spectrometer for Liquids (Appendix 2). This liquid particle counter is based on the principle of light blockage which allows an automatic determination of the size and the number of particles. The LiQuilaz is controlled by computer and reports the detected particle counts classified into six size channels (Table 3-1) detecting particles from 1 μm to 30 μm .

The diluted milk stream carries particles which periodically partially obscure the beam of the laser beam as they pass through the measuring cell. The resultant dip in light intensity at the detector is converted to a signal peak; the height of which is proportional to the size of the particles crossing the beam as illustrated by Figure 3-2. Peaks are counted and analysed to produce number and size distributions [Appendix 9; Janik, et al., 2008].

Table 3-1 Channels for the particles detected by counter

Channel No	1	2	3	4	5	6
Particle Size Diameter [µm]	$1 \geq x \leq 2$	$2 \geq x \leq 3$	$3 \geq x \leq 5$	$5 \geq x \leq 10$	$10 \geq x \leq 15$	$15 \geq x \leq 30$

Figure 3-2 Principle of a liquid particle counter

[Nogowski et al., 2007].

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3.1.1.4 Pulse Height Analyser

The PHA is a module made by MicroResearch Ltd., UK. It operates under computer control via an RS232 interface. The circuit diagram of the PHA is in Appendix 3. The PHA samples the input signal every microsecond and records the maximum value. A signal peak is when the signal exceeds a threshold and then some time later returns to below this threshold. The peak can be classified into one of 4096 channels based on the maximum height of the signal. The PHA includes analogue DC baseline removal. The PHA firmware can determine the optimum trigger threshold by analysing the statistics of the input signal, perform baseline restoration and ignore peaks that are too short or too wide to be form the particle counter [Hill, unpublished]. The PHA counts and classifies particles in range from 1 µm to 15µm.

3.1.1.5 Computer

The computer is a conventional PC with three RS 323 serial ports. The computer runs under the Linux operating system (Suse Version 10.2). The software is a bespoke application by Micro Research Limited in C++ using Trolltech's Qt 4 application framework.

3.1.2 Particle counter calibration

The LiQuliaz was calibrated in accordance with Facility Monitoring Systems standard procedures. All test equipment used is traceable to national or international standards. (Appendix 4)

3.1.3 Evaluation of accuracy of particle counter and PHA; latex sphere calibrations

Sample preparation

Latex spheres have been referenced as suitable particles for particle counter calibration [Hunt, 1993]. An accuracy test was carried with Polymer Latex Microspheres size 2, 5 and 9.9 μm obtained from Duke Scientific Corporation. The standard latex particles –were dispersed in 25 ml particle free water. Small particles tend to form large agglomerations of particulate which will affect the result. Therefore a small quantity of water (2 ml) was added to the latex standard and gently mixed with a spatula.

Microscopic particle count test

The concentration of latex particles was determined directly by using haemocytometer (Werber Scientific International Ltd). The haemocytometer grid is divided in large squares, which each consisting of sixteen smallest squares ($2.5 \times 10^{-3} \text{ mm}^2$). The number of particles in 1 cm^3 is calculated from formula below:

$$\mathbf{Z = N.4.10^6.a}$$

where: N – average number of particles in the smallest square

a – dilution factor

Particle counter and PHA counting test

The diluted sample, total volume 250 ml, was injected into the system and under stable flow rate (manual control) and the particles distribution measured.

The density of particles was calculated by multiplying the average number of particles (obtained from ten samples) by the dilution factor.

3.2 Monitoring the behaviour of latex particles in varying environments

The objective of this experiment was to measure the environmental effects and the handling procedure on particle size distributions as well as to account for possible variation in milk. The behaviour of latex particles was challenged in different environments including: influence of pH, particle concentration (dilution), time, salinity and flow rate of the sample whilst running through the particle counter. For this study latex microspheres of size 2 μm were used. As mentioned earlier latex spheres are recommended particles for calibration of particles counters therefore they were found to be most suitable particles to carry out a validation of potential behaviour of milk particulates in different environments. Below assays were design for assurance of the procedure which will be applied on second phase of experiment (monitoring of animals).

Measuring cross-contamination between samples

The standard sample of latex was diluted to the power of 10^{-6} , 10^{-7} and 10^{-8} . Firstly latex samples were run in decreasing dilution order; between every sample of diluted latex a sample of water free of particles was run. Secondly, latex samples were run in increasing order without samples of particles free water. This experiment was repeated three times. Samples were diluted manually:

- 10 μL of the standard sample were mixed with 10 ml of particles free water giving a dilution 10^{-3}

- This sample was diluted by: 10^{-6} by mixing 250 μ l with 250ml of particles free water; 10^{-7} by mixing 25 μ l with 250 ml of particles free water; 10^{-8} by mixing 2.5 μ l with 250 ml

Sample dilutions and their influence on particle count

The standard sample of latex was diluted by 10^{-6} , 10^{-7} and 10^{-8} . Dilution scheme is described above.

Sample pH and its influence on particle count

The standard latex sample was diluted by 10^{-6} , 10^{-7} and 10^{-8} in 10 mM phosphate buffer. Monosodium phosphate monohydrate and disodium phosphate heptohydrate were dissolved in particle free water, preparation is illustrated in Table 3-2.

Table 3-2 Buffer preparation

Buffer components	pH=5	pH=6	pH=7	pH=8
NaH₂PO₄* H₂O [g/l]	1.361	1.214	0.584	0.094
Na₂HPO₄*7H₂O [g/l]	0.036	0.322	1.547	2.497

For each pH three different dilutions were prepared. This experiment was repeated three times.

Salinity and its influence on particle count

Two concentrations of sodium chloride were used 1% w/v and 10% w/v by weight, dissolved in a phosphate buffer (buffer preparation is described above). From each salt concentration solution three dilutions were prepared. This experiment was also repeated three times.

Sample flow rate and its influence on particle count

In the first stage of the experimental work, the pump was not installed and the flow rate of the sample was regulated manually. The optimal flow rate amounted to 55-60 ml/s and for every sample run in this section it was measured and recorded.

Temperature and the age of the sample and their influence on particle count

The latex standard sample was diluted by 10^{-6} , 10^{-7} and 10^{-8} (as described above). Samples were stored at room temperature and at 3°C . The performance of particles was measured at time 0 and 5h.

Sample temperature and its influence on particle count

The latex standard sample was diluted to the power of 10^{-6} , 10^{-7} and 10^{-8} over a pH range from 5 to 8. Samples were put into a water bath and heated to 40°C and run through the particle counter.

3.3 Monitoring of the behaviour of milk particles in varying environments

Milk sampling techniques

Mastitic and healthy foremilk samples were collected manually before attaching the teat cups during morning milking (estimated time between 4 and 6 am) throughout the whole study. It was important to ensure that milking schedule was followed up as time and frequency of milking has a significant impact on milk particles number and size. Mastitic milk samples were collected from animals with clinical signs of mastitis such as abnormal texture (clots), discoloration of the milk, swelling of the udder, increased temperature of the quarter. Diagnosis of clinical mastitis was made by an experienced herdsman. Healthy milk samples were collected from animals which did not show above symptoms. Milk samples were stored under refrigerated conditions at 4°C , prior to transport to a laboratory where they were stored at 3°C .

Cleaned and labelled glassware were prepared ahead of time and delivered to the farm. Sampling procedure included the following steps: wash hands in soap and water, discard two-three squirts of milk from teat, collect about 50ml of milk into the bottle, close glassware before removing from under udder, place the bottle with milk into the fridge.

Milk samples were examined in varying environment. Influence of temperature, time, dilution, salinity and flow rate had been investigated for both mastitic and non mastitic milk. Additionally an enumeration of somatic cells was carried to confirm a diagnosis of mastitis.

Enumeration of somatic cells

Milk samples collected from the farm were diagnosed as mastitic only by visible symptoms. Healthy and recovered animals were assumed to have less than 200 000 somatic cell/ml [Dohoo and Leslie, 1991], each sample of milk was tested for SCC. Enumeration of somatic cells was determined by microscopy [International Dairy Standard, 148:1995]. Principle of method: milk (0.01ml) was spread over slide, then dried and stained. From each tested sample of milk two films were prepared and counted.

Cross contamination between samples

The purpose of this experiment was to understand if there is a possibility for the examined sample to be contaminated with particles from previous sample. In this study mastitic and healthy milk samples were diluted by 10^{-4} , 10^{-6} , 10^{-7} and 10^{-8} in particle free water. Diluted milk samples were measured in increasing order of dilution.

Sample dilutions and their influence on particle count

Results of this experiment will allow to establish the most suitable dilution for milk samples. The approximate density of milk fat globules was determined by using the haemocytometer method described by King (1969). Mastitic and healthy milk samples were diluted by 10^{-4} , 10^{-6} , 10^{-7} , and 10^{-8} in particle free water and also measured by the particle counter.

Sample pH and its influence on particle count

In this experiment mastitic and healthy milk samples were diluted in phosphate buffer by 10^{-4} , 10^{-6} , 10^{-7} , 10^{-8} (buffer preparation is described in point 3.2).

Sample salinity and its influence on particle count

Mastitic and healthy milk samples were diluted by 10^{-8} in solutions of three concentrations of sodium chloride; 0.5%, 1% and 1.5%.

Sample age and its influence on particle count

Mastitic and healthy milk samples were diluted to 10^{-8} . Each sample was divided into two glass jars. One was stored in room temperature and the other at 3°C. Particle counts were measured at time 0h, 2h, 4h, 24h, 48h and 72h after sample preparation.

Sample temperature and its influence on particle count

Samples of mastitic and healthy milk were heated to 40° in a water bath. The milk was then diluted by 10⁻⁸ in particle free water and the particles count was measured.

In the second part of this study milk samples were diluted by 10⁻⁸ in particle free water and then heated in water bath to 40°C. The particle count was measured immediately after the sample achieved the required temperature.

3.4 Monitoring the behaviour of milk particles during mastitis outbreaks and recovery periods

The results obtained in studies detailed above enabled establishment of procedures and to develop equipment for the automated comparative measuring of the particle count in mastitic and healthy milk.

This study investigated the hypothesis that the particulate content of milk is correlated to physiological conditions of lactating dairy cow. Therefore monitoring and recording of its dynamics will allow a better understanding of the health status of the udder, particularly related to mastitis outbreak. In this study twenty animals at different stage in lactation and age, during different times of the year were monitored. Thirty five outbreaks of clinical and subclinical mastitis together were diagnosed and observed. Dairy cows were subjected to monitoring from the first day of clinical mastitis outbreak until complete recovery. The animals characteristics are illustrated in Table 4-1. All milk samples were collected from the Royal Agricultural College's dairy, Elkstone Manor Farm. Elkstone farm was situated 200m above sea level

3.4.1 Animals and sampling

Due to impact of mastitis, (high costs of treatment and production losses) over the period 2003-2010, the herd size was reduced by third down to 200 animals. Although the head count was reduced, mastitis outbreak history at Elkstone indicated an increase of cases as presented in Table 3-3. These facts made this farm a suitable source for milk for this study.

The population of animals in this experiment represent the three different stages of lactation, age and mastitis history. The animals were self selected by developing

clinical mastitis. Monitoring was carried out for more than a calendar year (October 2007 – June 2009).

Table 3-3 Mastitis outbreak and milk production on Elkstone Dairy Farm

	Dec 2008	Dec 2007	Dec 2006	Dec 2005	Dec 2004	Dec 2003
Herd size	204	247	234	260	288	292
Milk production[L]/cow	8858	8690	9174	9314	9695	8804
Mastitis cases per herd	21	13	14	12	21	17
Mastitis cases per per 100 cows	10	5.5	7	4.5	7.3	5.8

Milk samples were taken from every quarter (as described in 3.3.) during morning milking. Milk samples were taken from animals with visible signs of mastitis (see point 3.3.), which was noted as the first day of clinical mastitis. Over the period of this research to ensure the consistency of sampling and best possible hygiene practice only two people collected milk samples for analysis.

Samples were collected until a complete recovery had taken place; which was defined by SCC below 2×10^5 and no presences clinical signs of mastitis. If any cow developed a further mastitis outbreak while under study then monitoring and investigation was extended until recovery from the new infection was complete. Following veterinary advice all animals on the farm, including study subjects, were treated with antibiotics from the first day of clinical mastitis outbreak

Healthy animals – heifers (2 in sample population) which have never suffered from any clinical infections – were used as for comparison with infected cows (it was expected to observe significantly different results for animals which have never suffered from mastitis when compared to animal which have developed this disease). Samples of foremilk were taken on regular basis during the lactation cycle.

3.4.2 Laboratory procedure

Immediately after collection the foremilk samples were cooled to 4°C and preserved with MMB Preservative Solution (Wychem Limited, Suffolk CB8 8YN). In the laboratory milk samples were stored at 3°C. The foremilk samples were measured for the density and the size of particles, diameter of milk fat globules, fat content, lactose concentration, protein concentration and electrical conductivity.

3.4.2.1 Determination of the number and the size of the particles in foremilk samples

Five samples of foremilk: back left (BL), back right (BR), front left (FL), front right (FR) and mix of them were measured by the particle counter. Samples were automatically diluted by 10^{-9} in the closed system with particle free water. Flow rate of samples was controlled by a pump and remained stable between 55-60 ml/min. Measurement of each sample was repeated ten times (two sets of five measurements) but only the last eight results were used in future calculations.

Raw data were converted into a Microsoft Excel compatible file format (Comma Separated Variable). To obtain the number of particles in a sample and number of particles in each size group an average of eight results was taken.

3.4.2.2 Determination of milk fat globules diameter by using direct microscopy

Milk samples were diluted by a factor of ten and carefully mixed with a rotary shaker. For each sample at least two microscope slides were prepared.

Images of milk fat globules were taken by using phase contrast microscopy (PCM), recorded and analysed with Infinity Analyse Program.

PCM depends on diffraction and scattering and differentially enhances the visibility of the light scattering edges of extended particles. This method was developed by Frits Zernike (1888-1966). The optical design is able to transform differences in phase to differences in amplitude [Oldfield, 1994; Murphy, 2002].

This study collected approximately 28 000 images of milk fat globules.

The diameter of MFGs was determined by using two independent programs: ImageJ Program (<http://rsbweb.nih.gov/ij/download.html>) and software written by Micro Research Ltd UK.

ImageJ Analysis

Scale was set up with eyepiece Stage Micrometer, 0.1 and 0.01 mm, Flatters and Garnett Ltd.

Reproduce of this experiment was depended on the parameters which were set as below:

Settings for ImageJ: size (pixel) – 0.25 infinity
Circularity 0.5 -1. Show – outlines

The particle radii were determined by ImageJ analysis. Bespoke software was written by Micro Research Limited, UK [B. Hill, unpublished]. The principle of operation is as follows:

- 1) Background threshold is determined using the statistics of the intensity distribution
- 2) All pixels in the image below background threshold are set to zero
- 3) The image is processed as a raster. When non-zero pixels are encountered a flood fill is performed. The number of pixels to fill the item is the particle's cross-sectional area, the number of pixels bordering the background measures the circumference. The size and circularity of the item can then be calculated.
- 4) Items that lie within the acceptable ranges of size and circularity are recorded and used in the calculation of volume median diameter (VMD) and number median diameter (NMD).

Results from ImageJ were obtained as area (A) for every single particle and converted into a Microsoft Excel file.

MND and VMD were calculated for every quarter for each day of monitoring using the following formula:

$$V_{md} = \frac{\sum_x \frac{V}{n}}{2\sqrt[3]{\frac{3}{4}\Pi}} \quad N_{md} = \frac{\sum_x A}{\sum_x n}$$

$$A = \Pi r^2 \quad d = 2\sqrt{\frac{A}{r}} \quad V = \frac{3}{4}\Pi \left(\sqrt{\frac{A}{r}}\right)^3$$

where: A – area of the particle calculated by ImageJ

d – diameter of single particles

V – volume of single particle

Micro Research Ltd Analysis

The software was written in C++ using the Nokia (Trolltech) application framework Qt 4. The application ran under the 64-bit Linux operating system (Suse 11.0) on a bespoke personal computer. The source code is available from the authors Micro Research Limited, UK [Hill, B., unpublished].

The software operated as follows:

Images were read from the hard disk and converted to 8-bit grey scale images.

The background level was determined as follows.

The intensity distribution was determined. The resulting histogram was evaluated and the first estimate of the background level was taken to be the intensity at which 70% of the pixels were less than this value.

The maximum intensity value was determined and the second estimate of the background level calculated as follows.

$$Threshold = \frac{\left(FirstEstimate + \frac{(PeakIntensity - FirstEstimate)}{2} \right)}{2} \quad \text{Equation 1}$$

This assumes most of the image is background, which it must be to resolve separate particles. This accounts for differences in image contrast.

- All pixels less than the background value were set to zero
- The image was analysed in a raster manner from the top left to bottom right. When a pixel that was neither background (0) nor filled (1) was found, a recursive flood fill was performed. The area was filled with a value (1) to indicate the area has been filled but also that it is not a background value.
- The number of pixels in the fill was the area of the particle while the number of pixels that shared a boundary with a background pixel was a measure of the particle's circumference. Knowing both the particle's area and circumference then the particle's circularity can be calculated as follows:

$$Circularity = \frac{(AreaObject)}{(AreaCircumference)} \quad \text{Equation 2}$$

Where the *AreaObject* is the number pixels used to fill the object.
AreaCircumference is the area of the circle whose radius is equal to:

$$\frac{(NumberofBoundryPixels)}{(2\Pi)} \quad \text{Equation 3}$$

A circularity of 1.0 implies a perfect circle. Lower values imply a rod. However single pixel wide lines will produce perverse results. As MFG particles appear as circles then items with circularities much less than one can be rejected.

When the image was scanned the VMD and NMD were calculated and saved in a

Comma Separated Value (csv) file such that each file contained only records for one animal, indexed by the date of measurement with the average of the values for NMD and VMD.

Errors (uncertainties)

In these experiments the relationship between pixel size and particle size was found to be 12 pixels per micron by using a reference graticule. A 2 μm diameter particle would be estimated as being 24 pixels wide. As pixels are quantised the uncertainty in measurement would be no more than 1 pixel in 24 or 4.1%. Because the radius is measured from the area the uncertainties were expected to be further reduced.

3.4.2.3 Fat content

The fat content of the milk was determined by using the Gerber Method, British Standards Institute BS 696: Part 2: 1989. Principle of method: sulphuric acid (91.0 g of H_2SO_4 per 100 g), milk and amyl alcohol were added into butyrometer. Immediately after mixing the butyrometer, closed with the stopper, was placed in a centrifuge. After adjusting the temperature in a water bath fat content was read from the scale.

3.4.2.4 Somatic cell count, protein content, lactose content

During the second phase of this research work somatic cell count, protein and lactose concentrations were determined by an independent laboratory – National Milk Records (NMR) (Harrogate, North Yorkshire, HG1 4LG). NMR used instruments from Foss of Denmark.

(<http://www.foss.co.uk/Solutions/ProductsDirect/CombiFoss%20FT+.aspx>).

3.4.2.5 Determination of electric conductivity

Electric conductivity of milk was measured with conductivity meter (Jenway model 4070). Conductivity standard was calibrated with 0.01N solution of potassium chloride with conductivity 1413 μS at 25°C. The conductivity data were used at the early stage of the research to confirm presence of disease.

3.5 Microscopic characteristics of milk particles

Foremilk samples were taken from animals on the first day of mastitis outbreak and after recovery process (see point 3.3).

In order to improve the visibility of milk particulate in foremilk, samples were mixed with Scarlet R (Sudan IV) – recommended stain for fat and methylene blue stain for cytoplasm [Gurr, 1953]. 20% Scarlet R, milk and methylene blue were mixed in proportion 1 : 1 : 0.25. Images were taken with 100 times magnification.

Milk fat globules appeared dyed in pink-orange shade and all cytoplasm in light blue. For each milk sample 10 to 20 images were taken.

Methods used to determine somatic cells count, fat content, lactose, protein concentration, electric conductivity and MFG size are described above.

3.6 Data analysis

Data collected in this research was a historical record of various parameters noted during mastitis and the recovery period. Significant changes and symptoms to milk particulate occurred before, during and after mastitis outbreaks. As the measured data varied gradually with time in any case with large, brief excursions indicating mastitis, statistical tests are not helpful. Further as these studies are limited to identifying indicators of mastitis on a relatively small sample of animals, each over short periods of time, it was not possible to construct statistical models to evaluate the sensitivity or specificity of these indicators.

Each particle count data point obtained for particle count for each day from each quarter was based on 8 readings. Each data point obtained for diameter of MFG was calculated from 10 to 15 results (over 10^3 particles detected per sample). The expected standard deviation in any one measurement is expected to be similar to the Poisson error, which is the square root of the sum of the counts. Typically the count was of the order of 1000, giving an expected uncertainty of approximately 3%. This was what was observed.

Some particle count samples were measured with a standard deviation of approximately 12% of the means. This was greater than expected from counting

statistics and was attributed to inaccuracy in the dilution process and non-homogeneity in samples. In particular samples of mastitic milk had clearly visible clots, which partly explain why samples from animals with mastitis had measurements with larger standard deviations.

The observed changes in particle counts with the onset of mastitis were found to a range over a factor of ten. Therefore the observed uncertainties as a result of the measurement process can be considered insignificant.

Microscopy Measurements

The VMD measurements were normalised so the results from each quarter mapped on to the range 0.0 to 1.0. This was to make comparisons between different quarters easier. Indeed it is remarkable that although the absolute values of VMD measurements of each quarter for a given animal differed noticeably after being normalised they were found to almost equal. The changes to VMD in one quarter (when used normalised data) were matched by changes of the same proportion in the other quarters.

The VMD results were examined to see if there were any obvious patterns in changes close to a known mastitis event. It was observed that prior to a mastitis event the VMD fell, usually to the baseline level, for typically three days, about 1- 5 days before mastitis was detected. As with the particle count measurements these are qualitative observations that require further investigation and modelling.

The next chapter will reveal results obtained by the procedures described above. Presented data will follow the subsequent order: (experimental protocol has also been illustrated in Appendix 11)

- Monitoring of latex particles in varying environments (latex spheres used as a standard) to establish their impact on latex behaviour / profile.
- Monitoring of milk particles in healthy and mastitic milk in varying environments to establish their impact on MFG behaviour.
- Monitoring of milk particle dynamics during mastitis and recovery period to establish influence of an inflammation on MFG behaviour.
- Review of the potential effect of the environmental factors on the above.

4 Chapter IV Results

Experimental and operational research work involved in developing the apparatus was successfully used to determine particle size and number in healthy and mastitic milk. Initial laboratory work established the behaviour of latex particles and milk particles in varying environments as well as data illustrating the influence of mastitis on milk particle characteristic by affecting their dynamics. This also showed that liquid particle counting technology can be used to investigate the onset of mastitis infection in dairy cattle and the physiology of the milk production, and secretion in the udder.

4.1 Monitoring of the behaviour of latex particles in varying environments

In order to achieve the best understanding of particle behaviour in the disperse system, the research work started with the monitoring of polystyrene latex particles (PLP) in varying environments (section 3.2). The latex spheres are widely available as uniform, regularly shaped and monodispersed particles in size ranges from 20 to 900 nm. Density of spheres is approximately of 1.05g/cm^3 and refraction index of 1.59 at 589 nm measured at 25°C . These characteristics have made PLP the ideal standard and they have found applications in light scattering studies and colloid system research. Moreover in this research similar size and regular shape of PLP are convenient for the comparison and better understanding of properties, and the behaviour of milk's main particles - milk fat globules.

Milk is a most complex fluid and the concentrations and interactions of its components influence osmotic pressure, pH, ions equilibrium and micro-activity. During the development of this research work it became clear that milk samples could not be analysed by the particle counter accurately without dilution. Moreover mastitic milk characteristics were expected to be different in comparison to the sample from healthy animals. Therefore PLP assays were necessary to establish correct dilution of the sample and investigate the effects of variations in pH, salinity and temperature.

The results obtained for latex particles are illustrated in Appendix 5A and 5B.

Effect of dilution and measurement of cross-contamination between samples

It was established that a dilution ratio of up to 10^8 is needed for consistent counts of latex particles (illustrated by Appendix 5). Particle numbers obtained by using particle counter were confirmed by results from direct microscopy. The liquid particle counter has a concentration limit of 4000 particles per ml; when the concentration exceeds this value, coincidence counting errors increases noticeably, when more than one particle is in the viewing volume of the particle counter at any one time, giving the illusion of fewer and/or larger particles. Samples were run through the particle counter in the decreasing order of dilution which did not affect particle count, cross-contamination between was not observed. The standard procedure required a sample run of particle free water in between the two tested samples; however, this experiment provided evidence that the samples of different particle counts can be examined (after dilution) one after another without influencing each other's results.

Effects of salinity, temperature and sample age on particle count

Results obtained showed the identical behaviour of particles in different pH environmental solution (range from 5 to 8) and different sample age. However, inconsistent readings were obtained when latex samples were heated to $>40^{\circ}\text{C}$ and when also diluted in a 10% sodium chloride solution. These preliminary investigations, described above, established that the experimental equipment gave poor results only in extremely severe circumstances. Reliable and accurate results were found in conditions likely to be used for milk analysis.

4.2 Monitoring of the behaviour of milk particles in varying environment

4.2.1 Milk from healthy animals

The operational protocol was applied to latex study as described above. Particle numbers in milk obtained by using particle counter was confirmed by results from direct microscopy. The preliminary experiment conducted that high rates of dilution;

in the order 10^8 to 10^9 were required to obtain accurate particulate counts. Appendix 6 illustrates the outcome of this part of the experimental work.

Effect of cross-contamination between samples

The milk samples initially diluted by 10^{-4} to 10^{-8} were run through the apparatus in an increasing dilution order. No effect of cross contamination was found during the processing of milk samples; which allowed a reduced time on washing-out procedures between replicates of the same sample of milk (please see Figure 4.1).

Effects of salinity, temperature, pH and sample age on particle count

No adverse effects were seen over a range of pH (pH 5 to 8) or with variations in salinity (0.5 to 1.5% w/v). The stability of milk at high dilutions needed for the optical resolution of particles indicates that the FMS measurement system was suitable for milk analysis. This finding allows the use of particle free water for analysis of milk. Temperature was found to depress milk particle counts (MPC) particularly over 40°C which is above physiological temperatures; it would be unlikely to approach this within a farm environment. MPC were found to be stable over periods up to 4 hours at room temperature and up to 44 hours (maximum times studied) when stored at 4°C . No effect of cross-contamination was found during the processing of milk samples; which allowed a reduced time on washing-out procedures as explained above.

4.2.2 Milk from animals diagnosed with mastitis

In order to minimise the handling error the operational protocol was identical for samples obtained from healthy animals. Furthermore the same rates of dilution were used. Appendix 7 informs the results of this part of the study.

Effect of dilution and measurement of cross-contamination between samples

Particle numbers in mastitic milk obtained by using the apparatus was confirmed by results from direct microscopy method (haemocytometer).

Mastitic milk samples required a high rate of dilution; in order 10^8 to 10^9 , to obtain accurate particle counts. These findings show that preparation for both healthy and mastitic milk samples can be carried out the same way; which will reduce the likelihood of human error by not requiring specific methods of assessment.

There was no cross-contamination identified in between different dilutions from milk samples collected from animals diagnosed with mastitis.

Effects of salinity, temperature and sample age on particle count

There was no effect on particle counts over a range of pH 5 to 8 or with variations in salt concentrations (0.5 to 1.5% w/v). Milk from untreated infected quarters retained a stable particulate count for 72 hours (maximum time studied) when stored at 4°C. Mastitic milk heated up to 40°C gave rise to a highly modified particle size distribution with fewer counts of larger particles. Mastitic milk remains stable for analysis in particle free water.

In conclusion, milk can be analysed without any concern that the preparation process may be interfering with measured parameters. Apart from dilution, milk does not have to be prepared for examination as particle count and dynamics remained stable under operational conditions explained above.

4.3 Influence of mastitis on particulate content of milk

The results presented above helped determine the operational, safe and reliable protocol which can be applied for the last part of experiment – a monitoring of milk particulate behaviour throughout the mastitis outbreak and recovery process. This provided opportunity to challenge the hypothesis that the particulate content of milk is correlated to physiological conditions of lactating dairy cow and also to bring new evidence about physiological relationship between neighbouring quarters. Results were presented in the form of figures and tables; the characteristics of animals for which results were obtained are presented in table below.

Table 4-1 Characteristics of monitored animals.

(* non-bold- clinical mastitis outbreak; in bold and underline subclinical mastitis outbreak).

Key BL back left, BR back right, FL front left, FR front right quarter.

Cow no	Age [years]	Parity	Day of lactation	Quarter *	Duration time [days]
28	11	9	1	BL/FL	9
323	8	6	195	FL	21
26	7	5	25	FR/ <u>FL</u>	20
71a	6	4	52	BR/FR	33
			65	FR	
			73	BR	
71b	7	5	62	FL/ <u>FL</u> / <u>FR</u>	28
4	6	4	32	FL	66
			47	FR	
			62	FL/FR	
29	5	3	25	FL	18
256	5	3	76	FR	22
			81	<u>BL</u>	
218	5	4	239	FR	20
1	5	4	127	BR	20
52	4	2	197	BR	20
51	4	2	222	BR	20
36a	4	2	15	FR/ <u>FL</u>	20
			24	FR/ FL	
36b	4	2	80	FL	20
128	4	2	47	FR	20
110	2	1	128	FL	200
			197	FR	
202	2	1	78	<u>BR</u>	210
			151	BL	
170	2	1	73	BL	18
			79	<u>FR</u>	
264	severe mastitis, animal culled during monitoring			BR/FL	12
109	8	6	81	FR	20

4.3.1 Results obtained by PHA and particle counter

Milk particulate count

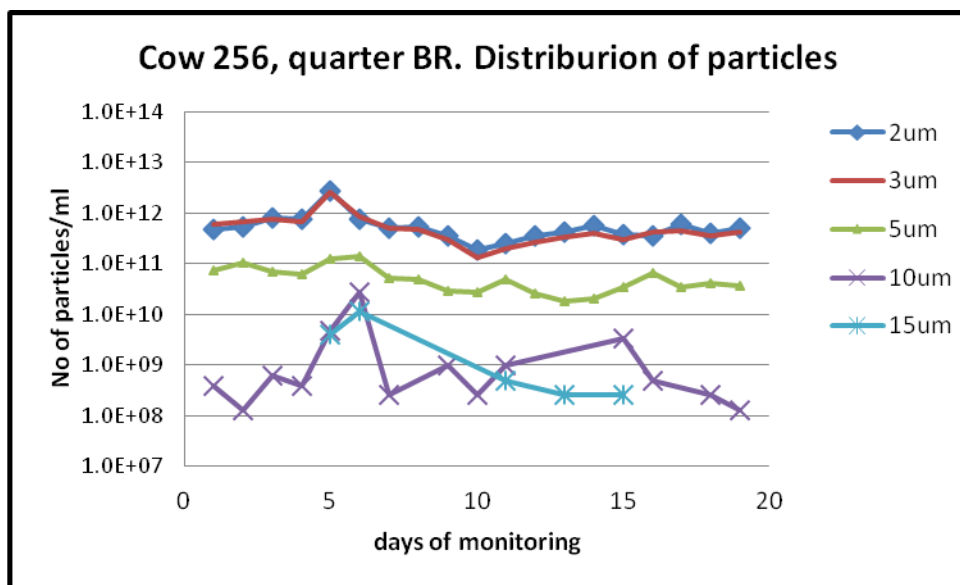
Particle count in milk obtained from PHA was found to be in the range of 10^{11} to 10^{13} particles per ml with an average at 10^{12} particles per ml. This is a greater number than previously reported by King (1957, 1969), where the concentration of MFG in healthy milk was calculated to be $10^9 - 10^{10}$ MFG/ml in the size ranges above $2\mu\text{m}$. However, our measuring system was able to detect particles as small as $1\mu\text{m}$, and since more than 80% of MFG are reported to have diameter smaller than $2\mu\text{m}$ this number is in broad agreement. The particle counter used in this study counted particles in the range from $1\mu\text{m}$ up to $30\mu\text{m}$ divided into six classes (section 3.1.1.3). The PHA classified particles in the range from $1\mu\text{m} - 15\mu\text{m}$. The technique used in this study allow to count a wide range of particle types to include MFG (main particulate of milk), leucocytes, bacteria, cellular material as described in Introduction and summarised in Table 4-2.

Table 4-2 Particles in milk and their behaviour during the monitoring process

Type of particle	Size	Detectable by PHA / particle counte	Observed behaviour
MFG	0.2-20 μm	yes	The total count of milk particles was found to be in the range from 10^{11} to 10^{13} During recorded mastitis outbreaks increase and/or decrease in total count were recorded.
Foreign matters such us leucocytes	14-15 μm	yes	During mastitis the number of somatic increased to over 10^6 /ml
Casein micelles	0.04-0.3 μm	no	n/a
Bacteria	<1-10 μm	yes	The total count of bacteria in monitored samples was not established. The number of microorganisms in milk might vary from 10^3 to 10^6 /ml (the second value is an indicator of poor udder hygiene / infection)

In all monitored cases the highest number of particles was recorded in range 2µm, closely followed by size 3µm, 5µm 10µm and 15µm. These particular examples illustrated on graphs below (4-1 and 4-2) show typical distribution of milk particles mastitic and healthy milk sample; the same pattern was observed for all examined cows. This result confirms the findings of other research workers (referred to in previous chapters) that the smallest milk fat globules contribute the highest number of milk particulate.

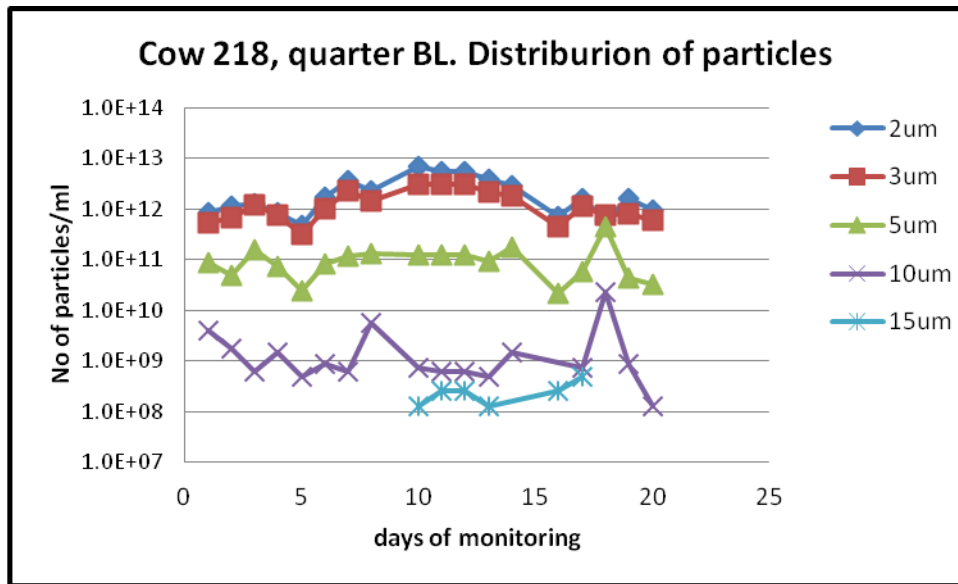
Figure 4-1 Cow 256 quarter back left. Distribution of particles



Average particle number for this quarter was calculated at 1.7×10^{12} . This animal was diagnosed with the second outbreak of mastitis on the day 6.

Standard error of the mean (SEM) values were left off the graphs for clarity. Calculated values were too small and not visible under the point marker. This was observed for collected date (particle count and size).

Figure 4-2 Cow 218 quarter back left. Distribution of particles.



Average particle count for quarter back left was calculated at 2.4×10^{12} . This quarter was not diagnosed with mastitis.

Particles count on the quarter and udder level

For all monitored animals foremilk samples from all four quarters and equal, physical mix of four quarters were analysed. Collected data were built into graphs which enabled comparison between the behaviour of particles in all four quarters during monitoring.

It was observed that the range of the particle numbers remained the same over the monitoring period for all four quarters within the udder. Moreover a high correlation between the behaviour of particles in all four quarters was characteristic for all results in this research work. These results are illustrated by examples below (Figure 4-3 and Table 4-3). Monitored animals were at different ages and stages of lactation, they were also examined at different time of the year (season). However similarity between the particle numbers on daily basis was extremely high; if the number has changed in one quarter the count change was noticed in others. It was remarkable to observe this kind of behaviour of milk particles in four anatomically separated parts of an animal body.

High correlation between particle counts in all four quarters has not been described previously.

Most of the mastitis cases in this study were observed from the first day of the clinical outbreak. SCC were measured to confirm presence of disease. It was expected for SCC to be elevated in the infected quarter; which was recorded. However it was not expected to find that density of particles changed significantly in all four quarters, even if only one was infected with clinical mastitis. Samples of foremilk from infected quarters had significantly different appearances if compared to the milk sample from healthy quarter/quarters from the same cow. Visual presence of clots, discolouration (yellow colour break or colour of diluted milk), and jelly consistency was observed; also foreign, unpleasant smells were noticeable. The foremilk samples from the quarter with healthy SCC readings looked normal. Therefore highly similar particle count/range found in all four quarters was extremely surprising. Over twenty animals were constantly monitored for a minimum of twenty days and each of them developed at least one outbreak of clinical mastitis. Described above behaviour of MFG was found to be consistent throughout the whole study.

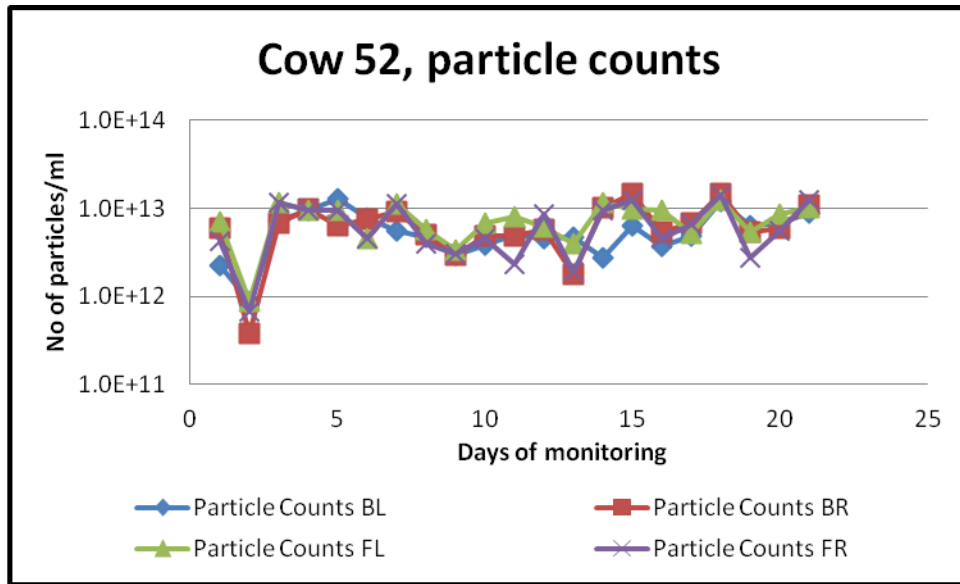
Table 4-3 Correlation coefficient calculated for PHA results for each quarter

(r value 0.497 **p=0.05**; r value 0.623, *p=0.01*; r value 0.742, *p=0.001*)

Correlation coefficient calculated for PHA results for each quarter;										
probability level:		0.001		<i>0.01</i>	0.05					
COW NO	1	4	71a	110	323	52	29	51	256	264
BL/BR	0.84	0.52	0.64	0.68	0.57	0.56	0.75	0.90	0.81	0.33
BL/FL	0.86	0.30	0.59	0.70	0.60	0.56	0.48	0.60	0.80	0.76
BL/FR	0.87	0.22	0.62	0.12	0.45	0.61	0.67	0.88	0.44	0.22
BR/FL	0.88	0.24	0.64	0.76	0.90	0.78	0.45	0.70	0.75	0.36
BR/FR	0.83	0.47	0.50	0.28	0.55	0.87	0.79	0.86	0.66	0.51
FL/FR	0.92	-0.02	0.54	0.22	0.63	0.83	0.52	0.89	0.56	0.05
COW NO	218	36a	71b	36b	26	128	109	28	202	
BL/BR	0.71	0.44	0.31	0.90	0.33	0.97	0.54	-0.21	0.52	
BL/FL	0.76	0.50	0.44	0.88	0.32	0.97	0.47	-0.11	0.34	
BL/FR	0.66	-0.29	0.05	0.91	0.24	0.92	0.42	-0.05	0.55	
BR/FL	0.76	0.24	0.23	0.94	0.40	0.98	0.64	0.69	0.32	
BR/FR	0.84	-0.18	-0.13	0.90	0.05	0.93	0.09	0.76	0.60	
FL/FR	0.66	0.22	0.43	0.78	-0.05	0.91	0.12	0.70	0.48	

Table 4-3 above illustrates the coefficient of correlation for particle counts for neighbouring quarters. The calculated correlation has been found to be significant for majority of animals with probability level from 0.001 to 0.05; No significant correlation was observed in only two animals. Figure 4-3 below demonstrates the same correlation between particle counts based on day-to-day monitoring.

Figure 4-3 Particles pattern obtained for cow 52.

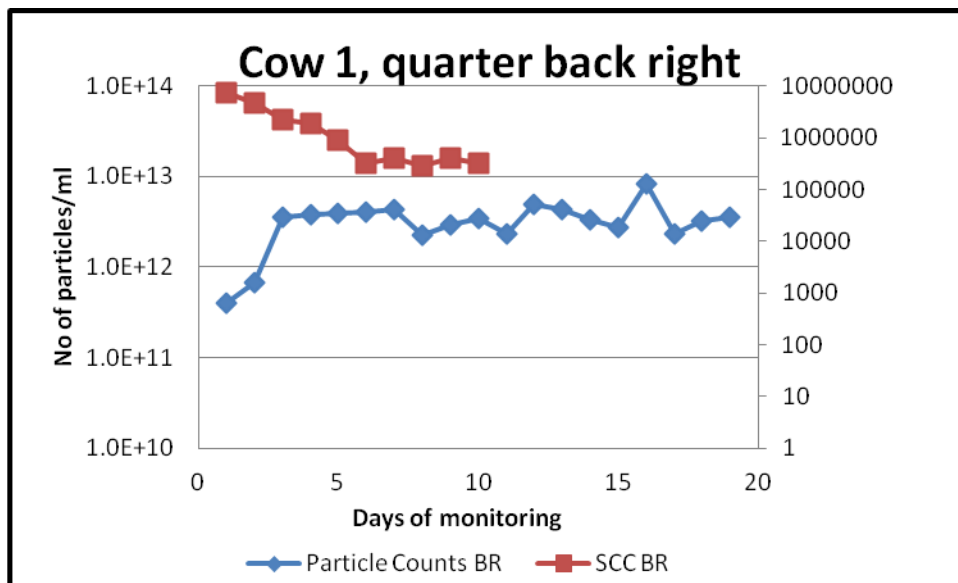


Clinical mastitis was diagnosed on the first day of monitoring in quarter back right. However particle counts in all four quarters remained highly similar. Data for this particular animal was selected to illustrate high correlation between the number of particles between all four quarters.

Changes in particle counts during inflammation of the udder

As mentioned earlier the majority of observed mastitis outbreaks were diagnosed on the farm and the monitoring was initiated following the clinical signs. Therefore the collection of data for most of the animals started on the very first day of clinical infections. Careful study of density of particles on a daily basis indicated that during clinical mastitis outbreak the particle count was significantly affected. Figure 4-4 below illustrates the behaviour of particles in foremilk samples taken from cow no 1, during twenty days of monitoring. A clinically diagnosed mastitis outbreak occurred in the first day in back right quarter. The comparison of particle counts clearly points out that the presence of infection affects MFG production and changes their number and distribution.

Figure 4-4 Cow 1, quarter back right. Clinical mastitis was diagnosed on the first day of monitoring.

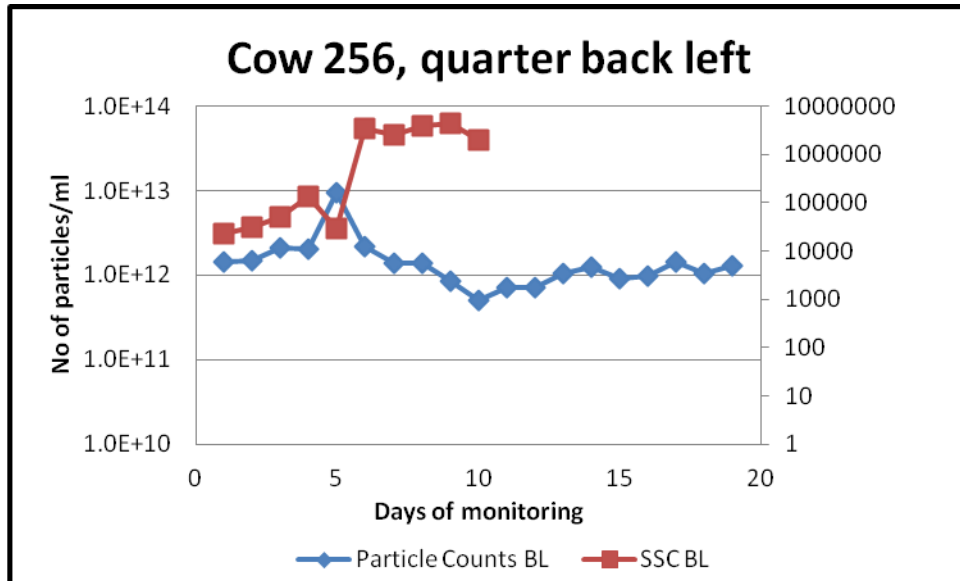


Results obtained for cow 1 showed lower density of particles over the period of infection (see graph above). These results are in accordance with King's (1957, 1969) work. However these changes were not consistent for all observed animals; in some cases milk particulates during the first day of the infection increased and the others decreased. Obtained results for cow 4 showed an increase in particle counts during the period of disease. Moreover there are examples of animals showing both behaviours in particle counts. On the other hand, when cows are not diagnosed with mastitis, the results are relatively stable throughout the year and lactation; in comparison to results that clearly have significant and obvious peaks/dips indicating mastitis.

In order to better understand the mastitis influence on the udder, animals were observed for a period of approximately 20 days which allowed time to collate data describing the recovery process. Surprisingly, for some animals (quarters) a significant increase or decrease in particle count was observed. These quarters did not show any changes in milk appearance such as clots, change in colour, swelling and/or evidence of udder pain. Conversely it was found that SCC were elevated to over $2 \cdot 10^6$ cells per ml. Seven cases of subclinical mastitis were documented, some of them had developed in quarters which have recovered from clinical mastitis; some developed in neighbour quarters which were not diagnosed with the clinical type of disease.

Results obtained for cow 256 (Figure 4-5), quarter back left; this quarter was regularly examined but not diagnosed with clinical mastitis. The particle count rose sharply on day 5 and on the next day SCC became elevated, which indicated an outbreak of sub-clinical form of the disease.

Figure 4-5 Cow 256, quarter back left. Sub-clinical mastitis outbreak.

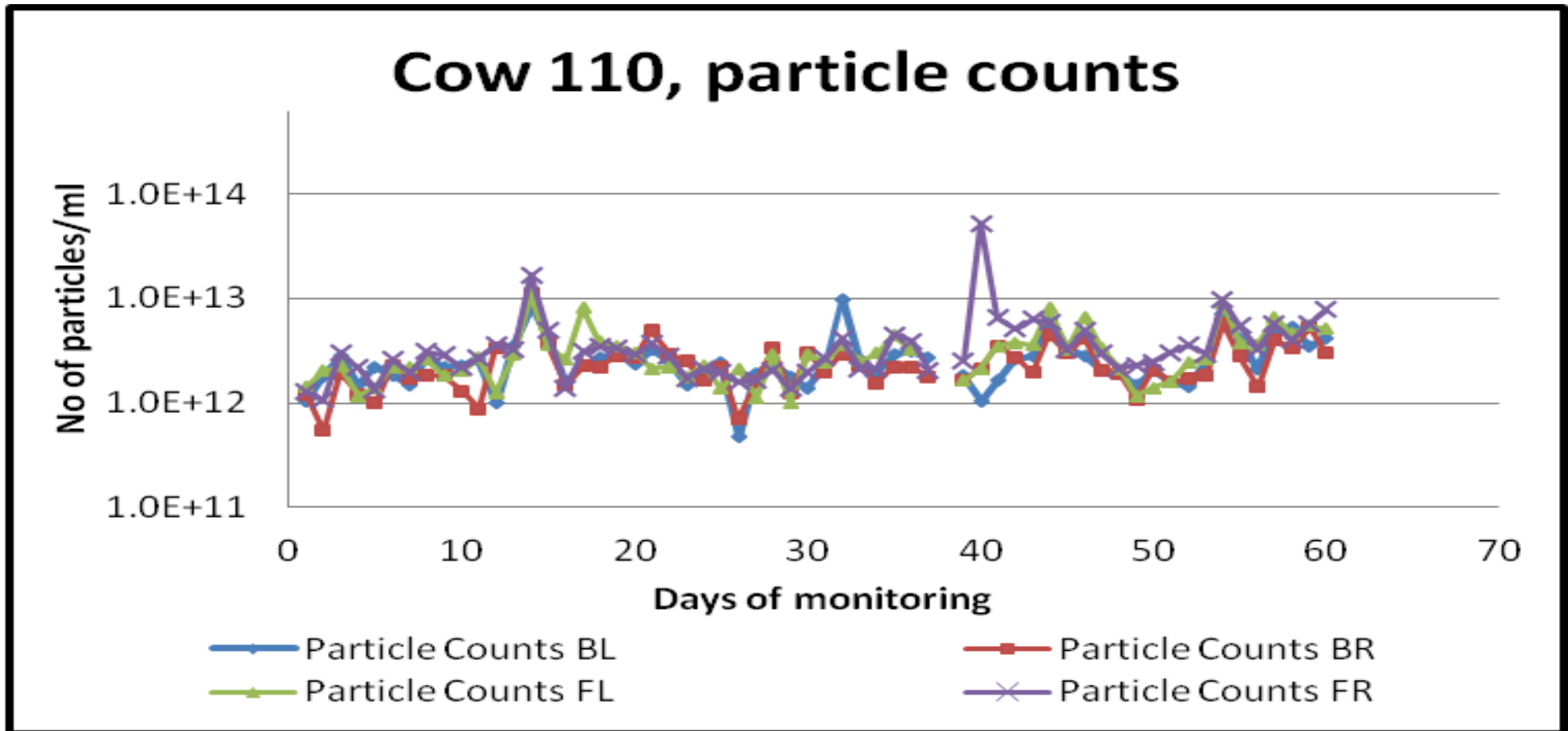


Equality to clinical outbreak of disease, particle count was clearly affected in all four quarters, even though SCC increased only in the infected one. This was behaviour was observed in all seven cases.

Further outbreaks of clinical mastitis were also diagnosed throughout this study. The clinical onset of disease was confirmed by elevated SCC and followed by visible changes to the foremilk samples and animals.

In animals that developed mastitis during the course of the monitoring series, particle concentration changes could be detected in 75% of cases up to three days before clinical signs of mastitis were present or SCC were significantly elevated. This suggests that particles change during pre-clinical mastitis as bacteria become established, but before the SCC rises.

Figure 4-6 Cow 110, particles counts.



Cow 110, Figure 4-6 was diagnosed with clinical mastitis on days 17 in quarter front left and on day 41 in quarter front right. Particle count remained similar for all four quarters even though two of them had not developed mastitis during the course of monitoring. Particle units rose sharply on day 15 and 40; two days before clinical signs of mastitis for the front left quarter and one day before for front right quarter. Although one quarter was presenting mastitis quite clearly; it was the remaining quarters where trends were evidently similar to the infected on and each other.

Particle count in composite sample

Similar particle variations were observed in the composite samples and these were also similar to changes obtained from the arithmetic average of the individual quarter counts (see Figure 4-7 and Table 4-4). This finding suggests that the detection of changes to particulate content of milk caused by mastitis (even for cases when only one quarter is infected with clinical or subclinical mastitis) can be achieved by monitoring particles in a combined milk-stream from an individual cow. Changes in the particles concentration during the monitoring period were in range 10^3 particle/ml, but the collected data showed that milk particulates could decrease or increase during the outbreak of disease, with the changes of sufficient size that they unlikely to be seen in milk from healthy animals.

Figure 4-7 Cow 323. Comparison between number of particles in composite sample and arithmetical average of individual quarters count.

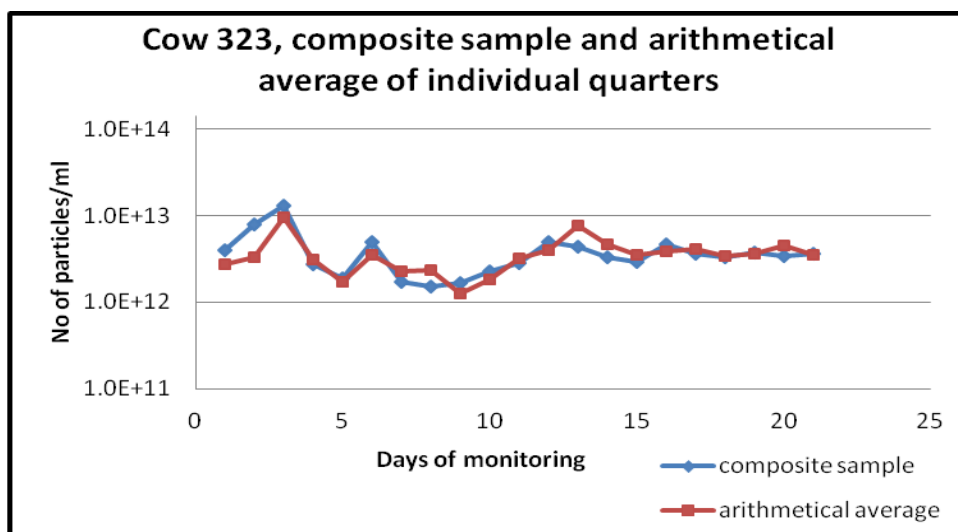


Table 4-4 Correlation coefficient calculated between composite sample and arithmetic average of the individual quarter counts (r value 0.497 $p=0.05$; r value 0.623, $p=0.01$; r value 0.742, $p=0.001$).

Correlation coefficient calculated for PHA results for composite and average;						
probability level:		0.001	<u>0.01</u>	0.05		
Cow No	110	202	29	71	323	4
composite/average	0.811	<u>0.741</u>	0.934	0.32	0.768	0.501
Cow No	1	218	52	109	71b	36b
composite/average	0.898	0.816	0.756	<u>0.74</u>	0.608	0.793
Cow No	26	128	28	36	256	
composite/average	0.216	<u>0.635</u>	0.52	0.614	0.937	

The table above illustrates the fact that significant correlation, between composite sample and arithmetical average of individual quarters count, has been observed for major proportion of monitored animals.

4.3.2 Results obtained from direct microscopy

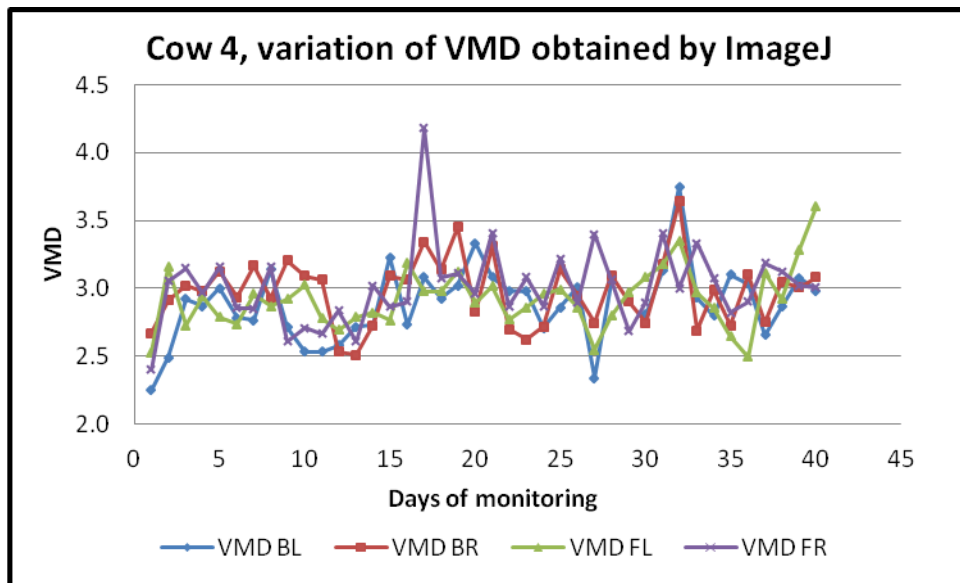
The particle counter itself has not been applied and established as a tool suitable for the monitoring milk particulate behaviour in order to predict the early onset of mastitis. Therefore a second monitoring process was essential to establish accuracy of PHA and particle counting technology.

The results presented below have been obtained by using the direct microscopy method. This method was chosen in order to monitor the changes in MFG dimensions during mastitis outbreaks. Analysis of MFG was by the estimation of both volume median diameter and number median diameter. VMD is the cube root of the mean of the particles diameter. This gives a value that is biased towards larger, less frequent, sizes. NMD is the simple mean of particles diameter. Conventionally in particle size measures the VMD is reported therefore all presented below results are for VMD.

ImageJ was used initially to measure the VMD of MFG from images of milk taken, under microscope. Collected images from all monitored animals were analysed. However obtained results indicated that that there is no correlation between the VMD of MFG and mastitis. This finding was not in agreement with results obtained by other researchers (King 1957, 1969). There was a concern that the manual method of

processing images (very time consuming and laborious) which required frequent adjustment for each slide of the background threshold levels caused unwanted variation in quality and quantitative analysis. All settings of the threshold level were based on human eye subjective assessment to which all obtained VMD results were subject to larger uncertainties. Therefore software was developed (B. Hill; Micro Research Ltd) to automatically process each of the images and apply an objective and consistent method to determine the image background threshold levels. Uncertainties were reduced and significant changes in the VMD values were noted as a result of automated image analysis. Graphs below (4-8 and 4-9) illustrate results for the same animal by using two different methods of analysing images; ImageJ (manual method) and software (automated method).

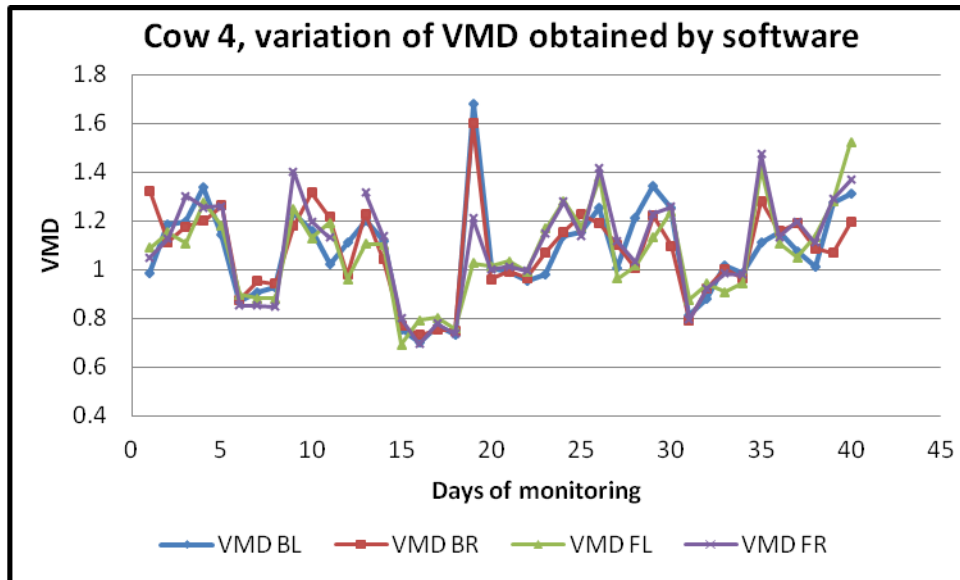
Figure 4-8 Cow no 4, variation of VMD values obtained by using ImageJ technique (manual).



Cow 4 was diagnosed with clinical mastitis on the 1st, 16th and 31st day in quarter front right and on 31st day in quarter front left. Data presented on figure 4-8 shows low variability between the results for each quarter. This suggests that the information in the data sets have not been resolved due to the greater uncertainties in the overall measurement method. The results from the software - automated method shows features which can be associated with observed mastitis outbreaks. Therefore all data

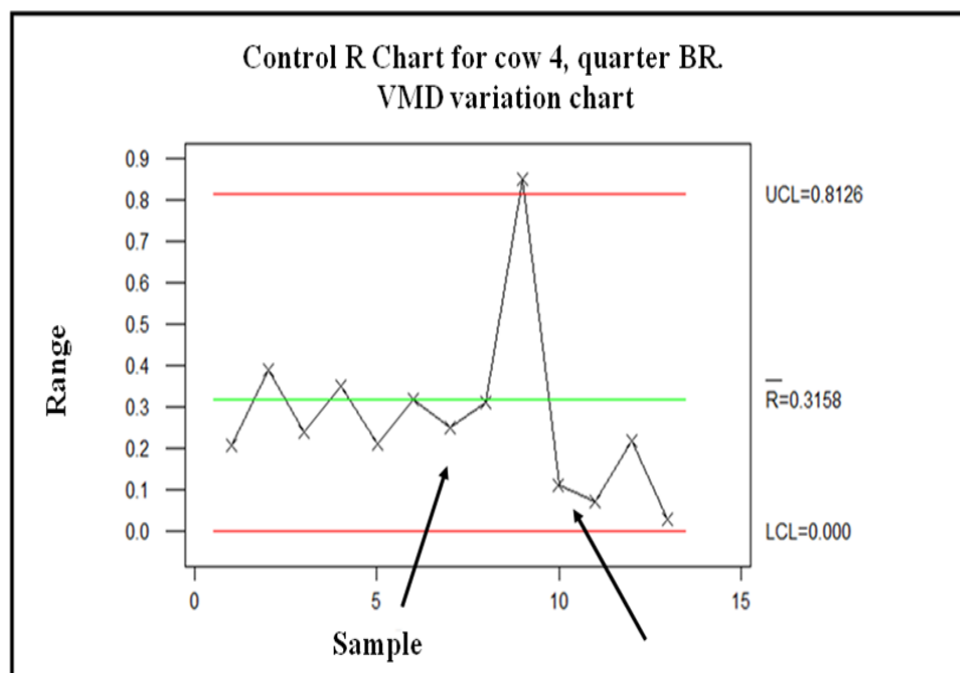
presented in this chapter is obtained by using automated method, as illustrated on Figure 4-9.

Figure 4-9 Cow no 4. Variation of VMD values obtained by using Micro Research Software technique.



The arrows present on figure 4-10 point out the minima observed prior to mastitis (this will be discussed in detail below).

Figure 4-10 VMD variation chart, cow 4, quarter back right. Subgroup size 3.

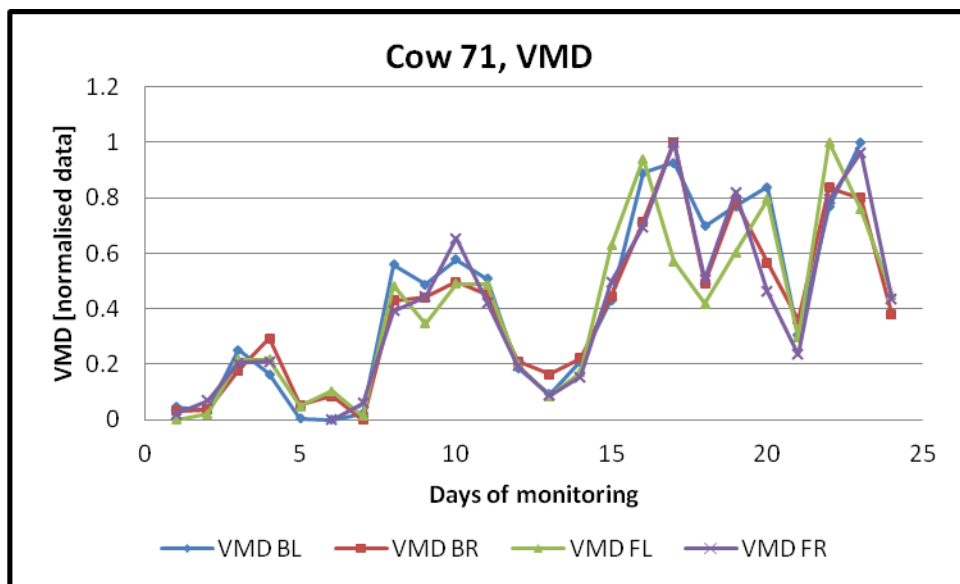


R Charts are commonly used to determine if process is stable and predictable, and to monitor variable's data (for samples collected in regular intervals). Horizontal lines are drawn at the mean range value (R), upper control limit (UCL) and lower control limit (LCL).

VMD variation on the quarters and udder level

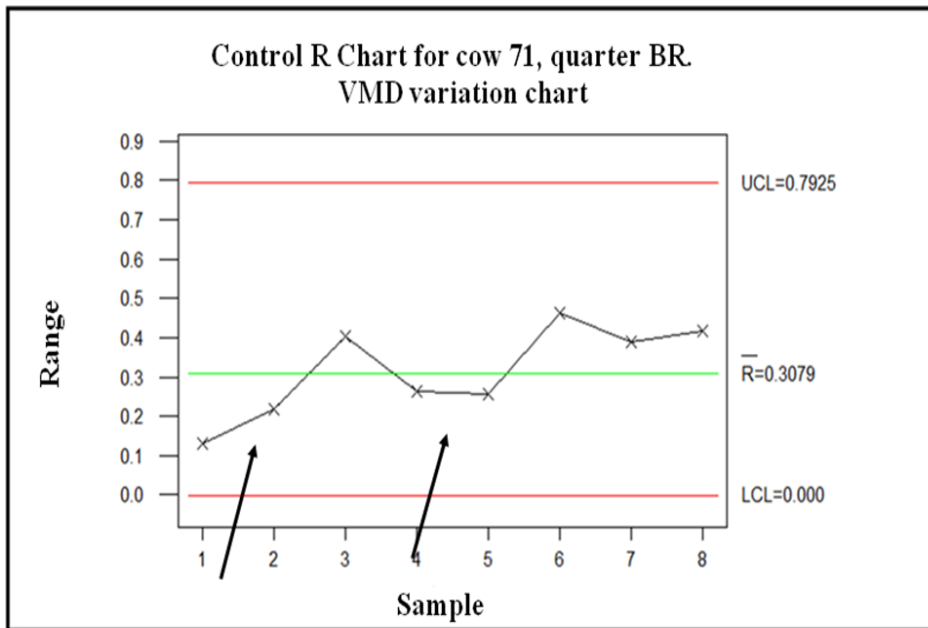
Variations identified in the behaviour of milk particles observed by using PHA, were confirmed with results obtained from dark field microscopy. The figure below demonstrates the pattern of the changes of particle size during monitoring for cow 71. Mastitis was diagnosed only in two quarters; captured data shows the interesting features - flat minima which corresponded with observed outbreaks of mastitis. The pattern of rapid fall in VMD to minimum and a similar rise after period of three days have been observed with other animals; please refer to figure 4-11 and 4-12.

Figure 4-11 VMD, Cow 71.



The figure above illustrates a comparison between the sizes of particles in foremilk samples. Mastitis outbreak was observed in quarter back right on days 1, 11 and 22nd and in quarter front right on day 11th. Quarter back left and front left did not develop an inflammation.

Figure 4-12 VMD variation chart, Cow 71a, quarter back right. Subgroup size 3



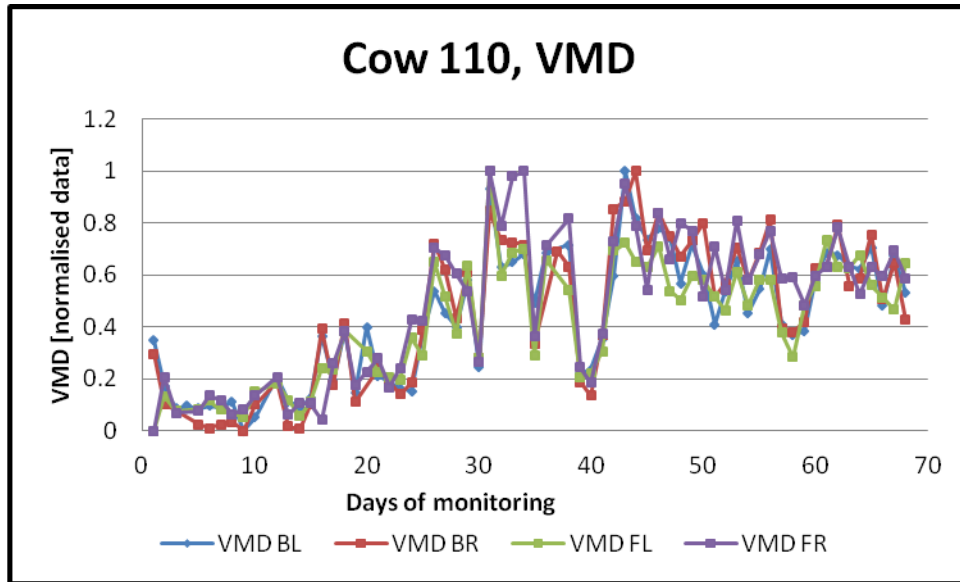
Horizontal lines are drawn at the mean range value (R), upper control limit (UCL) and lower control limit (LCL).

The arrows in above graph point out the flat minima observed before mastitis outbreak. As presented below this behaviour was also present in not infected quarters. Some animals developed further outbreaks of mastitis which allowed the opportunity to monitor all stages of this disease, from pre-subclinical stage to full recovery. The decrease in VMD values were observed for some animals on the day of mastitis outbreak, for others well in advance. These results are in accord with data collected from PHA/particle counter where particulate content of milk has been affected by the inflammation. Moreover they show that the image analysis method has delivered objective and consistent results with low enough uncertainties to see distinctive characteristic dynamics in the VMD of milk fat globules. In this case also particles size is affected which is in agreement with other research work (see Chapter Introduction).

The figures below illustrate an example of variation in milk fat globules size during monitoring of two heifers; cow 110 and cow 202. These two animals were monitored for the time of their first lactation. They were exposed to the various environmental

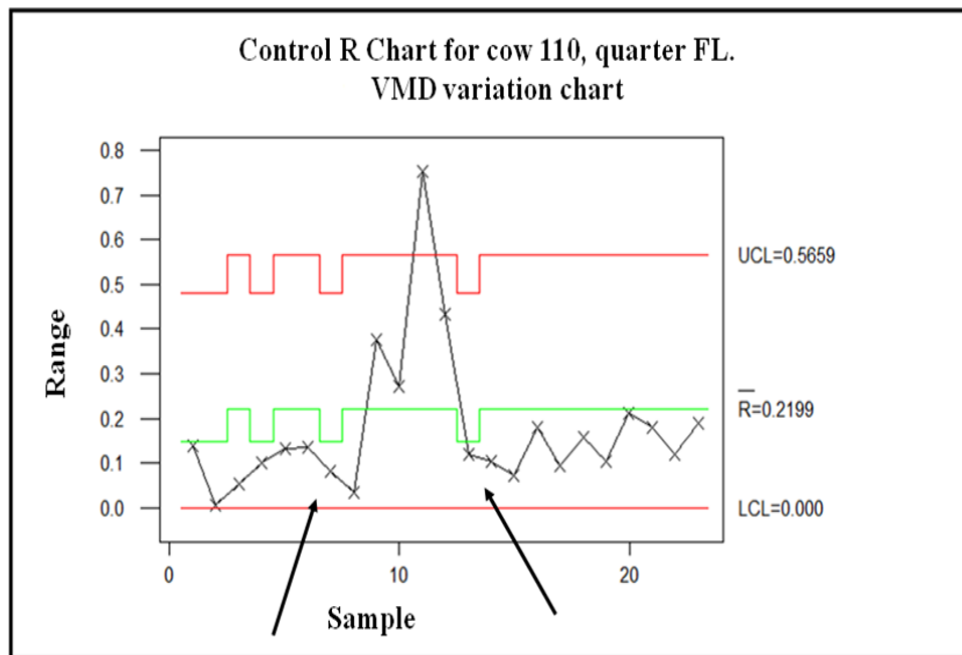
factors and different diet. Additionally their VMD values were recorded throughout the lactation cycle to which has been described to affect milk fat globules and total fat content.

Figure 4-13 Cow 110 changes in particle size during monitoring period. Mastitis was diagnosed in quarter FL (day 17th) and FR (day 41st)



Graph below illustrate variation of VMD.

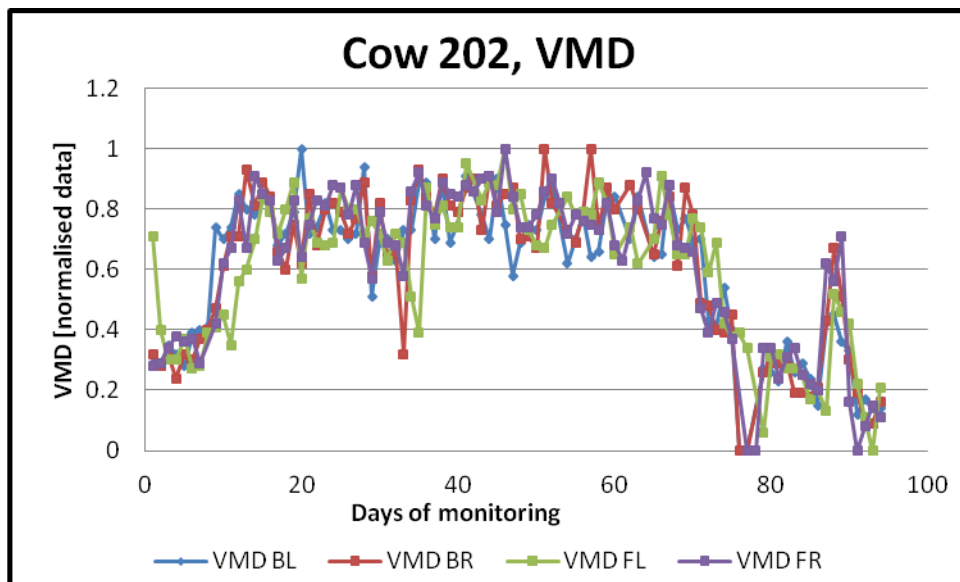
Figure 4-14 VMD variation Chart, Cow 110, quarter front left. Subgroup size 3



Horizontal lines are drawn at the mean range value (R), upper control limit (UCL) and lower control limit (LCL).

During the monitoring period of cow 110 two quarters were affected with mastitis; the other two had never developed an inflammation. In the case of cow 202, clinical mastitis was diagnosed only in quarter back right on the day one and subclinical mastitis (high SCC) in quarter back left on day 35. However, despite this pattern of mastitis development changes of the size of MFG were significantly correlated between all four quarters as illustrated by figures 4-13 and 4-14, 4-15 and Table 4-5.

Figure 4-15 Cow 202; changes in particles size during monitoring period



MFG size was affected in all four quarters even if only one of them was diagnosed with mastitis (clinical and/or subclinical). Indeed 16 of monitored animals showed a significant correlation of the VMD values between four quarters, irrespective of the number of quarters affected 2 showed correlations between three quarters and remaining 1 between two quarters.

Table 4-5 Correlation coefficient calculated for VMD results for each quarter for 19 animals (r value 0.497 **p=0.05**; r value 0.575, p=0.01; r value 0.679 **p=0.001**)

Correlation coefficient calculated for VMD results for each quarter;										
probability level:			0.001	<u>0.01</u>	0.05					
COW NO	1	4	71a	110	323	52	29	51	256	264
BL/BR	0.38	0.84	0.96	0.96	0.48	0.96	0.95	0.94	-0.17	0.83
BL/FL	0.52	0.69	0.90	0.92	0.83	0.97	0.88	0.97	0.34	0.93
BL/FR	0.49	0.54	<u>0.64</u>	0.89	0.85	<u>0.68</u>	<u>0.66</u>	0.95	0.52	0.83
BR/FL	0.29	0.69	0.90	0.92	0.73	0.97	0.87	0.96	0.01	0.84
BR/FR	0.44	0.56	<u>0.64</u>	0.90	0.69	0.75	0.77	0.90	0.02	0.93
FL/FR	0.54	0.72	<u>0.67</u>	0.92	0.88	0.74	<u>0.65</u>	0.97	-0.01	0.91
COW NO	218	36a	71b	36b	26	128	109	28	202	
BL/BR	0.89	0.89	0.79	0.79	0.94	0.88	0.30	0.20	0.93	
BL/FL	0.91	0.82	0.80	0.75	0.96	0.90	0.42	0.18	0.93	
BL/FR	0.80	0.86	0.85	0.81	0.96	0.85	<u>0.61</u>	0.35	0.91	
BR/FL	0.93	0.80	0.90	0.89	0.93	0.88	0.92	0.97	0.93	
BR/FR	0.91	0.91	0.93	0.88	0.97	0.79	0.96	0.94	0.89	
FL/FR	0.92	0.77	0.92	0.85	0.96	0.77	0.97	0.93	0.91	

In summary; the correlation found in both measurements (particle numbers and size) between four quarters was observed to be significant for majority of monitored animals. However particle counts are less correlated compared to size expressed as VMD (see Table 4-5 and 4-3). Both variants were also clearly affected by the event of mastitis outbreak, clinical and subclinical form.

4.3.3 Results obtained from total fat content, protein and lactose assay

The total fat content was found to be very low in comparison to the literature. However the fat content was measured in the foremilk samples of milk when text books and other literature refer to the fat content of the milk extracted during the whole milking. The foremilk is drawn during the first phase of milking and it has been reported that milk fat content increases during milking [Lollivier, *et. al.*, 2002; Vangroenweghe, *et. al.*, 2002; Ontsouka, *et. al.*, 2003].

Results obtained for lactose and protein do not indicate any evidence of association with mastitis outbreaks. There were no significant changes which could indicate the

physiological changes within the udder and/or disease. Table 4-6 illustrates the total fat correlation between all four quarters. In this study fat content was measured by Gerber method; for single milk sample only two readings (repetitions) were obtained. Therefore degrees of freedom, for correlation below, were low and the probability level $p=0.05$, was high (0.878).

Table 4-6 Correlation coefficient, calculated for total fat content for each quarter (for foremilk samples) by Gerber method

Correlation coefficient calculated for total fat content for each quarter;										
probability level:		0.001	<u>0.01</u>	0.05						
COW NO	1	4	71a	110	323	52	29	51	256	264
BL/BR	0.31	0.84	0.64	0.12	0.62	-0.29	0.99	0.72	0.81	0.86
BL/FL	0.97	0.44	0.71	0.22	0.63	0.39	-0.48	0.31	-0.24	0.24
BL/FR	0.18	0.3	0.34	0.33	0.56	0.62	0.98	0.59	-0.09	0.43
BR/FL	0.49	0.48	0.55	0.5	<u>0.99</u>	0.09	-0.52	0.68	-0.2	0.65
BR/FR	-0.23	0.18	0.51	0.26	<u>0.97</u>	0.24	0.98	0.89	-0.21	0.76
FI/FR	-0.02	-0.06	0.3	0.27	<u>0.96</u>	0.64	-0.51	0.76	0.09	0.89
COW NO	218	36a	71b	36b	26	128	109	28	279	
BL/BR	<u>0.95</u>	0.33	0.4	-0.18	0.64	<u>0.97</u>	<u>0.97</u>	-0.18	<u>0.99</u>	
BL/FL	0.92	0.03	0.51	-0.16	0.28	0.95	<u>0.99</u>	-0.23	0.92	
BL/FR	0.81	-0.15	0.18	0.04	0.52	0.96	0.99	-0.19	0.88	
BR/FL	0.86	0.16	0.42	<u>0.99</u>	0.6	0.97	0.96	0.97	0.93	
BR/FR	0.89	-0.2	-0.36	0.96	0.78	0.95	0.96	0.98	0.88	
FI/FR	0.74	-0.12	0.11	0.96	0.76	0.96	<u>0.99</u>	<u>0.99</u>	0.96	

4.4 Influence of other parameters on milk particulate behaviour

The particulate content of milk has been found to be affected by inflammation of the udder. All animals involved in this research project were exposed to the same environmental and dietary factors. As far as it is known none of them were taken out of the herd or were put under any stress.

Throughout the monitoring period seven diets were introduced to animals (example shown in Appendix 8) as illustrated in Table 4-7. The typical diet formulations were appropriate for the cow herd considering the time of the year. Diets implemented over the period of this research period did not change considerably – there were typical diet design to provide sufficient and balanced nutrient throughout the year.

Table 4-7 Diets introduced on Elkstone farm during research work (from June 2007 until April 2009)

Diet ID No	Start date	Planned Finished Date	Extended Date
1	June 2007	July 2007	Sept 2007
2	Oct 2007	Nov 2007	Feb 2008
3	Mar 2008	April 2008	June 2008
4	July 2008	Sept 2008	
5	Oct 2008	Dec 2008	
6	Dec 2008	Feb 2009	
7	Mar 2009	Mar 2009	

For this study the monitoring duration time for mastitis case for majority of animals was set at twenty days. Therefore it was not possible to establish the influence of diet on the particle counts. Cow 110 and 202 were monitored for several months; cow 110 from December 2008 until July 2008 and cow 202 from August 2008 until May 2009. The figures below (4-16 to 4-17) cross-reference changes to the nutrition and particles count, and size during observation time during monitoring of cow 202.

Cow 202 has suffered from subclinical mastitis on the second day of monitoring (quarter BR) and clinical mastitis on day 35th (quarter BL). This animal was monitored from August 2008 until May 2009; during this time four different diets were offered on Elkstone farm.

Figure 4-16 The relationship between diets and particle numbers. Cow 202

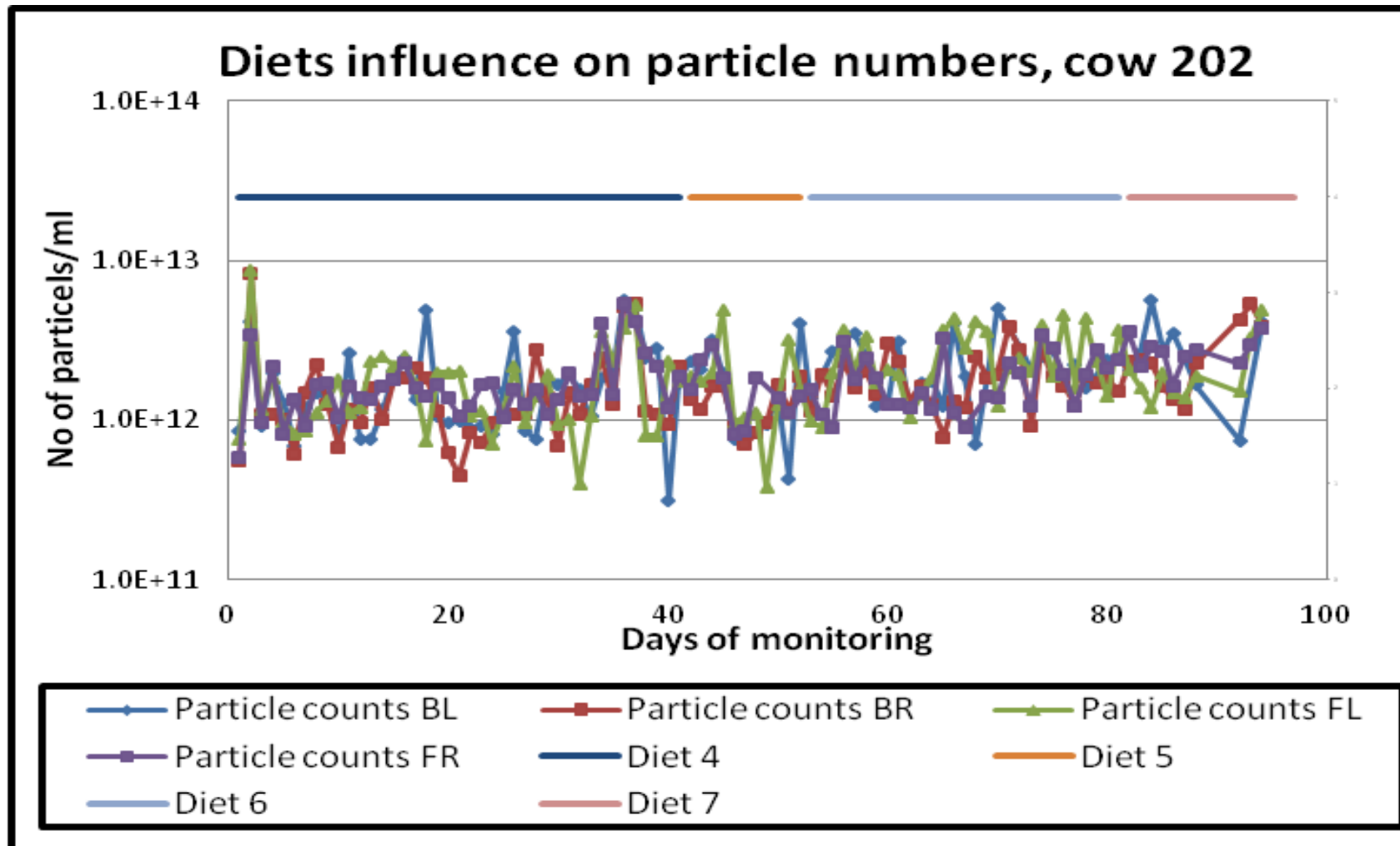
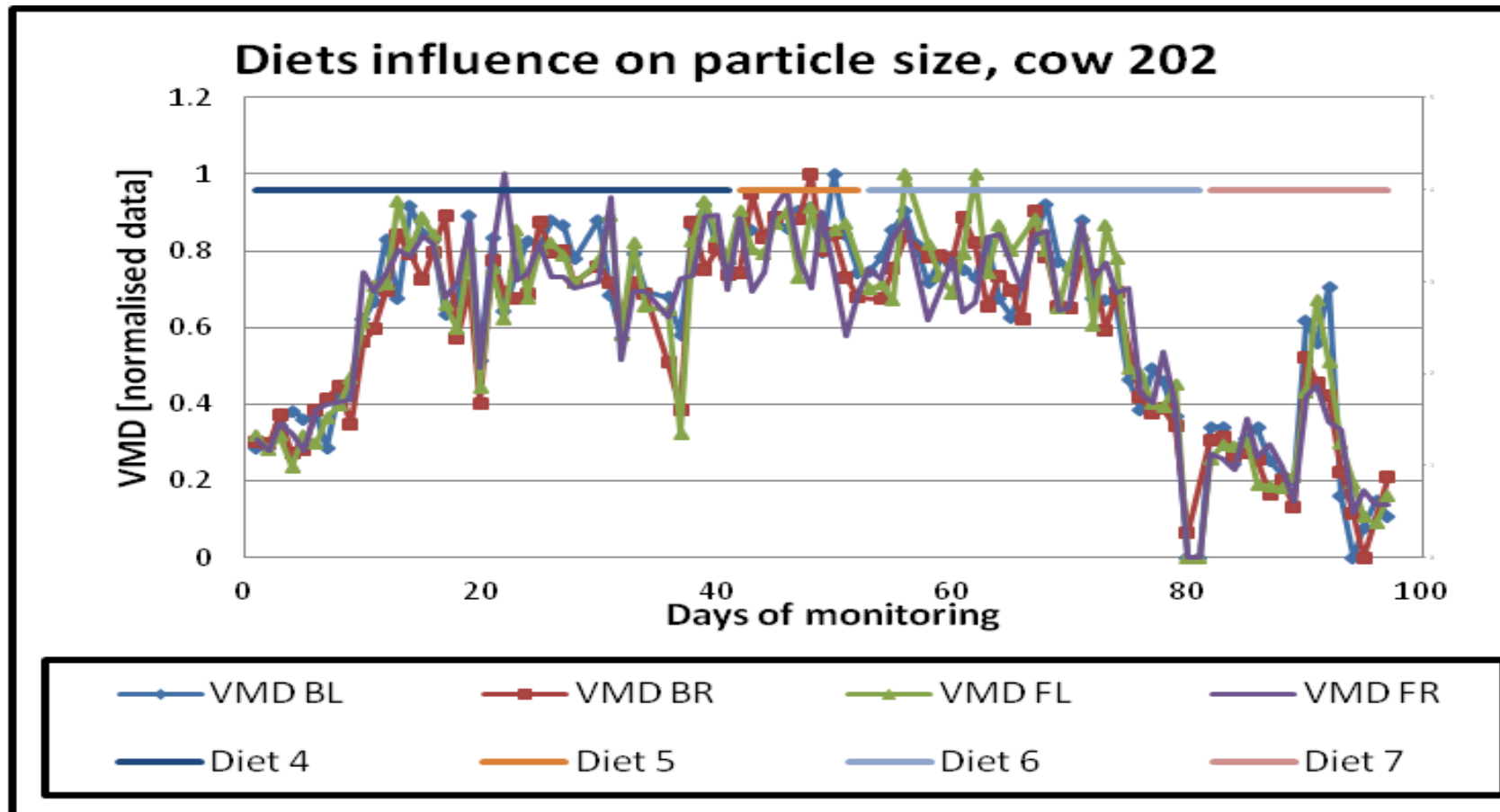


Figure 4-17 The relationship between diets and particle size; cow 202



The figures above illustrate the pattern of changes to particle size and number during the monitoring period. An observed animal was a heifer and the study took place throughout its first lactation cycle. At an early stage of this study, it was felt that heifer as an animal which has never suffered from mastitis would be a good base line subject to monitor behaviour of particles in milk and therefore was used as a control animal

Regrettably during this study only two animals were monitored for the time of the one lactation only; collected data was not sufficient enough to investigate any relationship between milk particulate and environmental temperature.

Collated results have brought very interesting findings and new facts with regards to mastitis and its impact on the physiology of the udder. The following chapter will review the outcome of this study, compare it with current knowledge and propose future developments for the pre-emptive detection of mastitis using particle counting technology.

5 Chapter V Discussion of results

Bramley et al., (1996) defined mastitis as “an inflammation of the mammary gland with the outcome of destroying and neutralizing the infectious agents and to prepare the way for healing and return to normal function”. Bovine mastitis is also an economically important disease due to its negative impact on the quantity and quality of milk production. Over the last two centuries mastitis has been widely studied by biologists and veterinarians. Many solutions to its detection and management have been presented and used. However many questions are still not answered and only a full understanding of the underlying causes, physiology and immune mechanisms can allow for mastitis to be successfully controlled. Moreover despite the ongoing research mastitis control in dairy cattle mainly depends on continuous monitoring and effort of the farmer and/or herd manager. The development molecular biology has significantly increased the knowledge regarding mastitis pathogens, transmission pathways and provided insight for a farmer on where to focus their efforts in above areas to minimise a chance of infection. On the other hand a lack of availability of an effective vaccine (which would allow a protection against pathological) leads to consistent use of antibiotic treatment leading to an increase of persistent antibiotic resistance.

Therefore current approaches to mastitis are rather reactive than proactive. Increased hygiene standards on the dairy farm reduce the chance of the pathogen transmission and development of infection. However when pathogen invades the udder the mastitis is diagnosed when clinical symptoms are visible and the infection is fully established. It is imperative for dairy farmer to have an access to an effective tool able to diagnose mastitis at its early stage, which will also reduce the cross-contamination between cows and minimise the potential cross-species transmission to humans. Moreover, prolonged use of can lead to the additional problem of emergence antibiotic resistant strains, which then can enter the food chain. Finally mastitis is a very painful disease therefore control of mastitis in the cow is a necessity being a major welfare requirement on the dairy farmer.

Previous chapters and a short summary above highlight an urgent need to develop effective strategy of prevention and control. As motioned before the technique used in this study allows monitoring health status of bovine udder throughout the lactation.

The combination of microscope and the particle counting technology permits to monitor single quarter and / or the udder as a one organ and deliver reliable results with regards to development of infections and other physiological changes. The operating protocol used in this work does not require additional reagent (only particle free water for dilution purposes), the prototype of on-line sensor is small and can be easily fitted into a milking parlour. The automation of the process will allow to deliver results in real time in order to act proactively in light of the onset of the disease as illustrated by the Table 5-2. Moreover results obtained in this study brought new, evidence supporting the theory of interdependence of the quarters and MFG production – each animal has their unique profile of milk particulate which can be described as a finger print. What is more this technique could be applied across other species as mastitis is affecting majority of domestic mammals.

The results obtained in this study, its limitations and also a potential of particle counting technology will be discussed in more detail in below paragraphs.

Particle counters as a tool detecting mastitis

The dairy industry urgently needs a new tool/method which will diagnose mastitis and alert early enough to allow time to undertake appropriate treatment leading to a high probability of a successful cure. The ideal sensor and equipment to detect mastitis on the farm would need to be non-invasive, cleanable, small, not requiring the use of reagents, cheap, and able to perform in real time whilst not wasting milk. Currently with a lack of rapid and successful methods to detect this illness, SCC still remains the main diagnostic tool for dairy farmers and the industry. The majority of UK farmers still use monthly SCC from a composite sample as an indicator of animal health. Clinical mastitis is usually caused by environmental pathogens and many incidences are transient, especially those regarding initial episodes for a cow and quarter. Hence, from an epidemiological perspective, an assessment of clinical mastitis can be judged on incidence of the disease and not prevalence. Therefore the standard methodology for monitoring subclinical mastitis which includes routine SCC testing and the culture of milk samples from cows with elevated SCC, are not good and insufficient indicators of clinical mastitis. Moreover cows with a high SCC as a result of a chronic mastitis may also display the signs of clinical mastitis, although the symptoms are usually mild. On the other hand, cows with a low SCC can also be

prone to developing clinical mastitis, especially those cases where the onset is considered acute. Table 5-1 below presents SCC results for two dairy cows, one of which was diagnosed with mastitis. These theoretical results indicated that mastitis can remain undetected especially if only one of the quarters is infected. Monitoring of SCC at the udder level is not efficient enough; low SCC in healthy quarters will conceal the infection as the average result will not alert the farmer.

In addition data that indicates the occurrence of clinical cases, and data from each past case may be able to help to establish risk factors associated with the development of the disease (e.g. season, age, stage of lactation, and previous outbreaks), therefore this information all together should be considered as a fundamental routine of any mastitis management control.

Table 5-1 SCC in composite sample from two animals (postulated data)

Quarter	Quarters cell count (000)				Average SCC (000)
	BL	BR	FL	FR	
Infected cow	50	500	50	50	162.5
Uninfected cow	150	100	150	100	125

Kovac *et al.* (2009) and also, Zeconui and Piccinini (2002) agreed that SCC cannot always be used alone as a marker of mastitis. In many cases *S.aureus* infection is characterised by as low as 200 000 SC/ml. Infection can develop into a chronic form of the disease and spread undetected between animals. Moreover the annual mortality rate from mastitis is 0.2 per cent [Menzies *et. al.*, 1995; Menzies and Mackie 2001]. Therefore it is understandable that research work has also been concentrated on other aspects/parameters of mastitis. Recent research indicated that mastitis can be diagnosed before SCC rise and inflammation signs are visible. According to Song *et. al.*, (2010) clinical mastitis can be detected up to two days before SCC is elevated. The continuous measurement of EC and milk colour at the quarter level, used on AMS farms, allow the detection of mastitis with sensitivity and specificity of 82%.

Another reason why milk should be analysed at quarter level is the fact that poor quality milk cannot be compensated for by processing. Bulk milk has always contained mastitic milk (clinical outbreaks failed to be noticed by herdsman and/or

subclinical cases) which increases the risk of unfavourable milk in the overall milk composition. Enzymes and SC present in mastitic milk will deteriorate the quality of bulk milk and the changes of fat and protein (at any point of the process) are of interest to the farmer because the economic value of the milk depends on these two components. It is clear that milk at dairy farms should be analysed at quarter level.

Table 5-2 presents cause and effect of bacterial induction into the gland. Monitoring EC and milk colour allows prediction of mastitis at the sub-clinical level of infection. This observation is based on the quarter level.

Table 5-2 Mastitis cause-effect spectrum (Adapted from Baines, 2009)

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In 1957 and later 1969 King observed that the number size distribution of MFG appeared to change with the onset of mastitis. However the available technology at that time made this finding of no practical use. The availability today of low cost computer and image hardware enables the rapid acquisition and processing of microscope images, so making it practical to study the variations in MFG size over long periods of time and for several animals.

An average density of particles measured by a particle counter was found to be in the order of 10^{11} to 10^{13} particles/ml. According to King (1957, 1969), Mehia (1995) and Michalski *et al.* (2001), the number of MFG in milk from healthy cows was estimated in the order $10^9 - 10^{10}$ particles per ml. However, the method used in this study allows to include the count of small MFG when methods used by others such as Coulter Counter and light microscopy cannot count particles in the range of $1\mu\text{m}$ and smaller. The particulate count of milk is dominated by MFG. For each somatic cell, there are approximately 1000 fat globules. Somatic cell population rises during mastitis and this property has been the basis of a variety of diagnostic tests for this condition. Research work started in the late 60's (King 1957, 1969) indicated some influence of mastitis on MFG. The particle counter used in this study allows the estimation of density of particles in range from $1\mu\text{m}$ diameter. 80% of MFG are in this range even though they contain only 1% of total fat content volume. Fröhling *et al.*, (2010) estimated the density of particulate content of milk by using a Coulter Counter and this technique gave a fast method of mastitis detection which could be used as a basis for sensor development in AMS application. Nevertheless foremilk samples had to be centrifuged before analysis to remove fat. A light blocking particle counter does not require any additional sample preparation or reagent. Clean, particle free, deionised water has been used in this research work. High dilution of the sample has been pointed out as a disadvantage of this method. However with the great number of particles present in one millilitre, a very small amount of milk is required to perform an assay.

In this study particle counts were monitored from the very first sign of clinical mastitis (identified by herdperson). Where there was no mastitis present, particle count as defined by PHA remained relatively stable with only a small variation observed. However, the concentration of particles changed significantly in all four quarters, even if only one was diagnosed with mastitis. Some of the animals

developed further outbreaks of disease during monitoring. Particle counts in these cases were affected for two to three days before visible signs and one day before a significant rise of SCC. Composite sample results were characterised by the same behaviour of particles and these were also similar to changes obtained from arithmetical average of the individual count. This observation suggests that changes in particulate content of milk can be achieved by monitoring particles in combined milk-stream from an individual cow.

Sufficient detection of mastitis based on monitoring of SCC must be carried out on quarter not udder level. Even a 10-fold rise in SCC for infected quarters might be hard to detect, as at the udder level it will be only a 2 to 4 fold increase of the count. Dilution of results can be seen as well for composite conductivity [Claycomb *et al.*, 2009]. Effectiveness of the method will be associated with the cost which is often the determining factor for the dairy farmer and the greatest challenge for inventors to overcome. Farmers will not invest in an automated system if it will be more costly (testing milk from each quarter) than the effect of mastitis itself.

Table 4-4 illustrates the coefficient of correlation calculated between composite sample of arithmetic average of the individual quarters counts. The correlation has been found to be significant for 17 out of 19 animals, at probability level of 0.001 for 8 animals. These data suggests that the sensor can measure particle count in combined milk stream (one sensor per milking per one milking station). SCC, EC and milk colour has to monitor at the quarter level in order to detect mastitis. Since interdependence of MFG synthesis between quarters has been observed, dynamic of MFG can be monitored at the udder level with successful rate of the inflammation detection at the quarter level.

The particle monitoring technique is able to detect secondary mastitis in combined milk-stream before clinical signs of disease occur. All animals were observed from the first day of clinical mastitis therefore the changes before primary onset were not recorded. However this methodology has a great potential also to identify primary infections. Moreover an abnormal density of particles were also observed for animals/quarters which have not been diagnosed with clinical mastitis; no characteristic signs such as clots, elevated temperature or changes in milk appearance were noted. Nevertheless SCC significantly increased up to the level normally indicating a mastitis outbreak.

Subclinical mastitis remains the biggest problem of the dairy industry but it is difficult to diagnose due to absence of simple indicators and subclinical effects are only assumed. Unidentified infection decreases the quality of milk and all dairy products, and affects animals welfare. Previously there were no therapeutic controls or indirect tools for the detecting of subclinical mastitis. Results presented in chapter IV clearly indicate that particle size and count are affected by sub-clinical infection and can be seen during the monitoring.

The idea for this research work was to use the particle counter to estimate SCC and therefore at the early stage of research it was assumed that MFG will need to be removed from milk samples. However, currently there is no method which will allow separation of MFG from the sample in on-line system as such. The dynamic of MFG became a major objective of this study. Particle counting technology has been commonly used since last century and automated monitoring systems (gas or liquid) found commercial use in the majority of industries. Liquid particle counter and PHA were successfully implemented in automated systems at the laboratory level. Direct microscopy has been used to shadow data obtained from the counter. The data collected over three years of research pointed out that a particle counter (combined with microscope) may be used as a tool for detecting both types of mastitis; Appendix 10 [Janik, *et al.*, 2010].

Early detection of mastitis

The change in milk particulates was found to be sufficiently well in advance of clinical symptoms to justify studies into the optimum method of managing an intramammary infection when identified at this very early stage. While this aspect was outside of the scope of this study, the opportunity to explore the possible improvements in animal welfare and the maintenance of productivity by more timely diagnose management is now evident. The particle detection and measurement technology may clearly detect clinical and subclinical mastitis at its early stage and help cut costs on the farm by preventing low quality milk entering the milk tank and spreading pathogens between animals.

The results obtained through direct microscopy indicated that the image analysis method has delivered results that are objective, consistent and with uncertainties low

enough to see distinctive changes in VMD (presented as normalised data). The results show that the VMD of MFG changes in a characteristic manner prior to the onset of mastitis in all recorded cases. These changes are well in advance of any changes in SCC. These modifications to the size of MFG reflect physiological changes in the milk generating structures that react to the early stages of an infection. According to Hillerton (2002) clinical mastitis on average is diagnosed by farmers up to five milkings after bacteria have entered the gland. Somatic cell counts rise up to the critical level indicating mastitis up to three milkings after pathogen intrusion into the gland. A summary of data published by Hillerton and MFG behaviour discovered in this study clearly indicated that mastitis causes significant changes to milk synthesis several days before it can be diagnosed with tools currently used on farms. All methods are passive rather than proactive and allow herdsman only to react to the disease when the disease is fully established.

A tool which combines direct microscopy and PHA allows to diagnose mastitis at its early stage when disease is not fully established yet. Early detection is to an advantage of the herdsman and it will help to act quickly – treat infection at its early stage, prevent from spreading and save the animal from unnecessary pain. The first attempt to build this type of sensor took place at RAC together with Microresearch Ltd in 2011. Samples of milk were successfully analysed at small scale. In the order to proceed with research work financial funding for ongoing research work is required.

Future research must include the greater number of monitored animals over a longer period of time to confirm these results and gain more information about milk particulates. It is important to understand and estimate mastitis natural self cure rate; in some cases udder inflammation can be overcome by the immune system which can reduce bacterial count sufficiently that the pathogen can no longer cause illness. Similar patterns are observed in nature e.g. not every bacterial/virus entering lungs will lead to a chest infection – the immune system is able to fight infection without medical intervention. Therefore pre-emptive detection should not be associated with early antibiotic treatment; firstly animals identified with mastitis symptoms (significant change to particle count and size) should be milked last (to avoid cross-

contamination); Secondly, bacteriological tests need to be carried out to confirm presence of pathogenic bacteria; thirdly animals should be monitored closely throughout all stages of the inflammation. False positive may be identified. At this stage of the research understanding of milk particulate behaviour is not sufficient enough to exclude any anomalies.

In addition, adequate amount of data will allow to identify of any potential similarities and relations in particles behaviour between animals at the herd or the breed level. We do not understand the impact of environmental factors on milk particulate and at this stage we do not know what impact the same factors will have on the herd population. It is also important that technology used in this study will allow observation of a natural self-cure rate at early stage of infection - before SC are attracted to the infected quarter and before blood-milk barrier will be bridged. Particle counter will allow for antibiotics to be a final choice in mastitis treatment and the emphasis can be placed on the preventive measures such as good housing controls and herd managements.

Pre-clinical detections will also allow prevention of the disease from spreading. Cross infections may account for as much as 40% of new infections [Bramley *et al.*, 1996], if the cow being milked has one or more infected quarters, pathogenic bacteria can be transferred to the surfaces of teats of other animals. After an infected cow has been milked, contaminated milking machine liner surfaces can carry bacteria originating from one infected animal to the next animal when the machine is applied. Automated system may detect changes to particle behavior long before the herdsman will identify first clinical symptoms. "Suspected" animal/animals should then be milked last to avoid potential contamination. Pathogens causing mastitis e.g. *S.aureus* are very difficult organism to treat and presently there are no intramammary infusion products available to successfully eliminate these pathogens. Therefore a particle counter can only be used to reduce the rate of mastitis outbreaks by pointing out animals at risk. As mentioned above false positive may occur, this is why the process of implementing new technology will need to include an assessment of costs and benefits before any intervention. It may be very hard to persuade a dairy farmer to initiate an antibiotic treat to a cow with no visible symptoms or discharge milk without clinical signs.

Theory of interdependence

Akres (2002) described an experiment in which dye was injected into the teat of one of the quarters. Only tissue of the injected gland was stained. This result suggests that there is no dependence between the quarters within the udder and quarters are fully independent units with regards to milk production and immunology. This assumption was also supported by results obtained across the globe - elevated SSC, EC, decreased yield and visible changes to milk caused by mastitis are present only in infected quarter/quarters. None of the above was recorded in healthy quarters. However these observations are a reaction to the infectious agent or the infection itself. SC are attracted by the presence of bacterial enzymes, toxins and cell wall components sent directly to infected quarters as they are attracted [Sordillo *et al.*, 1997], when elevated electrical conductivity is a result of the damage to the milk-blood barrier. Therefore changes to these parameters are only detected in infected quarters when the infection is fully established. Furthermore these changes are a direct consequence of the inflammation and the pathogenic microflora and for this reason they can be detected only in the infected quarter. Milk fat synthesis has been studied by many scientists [Bauman and Davis, 1974; Mather and Keenan, 1998; Heid and Keenan, 2005] and it has been described as unique pathway whose control mechanisms are not fully understood yet at both the cellular and the quarter level. Data collected throughout this study brought a significant finding regarding these control mechanisms at the udder level.

Results obtained showed a high correlation behavioural pattern of the milk particles in all four quarters during the monitoring term. Over twenty animals were monitored for a period of fourteen days; two animals were observed for the duration of their lactation. In all cases a significant measured similarity between particle behaviour in all four quarters were noted. SCC (in agreement with other researchers) was elevated only in the infected quarter; in the remaining quarters somatic cell composition was not affected. Results obtained by Adkinson *et al.*, (1993), Bansal *et al.*, (2005), Merle *et al.*, (2007), Hamann *et al.*, (2002) support the theory of interdependence of quarters; firstly by direct effects of mastitic quarters on the milk from healthy neighbouring quarters and, secondly, by finding differences in milk coming from healthy quarters from the same infected and uninfected udder. Berry and Meaney (2006) linked interdependence of quarters within a cow to the inherent immune

competency or to the pathogen specific effects and showed a strong interdependence between quarters for subclinical mastitis. Hogan *et. al.* (1990) obtained results suggesting that quarters within a cow are independent units and there is an equal possibility of infection in left or right quarters. Adkinson *et. al.*, (1993) proposed, based on a study record of 1630 Holstein cows, that quarters tend to be more alike with regard to clinical mastitis within a cow than expected. Sol *et. al.*, (2000) suggested that quarters within a cow are independent of each other and there is the same probability that either might develop mastitis.

Results obtained in this study indicate a strong relationship between all quarters during and outside infection periods. Particles in milk are represented by MFG, all types of cells, parts of epithelial cells, casein micelles and foreign bodies. During mastitis, migration of somatic cells disturbs the balance between milk components. Epithelial cells are damaged, the synthesis of fat (MFG), sugars and proteins is disturbed. Therefore it was expected the migration of somatic cells will affect the particle number in infected quarters only. However particle numbers were also affected in uninfected quarters where the barrier between milk and blood has not been breached. During mastitis, milk production is disturbed and the MFG distribution could be described as the best possible at that time. It would be expected that the healthy quarters will compensate in order to provide good quality nutrients for the calf. Conversely, as observed in this study, the uninfected quarters matched MFG production with infected quarter (with regards the size and number). Table 4.3 and 4.5 presents coefficient of correlation between quarters during infection. Coefficient of correlation calculated for particle number has been found to be significant in 80% of cases. However coefficient of correlation calculated for particle size was found to be significant for the majority of cases at the probability level of 0.001. These results strongly support the theory of the interdependence of the quarters within the udder, furthermore mechanisms behind it seems to not to be affected even by major infection. Obtained data suggest that system/systems overseeing MFG production (at the udder level) are centralised and are able to adapt even during excessive conditions in order to provide sufficient quality milk for a calf. The understanding of this system and factors affecting it might allow to influence MFD synthesis - desired size vs number; also to maintain the milk quality during infection.

According to Timmen and Patton (1988) composition of MFG depends on their size and their heterogenic production allows to provide balanced nutrient for the new born calf. Following this thought - if the genetic mechanism allows to produce different size of MFG for a purpose described above the same mechanism might control MFG secretion during the infection. Female mammals will continue to feed their calves throughout all phases of the inflammation therefore MFG synthesis might be redirected through different paths to allow the best possible and available (at the time) quality of milk.

Correlation in the size of particles between quarters was more significant than correlation between the numbers. It appears that the production of the similar size of MFG across all four quarters is more vital than synthesis of a similar total number, therefore emphasising quality over quantity. At this level of the research we can only speculate why this is happening. Referring to literature, damage to the mammary gland tissue has an influence on the fat droplets synthesis and fusion, and the availability of the apical membranes, which envelops droplets. The simple equation is that proportionally less membrane material is required to envelop one large droplet than three small ones of the same total droplet volume. In addition during mastitis the damage to epithelial cells reduces the availability of the membrane material. However collected data show that in 75% of cases reduction of that size of MFG was observed (for period of 2 to 3 milking before the rise of SCC) across all quarters followed by impulsive increase or decrease in size for time of 1 to 2 milking.

Mastitis cause the damage to the mammary gland tissue, and as a result secretory parenchyma is replaced by epithelium without secretory ability such as connective tissue. These histopathological changes lead to the reduction in milk production. Unfortunately the yield total per quarter was not recorded during this study and it is not known which monitored quarter might have been affected the most. However during the monitoring process a high correlation between MFG sizes across all four quarters was consistent throughout the whole monitoring process. One could conclude then that even the milk production is affected (post-mastitis tissue damage) in the quarter the mechanism of MFG synthesis remains the same within the udder. Milk lipid globule precursors appear as droplet in cytosol, their diameter ranges from 0.5 μ to 4 μ m (Dylewski *et al.*, 1984; Deeney *et al.*, 1985). These droplets fuse with each other and increase in size. According to Valivullan (*et al.*, 1988) the fusion process is

promoted by gangliosides, calcium and other high molecular protein fractions from cytosol. This experiment was performed in the cell free system. Valivullan (*et al.*, 1988) observed that the larger cytoplasmic lipid droplets do not fuse with each other. There are some differences in lipid composition of native MFG of different size, and some of them might be not compatible. The fusion process was described in detail by Heid and Keenan (2005), however it is not know if this is the only mechanism supporting/coordinating the growth of lipid droplets at the cell, quarter, and udder level. High correlation in MFG size between quarters during mastitis could suggest that other mechanisms may control fusion of droplets during infection at the udder level. However all above refer to healthy tissue and more research work need to be undertaken to understand this process during infection such as mastitis.

Data collected in this study suggests that the synthesis of MFG is synchronised in all four quarters; it also can be concluded that any impact put on one of the quarters will affect the other three. The mechanism behind this is unknown. Knowledge about the possible relationship or dependences between quarters within the udder during mastitis is very important (Adkinson *et al.*, 1993). More research work is required to better understand the mechanisms directing relationships between quarters and controlling mechanisms involved. It is clear at this stage that four quarters with the udder are working as one unit with regards to MFG synthesis.

The influence of other parameters on milk particulate

The geographic situation of Elkstone Manor Farm had a significant influence on the weather and microclimate. At a height of 200 meters above sea level the average temperature was up to 2 degrees lower than recorded in the neighbouring town of Cirencester. Elkstone Manor Farm was designed and built in 1980. Poor design of the cow accommodation building resulted in poor air circulation causing overheating during summer months and a constantly low temperature during the winter. Moreover inefficient air circulation may have allowed micro-organisms to spread easily contributing to the increased rate of mastitis in the herd. It must be noted however that good housekeeping and hygiene routines were maintained. The animals were kept clean and teat disinfection before milking and teat dipping afterwards were practised as routine. However the clinical mastitis outbreak rate had increased significantly since 2003. Majority of pathogens detected in mastitic milk on the farm were *E.coli*

followed by *S.aureus*. These bacteria represent two classes: environmental and contagious pathogens, both very difficult to treat and eliminate.

The stage of lactation has been established as a contributory factor affecting milk composition. Auldrist *et al.* (1998) investigated distribution of FA during lactation. Milk from early lactation (30 days) contained less 4:0 to 12:0 FA if compared with milk from middle (120 days) and late (210 days) lactation. Results were independent of seasonal effects and differences were attributed to inability to consume enough dry matter – decreased energy intake by cows in early lactation. Belyea and Adams (1990) pointed out that the initiation of lactation is characterised by negative energy balance linking to mobilisation of long-chain FA into milk fat.

Bitman and Wood (1990) investigated changes in milk fat phospholipids and cholesterol during lactation. Both of those milk lipids fractions are present in the milk fat globule membrane. Those results pointed out that, structural components of PL declined during lactation. However, at the same time, total milk lipids remained relatively stable. This observation might be explained by increased size of MFG in the late lactation (fewer number of MFG) or thinner membrane covering MFG as lactation progress (the same number of MFG).

As mentioned earlier only two animals were monitored throughout the duration of the lactation, cow 110 and 202. In both cases the number and the size of particles remained stable (excluding mastitis outbreak) when MFG size increased in the case of cow 110 and decreased in the case of cow 202. The sample size and the fact that both animals were heifers do not bring enough evidence to disagree or agree with the above. Some change to MFG size will occur during the lactation cycle. However, these changes will not be as significant and spontaneous as changes caused by a mastitis outbreak.

The diet on Elkstone Farm was designed to support the health and welfare of animals and increase milk production. The processes engaged in the conversion of feed to milk constituents are complex and significantly related between each other. The relationship between diet and milk composition and diet and mastitis has been described in chapter four. Lactose and protein share the same secretory pathway, therefore lactose; protein ratio may show a little variation in milk. However (as

described in Chapter I - Introduction) fat is secreted throughout a different route therefore fat: lactose or fat: protein ration is expected to show more significant variations. It has been agreed that data obtained for lactose and protein during this study did not bring any additional informative evidence and has not been analysed in relation to mastitis outbreaks. Both heifers included in this study were monitored from the very first day of their lactation cycle; during this time two diets were introduced to cow 110 and four to cow 202 (please refer to figures 4 20-23). According to König (1983) during the early phase of the cycle, lactational demands exceeds the feed intake, as lipolysis of adipose tissue exceeds lipogenesis causing accumulation of triglycerides, which increase the supply of lipid precursors. This fat mobilisation will slow down as lactation progresses and in later stage adipose tissue repletion will take place. Milk fat content in milk is usually high on high forage diets and it's unaffected up to moderate levels of concentrate intake. However the concentrate proportion exceeding 60% may decrease fat volume even though milk yield and protein fraction are stable. This is associated with the significant increase in the quantity of propionic acid produced in rumen (which leads to a decrease in the acetic acid) (Sutton, 1985) and with high plasma insulin levels (which reduce the availability of substrates for fat synthesis) (Sutton *et al.*, 1985).

The results obtained for both animals (cow 202 and 110) did not show any relationship between the nutrition and the number of milk particulates. However the size of MFG seemed to be affected by the changes to the diet. The change to the pattern has occurred as a shift; this process is more visible in case of cow 202. This change was found to be very different to the mastitis influence on milk particulate. The onset of disease caused significant peaks or dips in data which (following data review of all observed animals) were characteristic for mastitis and could not be mistaken with smooth shifts in MFG size observed for cow 202.

During the early stage of this work diet and its impact on the milk particulate was not one of the primary objectives of this research. Therefore the sample size is not sufficient to be certain of dietary effect, if any, on the milk particulate behaviour and at this point of research no further speculation should be made. Demand for milk and dairy products are vast across the globe and understanding of the role of animal diets in controlling all aspects of milk fat production has grown over the period of last 60 years. The dynamics of MFG have a significant impact on the quality of the dairy

products (this will be expounded in more detail in the next paragraph) therefore the knowledge of how they can be modified will be essential.

Regrettably, the impact of genotype and breeding effect on milk particulate was not investigated in this study. Record kept on Elkstone farm did not provide any information regarding genetics of monitored animal; therefore this work cannot provide any conclusion on relationship between particulate content of milk and genetic and/or breeding effect.

All observed animals were of Holstein-Friesian breed which dominates in milk production industry due to high milk production, lower feed cost if compare to other breeds, high ability to adapt to wide range of environmental condition and high reproductive efficiency. As illustrated in Figure 5-1, milk production and its composition depends on breeds.

Figure 5-1 Milk production, butter fat and protein content

(http://www.raw-milk-facts.com/dairy_cow_breeds.html)

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Holstein cows have a lower content of butter fat than other main breeds. During experimental part of this study it was observed that particle numbers are not consistent throughout the milk process and set dilution is only sufficient for foremilk sample. This brings a conclusion that operational protocol will need to be repeated for other breeds in order to establish correct dilution.

It should be emphasised that the dynamics of particulate levels in milk have not yet been fully established. The influence of age, reproductive status, season, and the effects of other disease or injury is yet to be investigated with this new technology. However the results obtained so far suggest that within a relatively stable production environment the distinctiveness of the particulate response to an acute infection of the mammary gland should remain detectable by particle characterisation and be identifiable as such.

It is also important to understand the distribution of milk particulates during milking process and milk intervals. It was reported by other workers that fat composition and volume change during milking. Milk removed at the start of milking contains less fat than milk removed at the end of milking. The last milk out of the gland has the highest fat content. According to Quist *et al.*, (2008) fat percentage has more milking

to milking variation that protein. Fat percentage has been higher in the evening by 0.32%. This finding can be explained by the fact that there is a longer interval between morning-evening milking compared to evening-morning milking [Weiss, 2002]. Moreover the level of phospholipids is greater in the fore milk samples compared to residual milk samples [Kernohan *et al.*, 1971]. An increase in the concentration of phospholipids can be associated with greater concentration of smaller globules in foremilk. During this experiment, samples were diluted by 10^{-9} before samples were injected into particle counter this protocol was established for fore milk samples from morning milking only. Data obtained in this study are in agreement with above. At the early stage of the experiment foremilk samples from afternoon milking and residential samples from both morning and afternoon milking were also collected. Following the procedures, samples were diluted by 10^{-9} and analysed by particle counter. Results were inconsistent and incorrect (data not shown) as an effect of not sufficient dilution because the concentration of milk particulates in mid milk and afternoon milk was found to be significantly higher and greater degree of dilution was required. Moreover the higher the fat concentration the grater size of MFG was found (foremilk, midmilk and post-stripping milk); therefore it is crucial to ensure that sample collection procedure is followed carefully to avoid false positive readings.

Potential applications for particle counters

Monitoring the particulate content of milk may also find application in the processing part of the dairy industry. The size of MFG is very important for the stability of milk emulsion. MFG size affects rheology, stability, appearance and physiochemical properties of milk e.g. creaming of dairy products. The large globules will coalesce faster than small ones. Moreover particle size is affected during thermal treatment. According to Raikos *et al.*, (2009) MFG diameter increased significantly when heated. For the first hour of heating there is no notable change however when heated to 50°C for periods in excess of one hour, milk fat globule size is affected significantly, which may be explained by flocculation or coalescence of MFG.

In 1988 Timmen and Patton reported that the FA composition and lipid content of the MFG depends on their size. According to Lopez *et al* (2011) size of MFG has a significant impact on the amount of molecules, pollar lipids and FA they contain. The finding above was also reported by Fauquant *et al.*, (2005), Michalski *et al.*, (2005),

Gallier *et al.*, (2011) and Lopez *et al.*, (2011). This is an evidence of heterogenic production and assembly of MFG. The production of the various sizes of MFG provides a balanced nutrition for a calf. According to Timmen and Patton (1988) different FA composition can be associated with a different secretion path; Large MFG fuse from smaller globules (some of them exceed the size of the alveoli epithelial cells); medium size MFG originate from the fusion of microdroplets; small MFG are produced in endoplasmic reticulum in form of microlipids.

The better understanding of MFG properties, physiological functions and structure might have a great technological potential to develop healthier dairy products with improved organoleptic properties. Milk, which contains small MFG will produce butter which is easier to spread (more intact MFG) compares to butter made of large MFG. However milk with large MFG has better properties for cream production (Walstra *et al.*, 1999). Small MFG have an increased surface area of MFGM therefore Camembert and Emmental cheese produced from milk containing small MFG are less firm (Michalski *et al.*, 2003, 2004). According to Gougedranche *et al.*, (2000) cheese produced from milk with small MFG is less firm as an effect of the ability of MFG to insert into the protein network allowing higher water capture. The size of large MFG is usually greater than protein pores causing the damage to the network. However as reported by Gallier *et al.*, (2011) the concentration of carotenoids increases with size of MFG which allows to produce cheese with more intense yellow/orange colouring. Currently value of the milk depends on the total fat and protein content and farmer is paid for their quantity not quality. Particle counter combined with microscope can provide a rapid profile of MFG distribution at the bulk of specific fraction (e.g. cream) level. This knowledge might allow to optimise the usage of delivered milk and improve the eating quality of the dairy products.

Milk and other dairy products can frequently be infected with mastitis pathogens as mastitic milk is mixed into bulk milk and enters the food chain (subclinical mastitis) and is potentially transmitted to humans. At the beginning of this century it was discovered that milk can transmit tuberculosis, brucellosis, diphtheria, scarlet fever, and Q fever to humans [Hameed, *et al.*, 2007]. Over the years this threat has been reduced due to increased hygiene standards of milk production practices and pasteurization techniques. According to Kluytmans *et al.*

(1997) and Gilmour and Harvey (1990) milk of infected animals is the main source of enterotoxigenic *S. aureus* of animal origin. Certain strains of *S. aureus* can produce heat-resistant enterotoxins, which are linked to nausea, vomiting and abdominal cramps when ingested by humans and is responsible for staphylococcal food poisoning outbreaks. Moreover the first case of transited *S.aureus* between cow (with subclinical mastitis) and human has been documented [Juhász-Kaszanyitzky, *et.al.*, 2006].

The presence of mastitic milk and pathogenic bacteria also reduces nutritional value of milk (due to the changes in its composition) and increases processing problems, off flavours and decreasing the shelf life of milk and its products, due to the growth of spoilage bacteria and presence of enzymes [Oz *et al.* 1985]. The above discussion clearly indicates that the particle counting technology used as an indicator of mastics can help minimise the transition of pathogens from milk and its products into humans. The early detection of mastitis and detection of subclinical mastitis will reduce the chance of “infected milk” to be mixed into bulk milk. Mastitic milk also brings the risk of antibiotic residue which can lead to allergic reactions and development of antibiotic resistance strains of bacteria.

The technique used in this study to monitor dairy cow milk samples could potentially be used to monitor health of other mammals. The lactation plays an essential part in all mammals reproductive cycle; milk provides a nutrition and disease resistant to the new born. However this balance can be easily interrupted by an infection and mastitis can affect essentially all lactating mammals [Sordillo and Streicher, 2002].

There are also many similarities between mastitis and its characteristic across all species. Lower susceptibility to mastitis in dairy cows was linked to periparturient periods when defence mechanisms are diminished [Oliver and Sordillo, 1988] the same correlation was observed for other mammals to include women [Fetherston, 1988]. The mastitis and the mammary gland defence has been studied and described more extensively for bovine than for any other mammal [Sordillo and Streicher, 2002]. The next step will be to use current knowledge and understanding of bovine mastitis and implement them for other species. It has been agreed the main mastitis pathogens causing mastitis in bovine also occur in human [Zakos, *et. al.*, 2011]. According to Spencer (2008) 10 percent of breastfeeding mother develop mastitis

when Kinlay *et. al.*, (1998) reported that this number can increase up to 20% and the diagnosis of mastitis is clinical (fever, tenderness in breast, pain), and the treatment includes change to breastfeeding technique and/or antibiotic treatment. In infective type of mastitis in human is usually caused by *Staphylococcus aureus* and *Staphylococcus albus* when E.coli mastitis is less frequent (Novy 1984; Riordan and Nichols, 1990). The treatments usually include encouragement to effective milk removal, symptomatic treatment (anti-inflammatory and pain medication), antibiotic and probiotic therapy.

As mentioned before mastitis is a common disease affecting lactating mammals, therefore particle counting and monitoring technology which was used in this study for dairy cows could be implemented for other species. The size distribution and numbers might vary however the basic physiology of lactating gland in the same across all mammals. There is no diagnostic tool which would allow early detection and therefore early treatment hence why the milk particulate behaviour should be taken into consideration during search for new method of early detection.

The next steps for research and future application of particle counting and sizing technology on dairy farms

This study is not the first attempt to use pre-emptive strategies for detection of infection for instance or example. Continuous cardiac output monitoring has been implemented for critically ill patients to monitor/detect subtle changes in the patient's ability to deliver oxygenated blood to the organs and tissues of the body following cardiac surgery. This method is an invasive form of monitoring which uses a sensor-tipped catheter threaded through either the internal jugular or subclavian vein and resting its end-point on the pulmonary artery. Cardiac output is calculated by multiplying heart rate by stroke volume (the amount of blood ejected into central system). Constant monitoring of heart pressure allows the overseeing of any systematic response several hours to even a day before clinical signs of sepsis occur. These results will identify the patient as "a patient at risk" and further examination will then be carried out.

Another example is system controlled temperature and humidity of the air of greenhouses. Automated systems constantly monitor changes within the micro-

climate and ensure that all of them stay within set up parameters. If humidity decreases then water sprinkles will release enough water to bring the balance back within specification.

It has been agreed that even though current knowledge about mastitis and the physiology of the udder has increased greatly since 1970's, many questions still remain unanswered. Mastitis is a complex disease and its full impact the physiology of the udder is unknown. Over that last fifty years comprehensive programs promoting animals health were introduced to farm mangers, herdsman and veterinarians. The incidence rate of mastitis is closely related to the management practice. Therefore, an improvement of the environment of the dairy cow through increased hygiene standards and implementing good milking procedures has decreased the rate of disease. The reduction of three major risk factors: susceptibility of the animal, exposure to pathogen and cure rate has been found to be negatively correlated to mastitis outbreak. However mastitis still remains the most costly disease for dairy farmer and dairy industry, and seems that there is no immediate solution available.

Particle counting and sizing technology as used in this study has brought new evidence regarding influence of mastitis on the lactating dairy cow. Firstly, the important relationship between four quarters has been observed; data collected during this work brought facts supporting theory of the interdependence of quarters within the udder (moreover the interdependence of the udder response to disease when only quarter is infected). Secondly, mastitis has great impact on the quarters and their physiology even at the very early stage of infection – at the point when pathogenic bacteria enter the gland, therefore dynamics of MFG can be used as an early indicator of the onset of mastitis (both clinical and subclinical). Particle counter combined with microscope can be the new tool which will allow to detect disease early enough to undertake appropriate treatment. Thirdly, the behaviour of MFG (size and diameter) and their reaction to mastitis are unique to each animal therefore healthy profile for one dairy cow could mean mastitis presence for another.

This study was performed on a relatively small scale and the next step is to design and build a particle counter combined with microscope in a form of small sensor. The first attempt has been undertaken by the scientific staff of Royal Agricultural College and Micro Research Ltd. The sensor must be tested in more a commercially scaled project

with sufficient number of animals/herds, including different breeds, implemented diets, time of the year, and longer monitoring periods. Moving the sensor to close proximity to the milking parlour will be very challenging as appropriate software and diluting system needs to be designed. In this study the experimental part and data analysis were undertaken and processed manually, the process was detailed but time consuming. Once a sensor is placed on the farm the automated software will collect and review the data on a daily milking basis. Data collated during this research shows that each animal studied had a characteristic and unique particle pattern and range. An automated particulate monitoring device would recognise the “particle-finger print” for each dairy cow. Ideally animals would be monitored for the entire duration of the lactation/lactations. Monitoring period over the time duration longer than one lactation cycle will allow to better understand behaviour of particles during periods of time outside infections. Moreover this will also enable to establish the influence of other parameters such as diet, time of the year and age (number of calves). A further project might benefit from concentrating only on one group of animals and follow them for a period of two years. This would then allow investigation impact of age, the number of calves and nutrient (quality and type) on milk particulate.

On the traditional dairy farm mastitis is diagnosed at the cow level by the dairy farmer, the introduction of an automated and reliable method will allow monitoring each animal at the point of milking (microtesting results confirming presence of bacteria take the minimum of 24-48 hours) for both traditional and automated farms. The new software will identify potential infection (the statistics calculating the rate of false positive and false negative events must be included) however for this to be achieved, the software needs to be taught how to identify the infection. The particles behaviour and range have been found different for each animal. A comparison between different animals does not show true results as each animal has a different dynamics of particulate pattern. Moreover, it is difficult to define precisely at which point a cow has mastitis. Referring to the current standard – SCC over 200 000 per ml is a cut out point for mastitis; however particle counter/microscope device can identify mastitis at the very early stage when the SCC is not yet elevated. The data will need to be continuously collected at each milking and interpreted by the system. The greater amount of information collected the more accurate the prediction should

be achieved (filtering the noise out and frequent update) and it should be possible to establish the definition of infection for each animal. The review of this information at the herd level will also allow to estimate sensitivity and specificity for the method, and the threshold settings for alerts. Once herd dynamic spectrum is recorded and deducted, the experiment can be moved to another herd (which should illustrate the impact of environmental factors) or breed (impact of genetic factors). Full analysis of milk particles dynamics at the animal and/or herd level has a great potential and will allow to better understand physiology of the udder.

The data collated through this study were based on results obtained from foremilk samples during morning milking only; as mentioned above, milk particle count differs throughout the milking process and milking times. It was found in recent studies that mastitis was not detected until after five milking had occurred; therefore researching more significantly into milking patterns and time of milking may provide more information regarding early onset of mastitis and changes to milk composition. It was observed that the total number of MFG in samples collected during morning milking (longer interval) had lower particles count at the same phase of milking when compared with sample collected during afternoon milking. When setting the research protocol for future study this need to be taken to the account as an accurate dilution is crucial in order to obtain the correct results. This will also need to include the breed as the levels of the fat content varies across different breeds; moreover the fat content is also related to the size of MFG – the highest fat content the greatest diameter of individual MFG. In addition Elkstone farm was not an average farm; secluded, situated at high altitude (lower temperatures and high humidity), and distressed by tuberculosis outbreaks – these parameters and events had a significant impact on the health status of the animals. Going forward this research needs to be repeated on several farms across the UK in order to better understand potential influence of the environment on the milk particulate studied in scale.

Regrettably, a genetic database has not been created or maintained on Elkstone farm. It has been scientifically proven that genotype is one of the contributing factors to the natural resistance of mastitis (self cure rate) and it will have a great impact on the ability of the animal of the milk production. The future research must cross-reference genetic information of observed animals.

The impact of nutrition on physiology of the udder, milk production and an animal immunity has been studied by many authors and described in a great detail. At the early stage of this work, when the research protocol was designed, the influence of nutrition on the obtained data was not included in the agenda. It is worth explaining, that the main focus was placed on the understanding of the particulate type and its behaviour. As the research progressed, it was concluded that MFG are the main fraction of milk particulates and their dynamics will be correlated with nutrient provided for animals. However, in order to understand behaviour of MFG larger group of animals (mastitis outbreaks) had to be recorded and analysed. Average time of monitoring was 20 days. Typical diet on the farm was designed for one and a half to four mounts. As explained earlier only two animals were monitored throughout the lactation cycle; however sample size is too small and collected results do not bring any evidence allowing to conclude on the influence of the diet on MFG behaviour. Milk fat production can be affected by diet (please see previous chapters) and therefore future research is required to investigate the direct and indirect relationship between nutrition and particulate content of milk. Moreover particular nutrients can enhance natural immune response – impact the self cure rate. Through the particle count we can observe (and potentially calculate) how successful different diets are against disease.

Limitations of this study

At the early stage of this work particle counter was expected to monitor SC, as explained earlier first assay brought evidence that MFG are the main particle group in milk and therefore this work investigated their behaviour. Milk sampling intervals were set at 24hours (morning milking). It is now clear, that collecting data in 12 hours intervals would allow to see pre-mastitis changes to milk particles in greater detail and possibly earlier in some cases (by 12 hours). The number of particles in fore-milk samples differs between morning and afternoon milking therefore firstly the correct dilution should have been established and collected data presented as normalised figures. Moreover setting a group of animals milked three times a day would be ideal. Data collected in this study brought new evidence regarding milk particulate and mastitis; however it could have been collected with greater precision.

During second phase of this study samples of milk were sent to accredited laboratory for SCC determination; regrettably this was performed only for the first ten days of experiment (unless an animal developed another outbreak of clinical mastitis within that period). It was found that in some cases SCC rose without visual signs of clinical infection which was interpreted as an indication of subclinical mastitis. The record of SCC for each sample would allow to better understand changes and dynamics of milk particulate, and associate them with disease (identification of false positive).

The record kept on for each animal on Elkstone farm was basic. Desirable more detailed information about monitored animals should have been collected; this information could have helped to uncover underlying relations between e.g. genetics, average SCC, particle numbers or size.

E.coli followed by *S.aureus* were identified to be the two main pathogens causing mastitis on Elkstone farm. During this work no microbiological tests were carried out and there is no knowledge of possible relation between pathogen, strength of natural immune reaction and MFG. These two bacteria have different mechanism of attack, unlike symptoms and established infections are treated differently. Microbiological tests should have been performed for each monitored animal to exclude or identify any correlation between pathogen and milk particulate as at this point of research impact of microbiological factors is not known.

During this research small number of animals could have been monitored – samples preparation and analysis were limiting factor. It was agreed that period of twenty days should be sufficient to observe infection and recovery time. Only during the interpretation process of collected data, it was discovered that particulates content of milk is affected before clinical signs of disease and also that some animals developed secondary outbreaks of mastitis (both types). In order to better understand mastitis impact on the relations and factors affecting udder physiology long term monitoring is required; preferably for the minimum period of one lactation. This will allow to record data prior to the primary onset of mastitis and better understand influence of environmental factors such as weather, nutrition type. Moreover the experimental protocol of this study had no influence on any day to day aspect of Elkstone Farm therefore ability to monitor impact of implemented changes e.g. diet, milking routine would allow to better understand physiology of the udder and its susceptibility to external factors. As discussed earlier particulate pattern is unique for each animal and

therefore data cannot be compared between each other. The monitoring over two lactations would allow to examine if number of locations and/or age have an impact on MFG production. This would also allow to understand the impact of physical damage to the tissue on MFG production and physiology of the udder over time.

As discussed above, this study brought new evidence regarding physiology of the udder and mastitis. The technique used in the experimental part can be now automated which will allow to continue this work in more timely manner. The further work should consider limitations of this study and based on its findings include wider range of factor which may have an impact on the collected data.

Concluding comments

It has been observed, that particulate number can greatly increase or decrease prior to a mastitis outbreak up to two to three days before clinical signs are visible. This observation has been published (please see Appendix 9 and 10) and a patent has been granted for a particle counter as a tool to monitor the quality of milk and to detect mastitis. Pre-emptive detection of mastitis will not only significantly reduce cost on the farms; it will also have a great impact on the improvement of the welfare of the dairy cows. Moreover, data obtained in this study brought new evidence supporting the theory of the interdependence of the quarters within the udder, demonstrating that physiology and milk synthesis in the four quarters is synchronised and controlled by the same physiological pathways (central controlled). These pathways are able to continuously work even during severe disease which can cause physical damage to the tissue. Furthermore this thesis also provide evidence that particulate characteristics are unique for each animal therefore they have to be treated as a separate cases and cannot be compared between each other. Since characteristics of MFG have a significant impact on the organoleptic properties of dairy products, particle counting and sizing technology might also find their applications in the dairy industry.

Hopefully, this thesis will be an appropriate foundation for future research work associated with milk particles and animal health, and will bring research a step closer

to resolving difficulties faced by dairy farmers on the detection and treatment of mastitis.

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Appendices

1. The Syringe Injector Hamilton Microlab 500BIC
2. LiQuialz E20 – Liquid particle counter
3. The circuit diagram of the Pulse Height Analyser
4. Procedure of the calibration of the particle counter
5. 5A and 5B sample of the latex particulate behaviour
6. 6A and 7B sample of the healthy milk particulate behaviour
7. 7A and 7B sample of the mastitic milk particulate behaviour
8. Diets implemented on Elkstone Farm during the study
9. Janik *et al.*, (2008)
10. Janik *et al.*, (2010)
11. The flow diagram of the research protocol

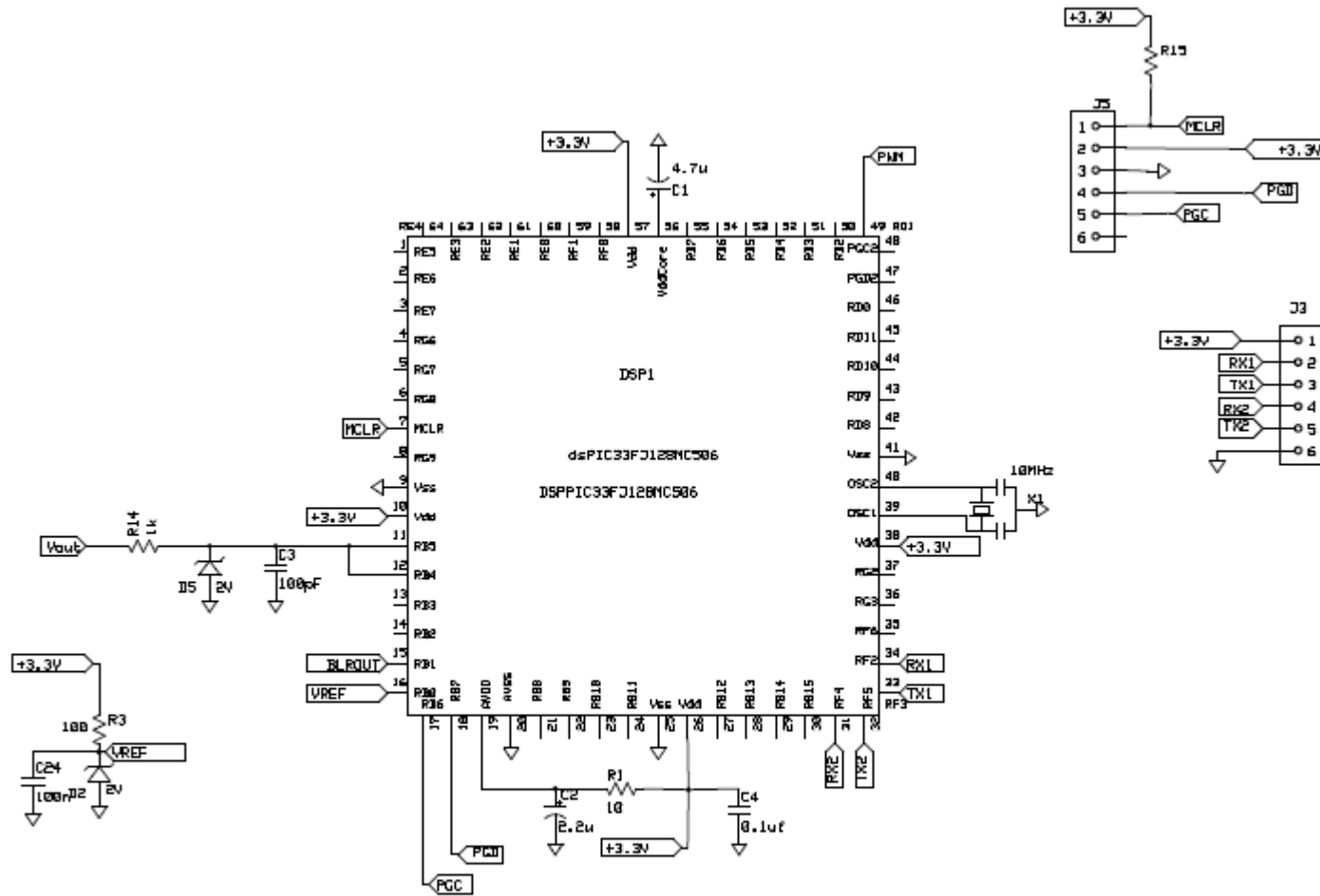
Appendix 1. The Syringe Injector Hamilton Microlab 500BIC

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Appendix 2. LiQuialz E20 – Liquid particle counter

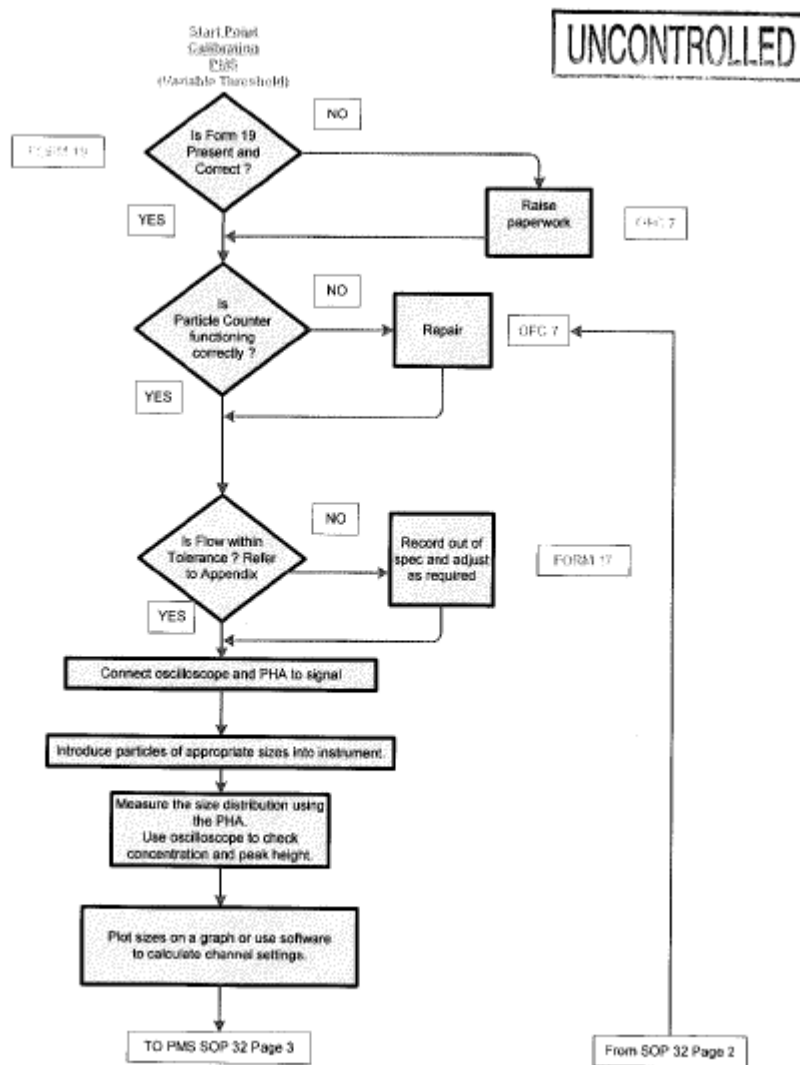
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Appendix 3. The circuit diagram of the Pulse Height Analyser

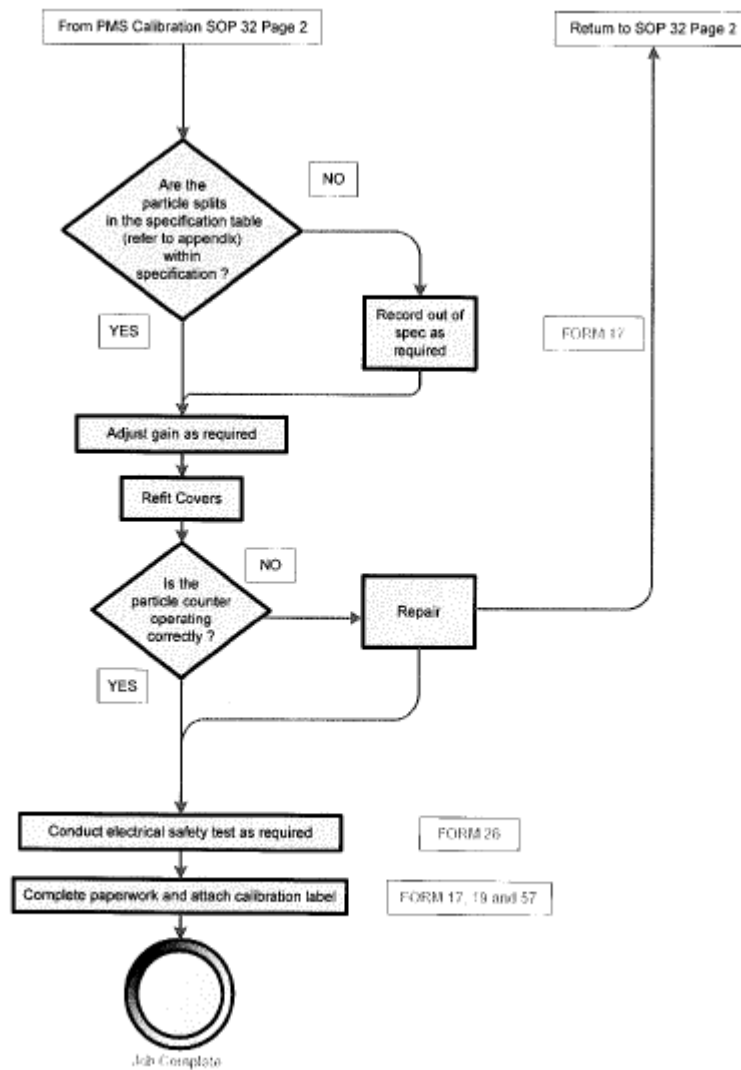


Company Name		
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Appendix 4. Procedure of the calibration of the particle counter



Appendix 4. Procedure of the calibration of the particle counter



Appendix 5A . Sample of the latex particulate behaviour

Figures below illustrate results obtained for latex particles by using liquid particles counter. Key aberration: water **w**, dilution 10^{-8} **8**, dilution 10^{-7} **7**, dilution 10^{-6} **6**.

Figure 1. Latex particle size 2 μm . Read by channel size 2 and 5 μm .

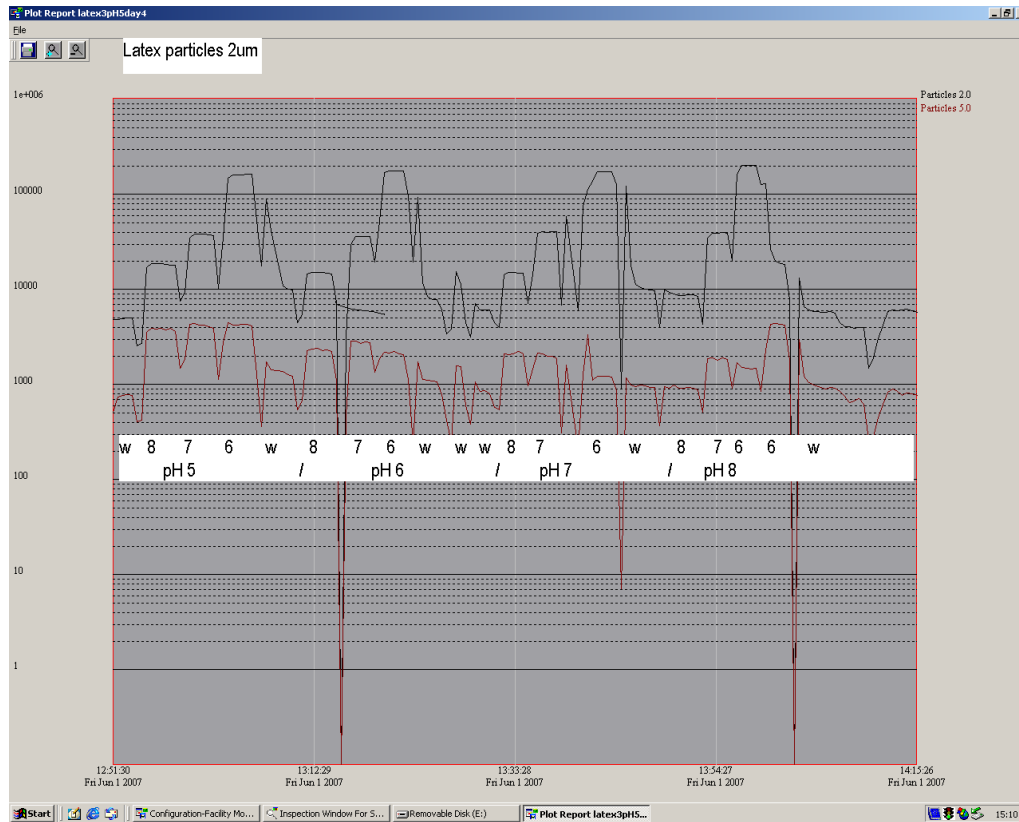


Figure 2 Latex particle size 2 μm cooled to temp 20°C after heating up to 40°. Read by channel size 2, 5 μm

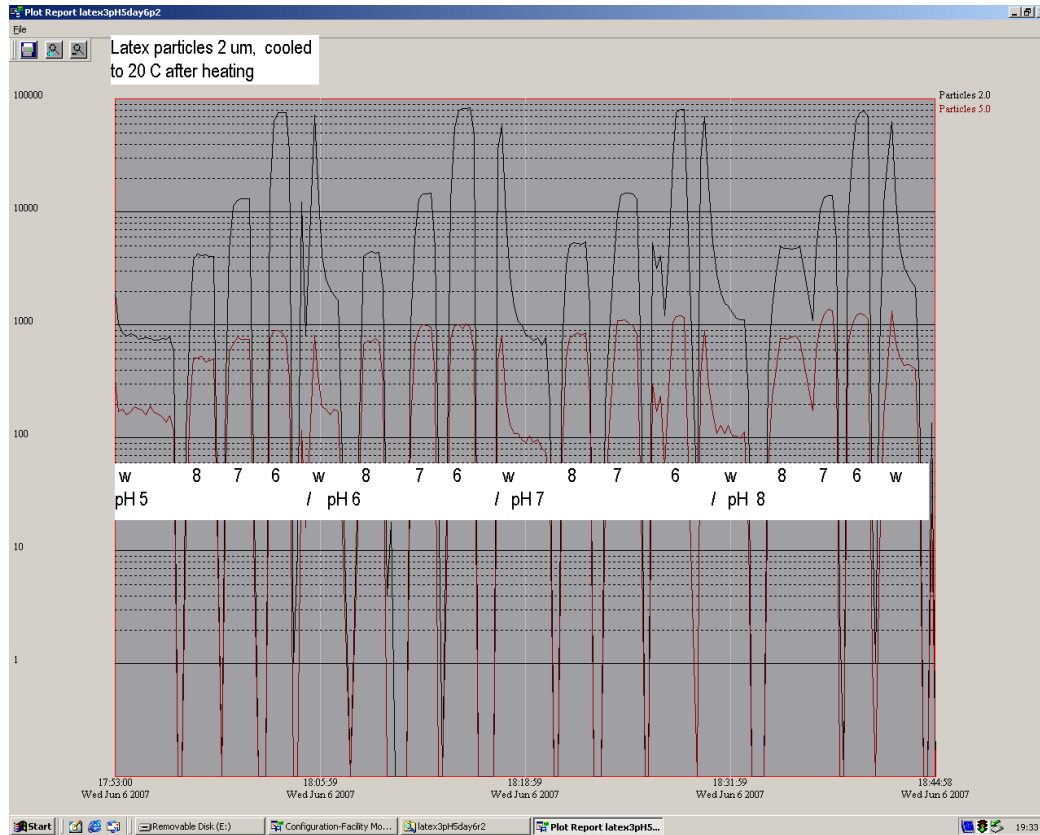


Figure 3 Latex particle size 2 μm in solution of 1% NaCl. Read by channel size 2, 5 μm



Figure 4 Latex particle size 2 μm in solution of 10% NaCl. Read by channel size 2, 5 μm

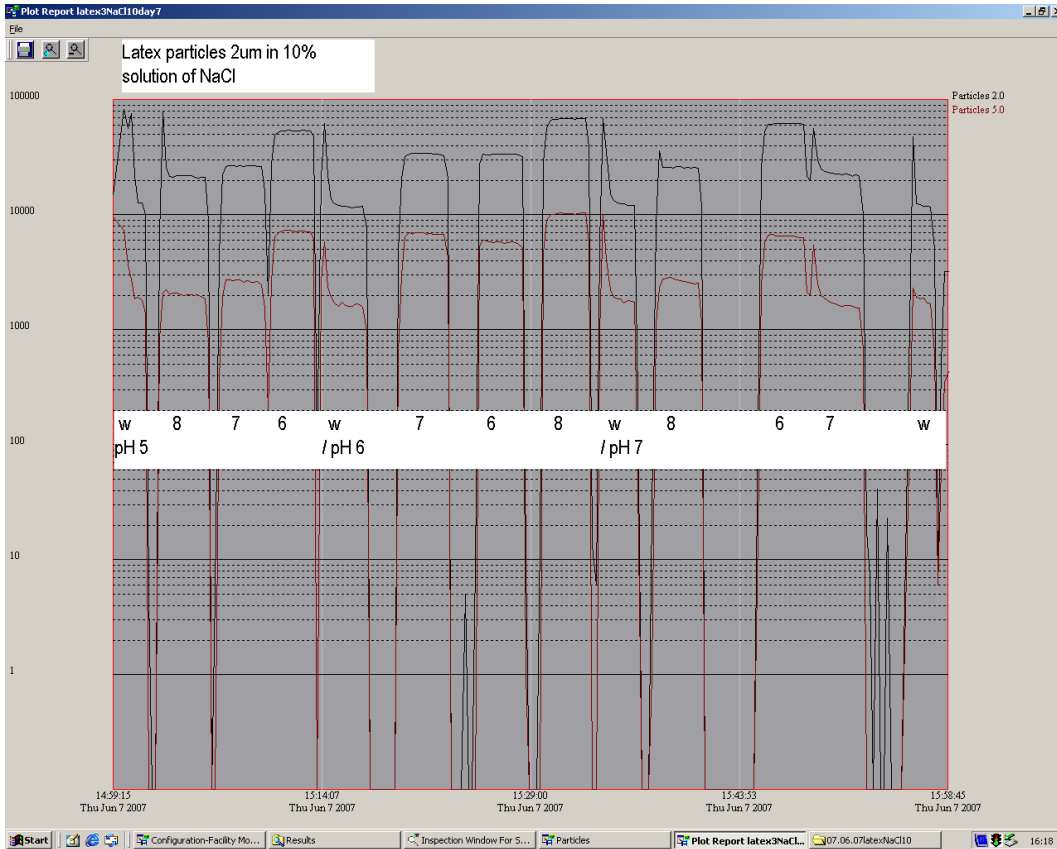
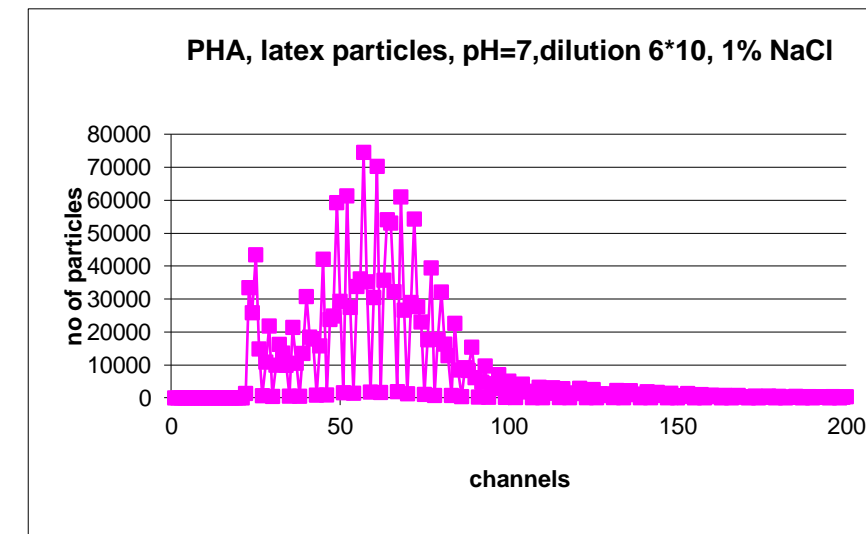
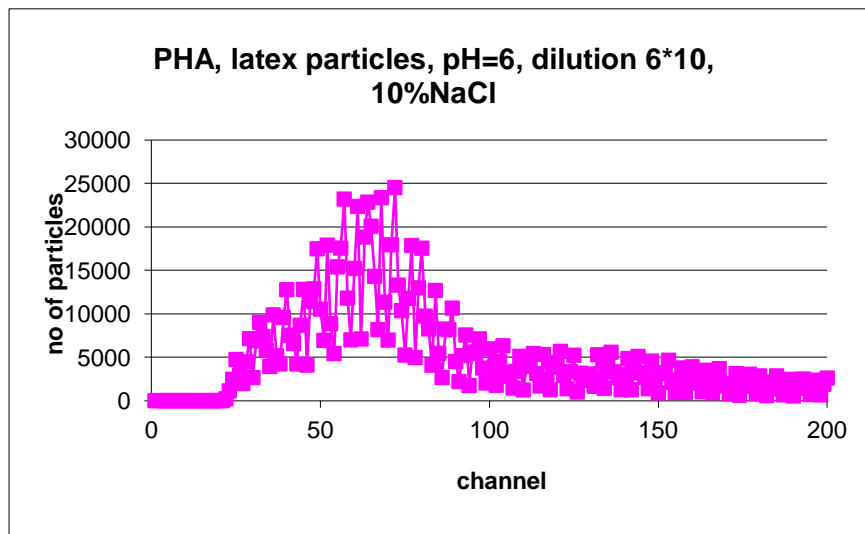
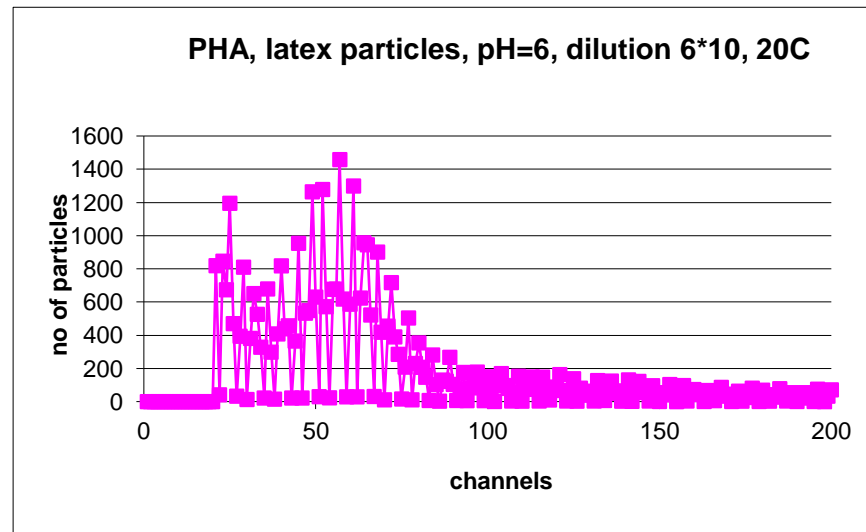
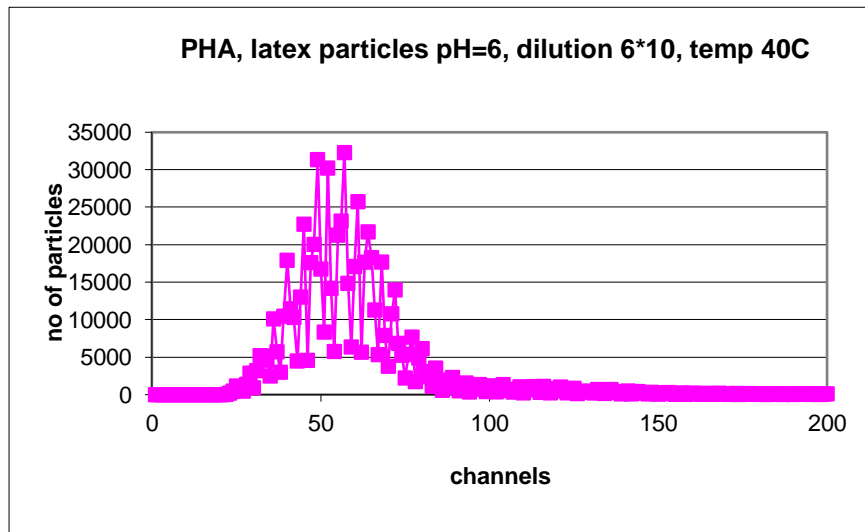


Figure 5 Latex particle size 2 μm , sample age 24 hours. Read by channel size 2, 5 μm



Appendix 5B . Sample of the latex particulate behaviour

Graphs belows illustrated behaviour of latex particles analysed by PHA



Appendix 6A . 6A and 7B sample of the healthy milk particulate behaviour

Figures below illustrate results obtained for non-mastitic samples of milk by using liquid particles counter. Key aberration: water **w**, dilution 10^{-8} **8**, dilution 10^{-7} **7**, dilution 10^{-6} **6**, dilution 10^{-4} **4**.

Figure 1 Normal (non-mastitic) milk sample. Read by channel size 2, 5 and 10 μm



Figure 2 Normal (non-mastitic) milk sample. Read by channel size 2, 5, 10, 15, 20 and 30 μm .

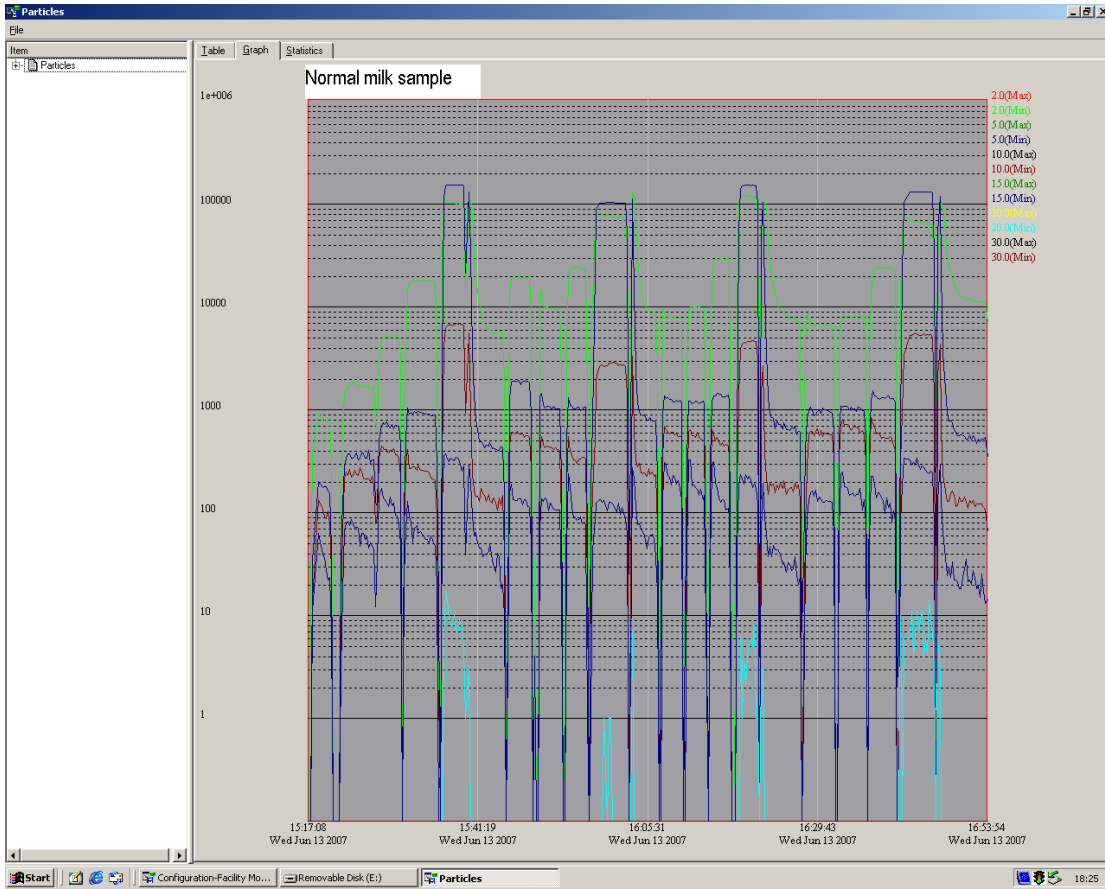


Figure 3 Normal (non-mastitic) milk sample, sample age 44 hours. Read by channel size 2, 5, 10, 15, 20 and 30 μm

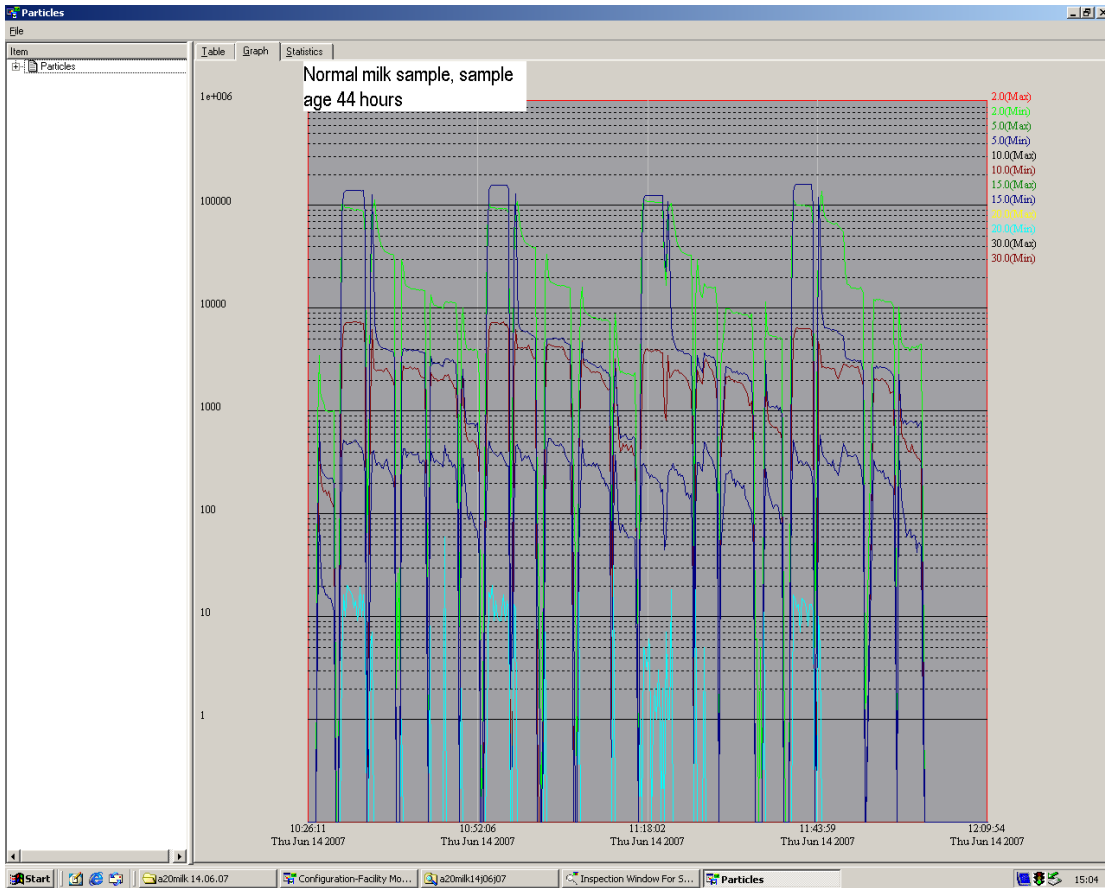
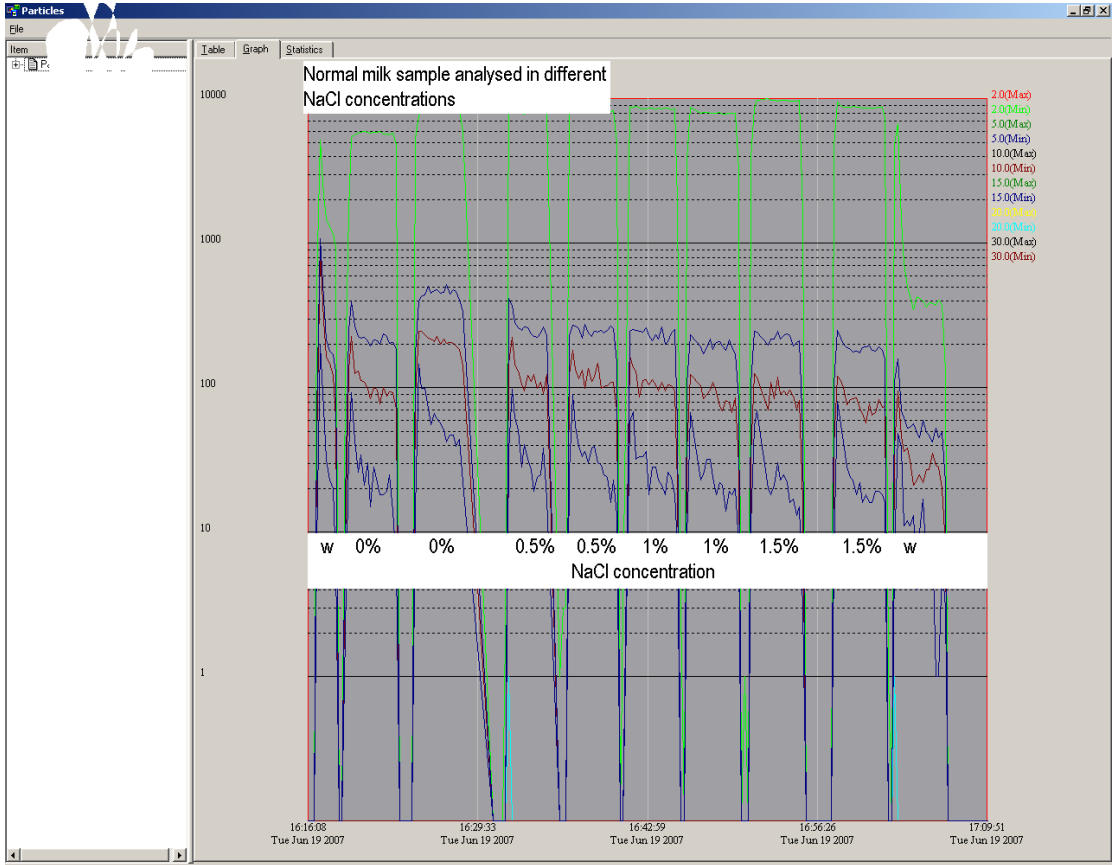
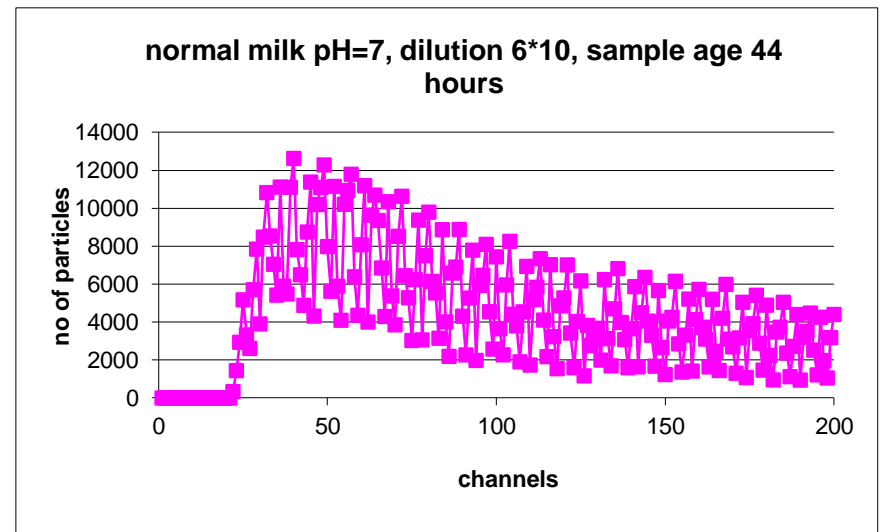
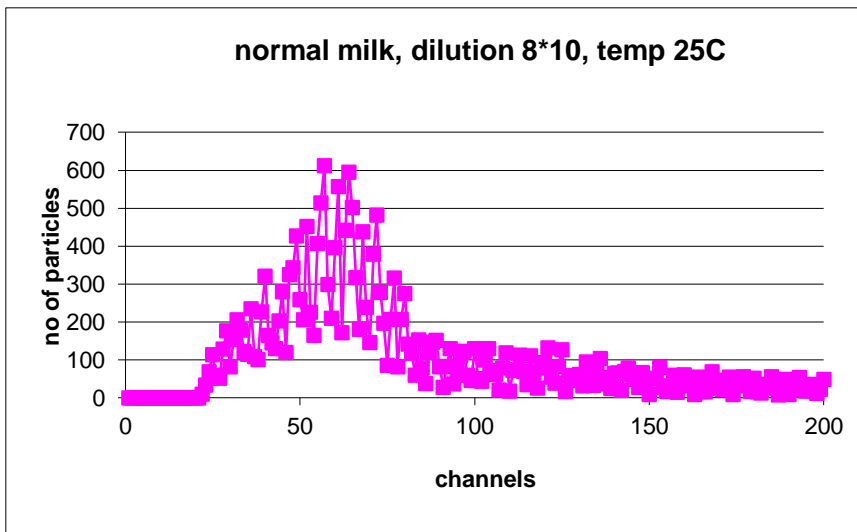
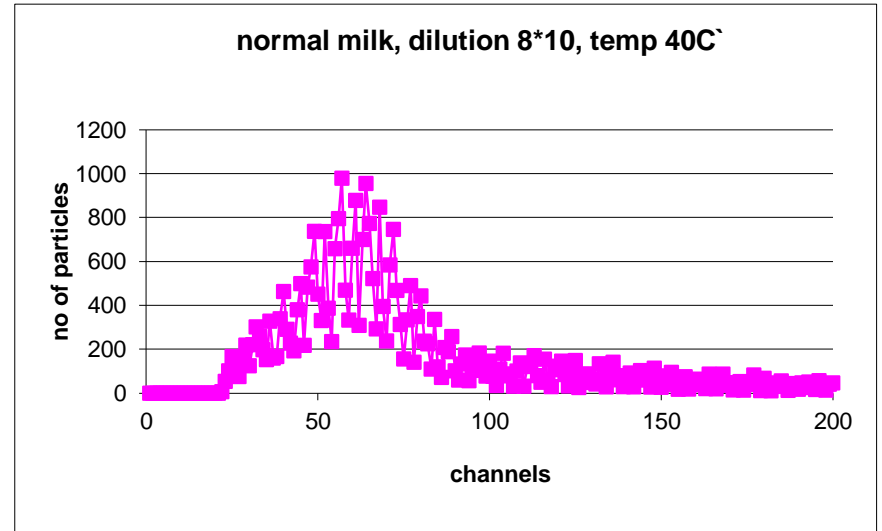
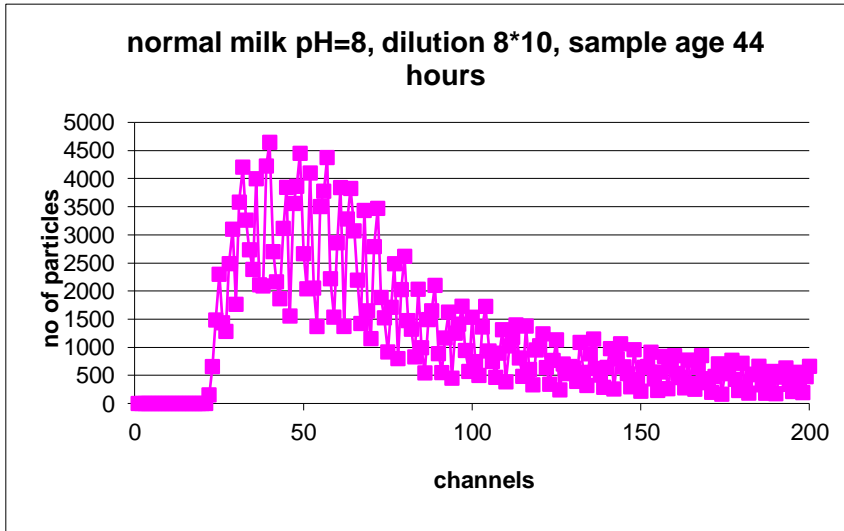


Figure 4 Normal (non-mastitic) milk sample diluted 10^{-8} and analysed in different NaCl concentrations. Read by channels 2, 5, 10, 15, 20 and 30 μm .



Appendix 6B sample of the healthy milk particulate behaviour

Graphs below illustrate behaviour of healthy (non-mastitic) milk particles analysed by PHA.



Appendix 7A sample of the mastitic milk particulate behaviour

Figures below illustrate results obtained for mastitic milk samples by using liquid particles counter. Key aberration: water **w**, dilution 10^{-8} **8**, dilution 10^{-7} **7**, dilution 10^{-6} **6**.

Figure 1 Mastitic milk sample. Read by channels 2, 5, 10, 15, 20 and 30 μm .

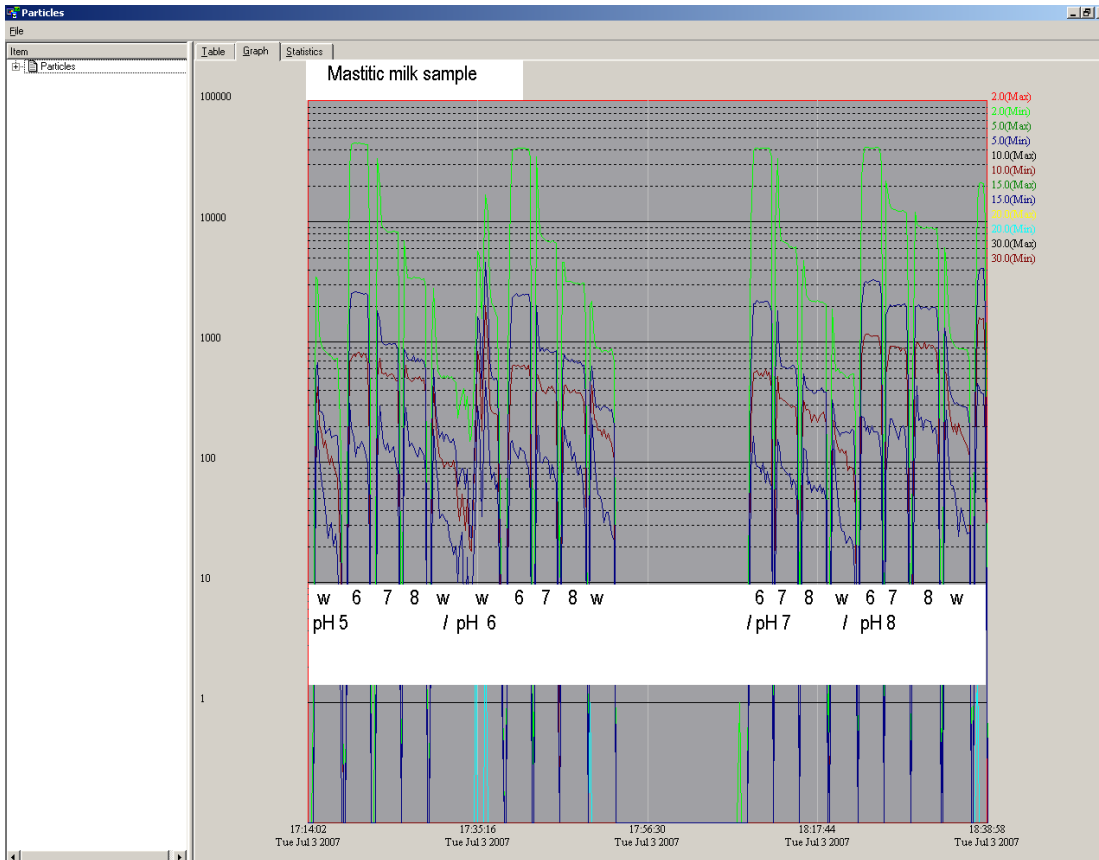


Figure 2 Mastitic milk sample, sample age 24 hours. Read by channels 2, 5, 10, 15, 20 and 30 μm .

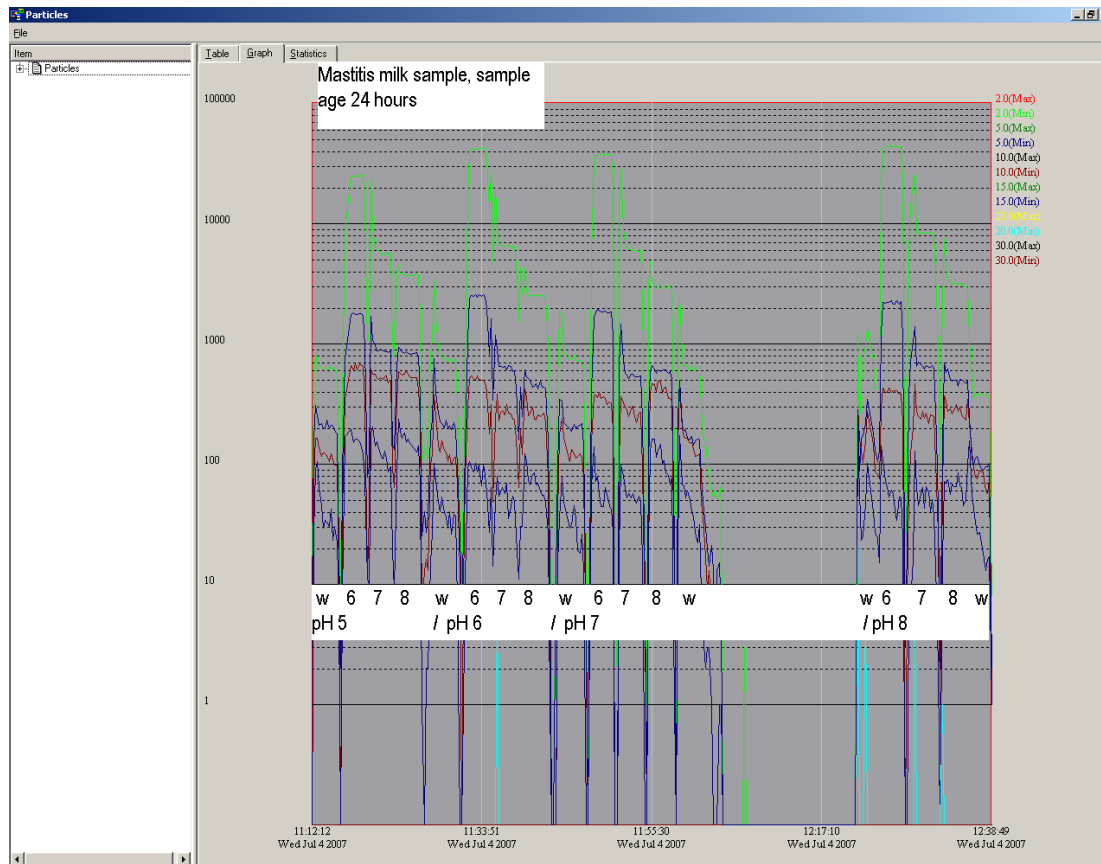
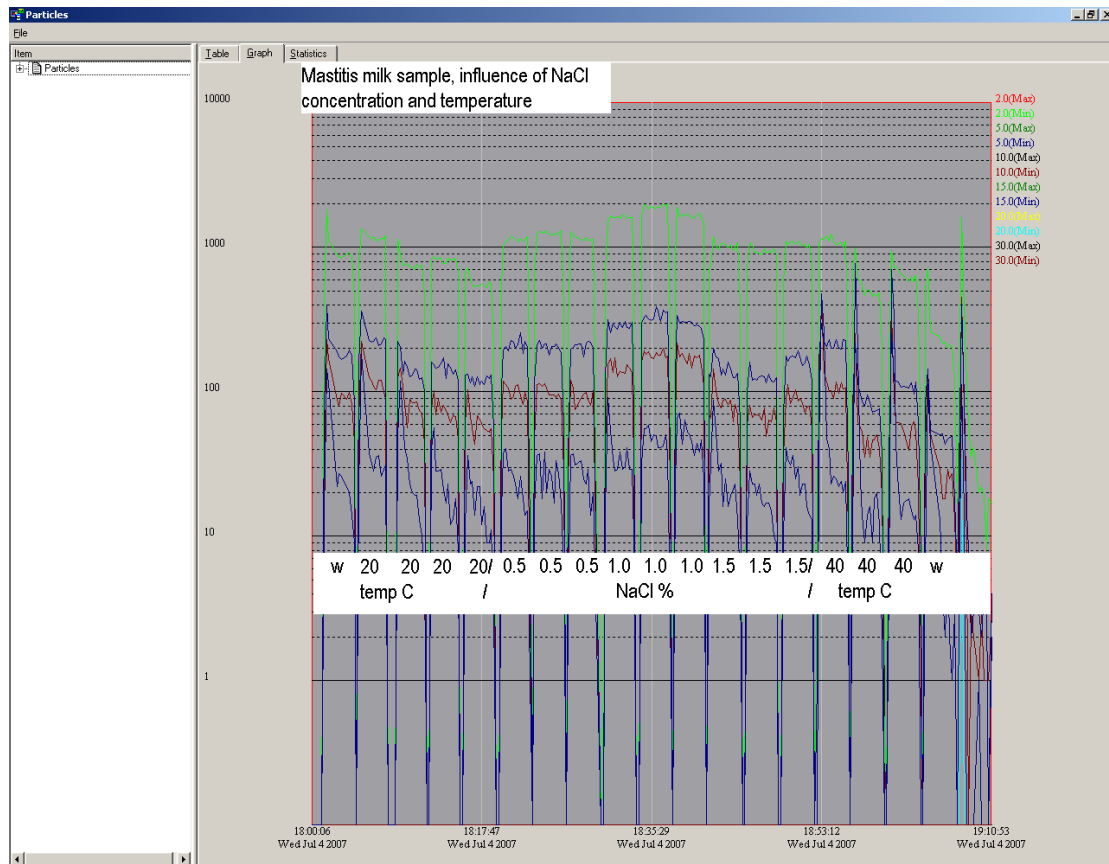
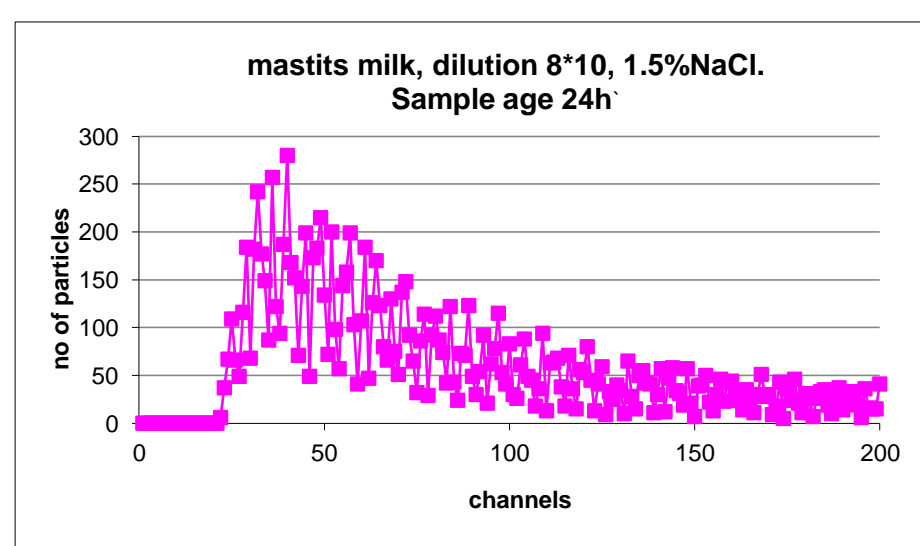
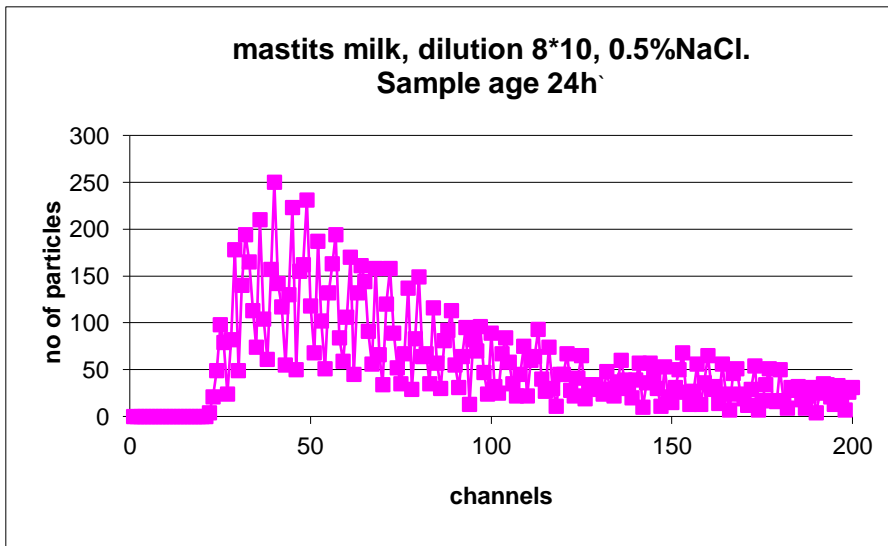
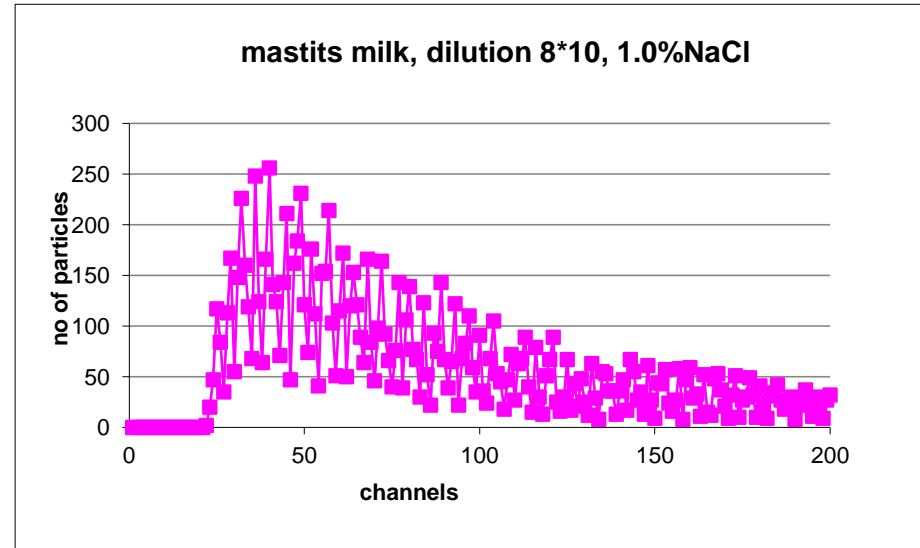
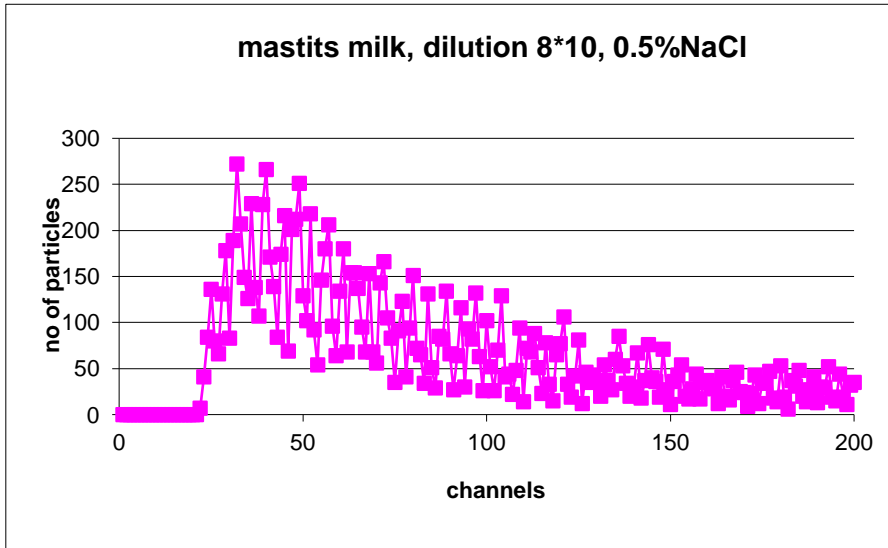


Figure 3 Mastitic milk sample, dilution 10^{-8} . Influence of NaCl concentration and temperature. Sample age 48 hours. Read by channels 2, 5, 10, 15, 20 and 30 μm .



Appendix 7B sample of the mastitic milk particulate behaviour

Graphs below illustrated behaviour of mastitic milk particles analysed by PHA.



Appendix 8 Diets implemented on Elkstone Farm during the study (example)

Green & Kelly Farm Business Consulting

Diets prepared for Mr D Eldred, Elkstone Dairy

Start 20 Oct 2007

Butterfat 4 %

Finish Nov 2007

Milk Protein 3.4 %

Group name	Transition diet	15 Oct	from 20 Oct	Nov when no C wheat	MY
Milk yield		42 litres	42 litres	42 litres	28 litres
Weight change (kg/d)	1	-0.2	-0.2	-0.2	0.25
Bodyweight (kg)	700	650	650	650	650

Feeds	£t	kg fresh	kg fresh	kg fresh	kg fresh	kg fresh
2007 Maize	20	9.00	15.00	24.00	24.00	20.00
Spring grass (DM)	8					
Second cut far pit	16	9.00	32.00	24.00	24.00	30.00
Straw	40	4.00	0.50	0.50	0.50	
1st cut (17/7 test)	16					
25% blend with wheat	141					
Non GM summer blend	145	3.00	10.00	10.00	11.50	6.00
c18	620	0.20	0.40	0.40	0.40	
Wheat - caustic	130		3.00	2.00		2.00
Wheat -rolled	160					
Maize mineral	384		0.15	0.15	0.15	0.15
Mag Cl/Amm Cl mix	170	1.50				
Dry cow mineral	300	0.15				
Salt (Sodium Chloride)	160					
Bicarb	280		0.20	0.20	0.20	
Total intakes		26.9	61.3	61.3	60.8	58.2

Nutrient supplies

Energy required (MJ)	111	291	291	291	223
Diet energy (% of req.)	105	101	100	99	105
Protein required (g)	896	2350	2350	2350	1792
Diet protein (% of req.)	135	100	97	101	97

Diet Costs

Purch feeds £/head/day	0.86	2.20	2.07	2.03	1.19
		5.18 p/l	4.91 p/l	4.9 p/l	3.97 p/l
All feeds £/head/day	1.34	3.04	2.96	2.92	2.07
		7.2 p/l	7 p/l	7.1 p/l	6.9 p/l

Concentrate use

kg/hd/day at 87% DM	3.8	13.4	12.6	12.4	7.9
feed rate at 87% DM		0.32 kg/l	0.3 kg/l	0.3 kg/l	0.26 kg/l

Comments

Three diets for Hy group and one for MY/LY group while you still have caustic wheat.
Gradually increase maize from diet shown as 15 Oct to 20 Oct during the week.

Green & Kelly Farm Business Consulting

Second premix preparation for Mr D Eldred, Elkstone Dairy

Premix Specification

Feed and Price/T	kg fresh	kg DM	% Fresh	% in DM	DM %	DM basis	As fed
Maize screenings £ 110					ME (MJ/kg DM)	86.95	86.95
Soya (Braz) £ 145					FME (MJ/kg DM)	12.65	
Rapeseed meal £ 115	10.00	8.80	10.00	10.12	Total NDF (%)	11.63	18.90
Distillers Grains (Maize) £ 120					Effective NDF (%)	21.74	5.57
Dried beet pulp £ 120	20.00	17.40	20.00	20.01	ADF (%)	6.40	9.83
Urea £ 240	0.80	0.79	0.80	0.91	Starch (%)	11.31	20.76
Molasses (Molaferm 20) £ 90	3.00	2.19	3.00	2.52	Sugar (%)	23.87	9.19
Limestone £ 70	1.00	0.95	1.00	1.09	Oil (%)	10.57	2.34
wheat distillers £ 120	15.00	13.35	15.00	15.35	CP (%)	2.70	24.42
Non GM soya £ 170	16.00	14.40	16.00	16.56	Rumen Stability Value	28.08	
Wheat -rolled £ 100	34.20	29.07	34.20	33.43	Calcium (%)	10	
Oats £ 100					Phosphorus (%)	0.81	0.70
£					Magnesium (%)	0.51	0.45
£					Sodium (%)	0.20	0.17
£					Sulphur (%)	0.17	0.15
Total premix weight	100.00				Chloride (%)	0.63	0.55
Mixing charge (£/T)	25.00				Potash (%)	0.54	0.47
Premix cost (£/T)	145.20				Copper (mg/kg)	1.29	1.12
Premix name	Non GM summer blend				Selenium (mg/kg)	7.46	6
					Manganese (mg/kg)	0.12	0.10
					Zinc (mg/kg)	31.25	27
					Iodine (mg/kg)	25.92	23
					Vitamin A (i.u.)		#VALUE!
					Vitamin D (i.u.)	#DIV/0!	#DIV/0!
					Vitamin E (i.u.)	#DIV/0!	#DIV/0!
						#DIV/0!	#DIV/0!

File ref

Milk particulates and animal health: mastitis in dairy cows

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Summary

This project investigates the hypothesis that the particulate content of milk, as detected by a liquid particle counter, is related to health status of a lactating cow. Thirteen Holstein cows were monitored from the first day of a clinical mastitis outbreak. During the experiment, changes in all four quarters and the mix of milk from each of them were measured. For each sample, the following parameters were collected: somatic cell count (SCC), fat content, number and distribution of milk particles and diameter of milk fat globules (MFGs). The number of particles, SCC and MFG diameter remained stable during periods without mastitis. Clinical mastitis was associated with significant changes in these parameters.

Key words: Mastitis, particles count, somatic cell count

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Advances in the automated pre-emptive detection of mastitis in dairy cattle

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Abstract

10 Liquid particle counting and sizing technology was used to investigate the relationship between the particulate content of milk and the onset of mastitis in Holstein dairy cows. Foremilk was collected during routine milking and examined for particles in the range 1 to 30µm, somatic cell count (SCC), fat content, protein content. Direct microscopy was used to measure the diameter of milk fat globules (MFG). The concentration of particles, SCC and MFG diameter remained stable during
15 periods without mastitis. Clinical mastitis was associated with significant changes in these parameters. It was found that changes in milk particle characteristics preceded the onset of mastitis in both clinical and non-clinical instances. The potential of liquid particle technology is discussed in relation to the early and rapid detection of mastitis.

20 **Key words:** Mastitis, particles count, milk fat globules, somatic cell count

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