THE EFFECT OF PLASMIN ON AGE GELATION IN UHT MILK

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DECLARATION

I declare that the thesis hereby submitted for the qualification of Doctor of Philosophy in Consumer Science at the University of the Free State is my own independent work and that I have not previously submitted the same work for a qualification at another University or Faculty. I furthermore concede copyright of the thesis to the University of the Free State.

ABekker

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LIST OF ABBREVIATIONS

Acetonitrile	-	CH_3CN
Alpha	-	α
Alpha-s_1	-	αs_1
Alpha-s_2	-	αs_2
Beta	-	β
Beta-lactoglobulin	-	β-LG
Bovine serum albumin	-	BSA
Calcium	-	Ca
Casein hydrolysates	-	CNH
Cleaning in place	-	CIP
Colony-forming unit	-	CFU
Dalton	-	Da
Distilled water	-	DH_2O
ε-aminocaproic acid	-	EACA
Ethylenediaminetetra-acetic acid	-	EDTA
Fluorescein thiocarbamoyl casein	-	FTC
Gamma	-	y
High-performance liquid chromatography	-	HPLC
Hydrochloric acid	-	HCl
Innovative steam injection	-	ISI
Iso-electric point	-	pI

Kappa	-	K
Kilo Dalton	-	kDa
Millimolar	-	mM
Molar	-	M
Nanometer	-	nm
Normal	-	N
Phosphate-buffered saline	-	PBS
Potassium iodate	-	KIO_3
Pre-activation peptide	-	PAP
Reverse-phase high-performance liquid chromatography	-	RP-HPLC
Revolutions per minute	-	rpm
Sodium chloride	-	NaCl
Somatic cell count	-	SCC
Species	-	spp.
Sulfhydryl group	-	SH-groups
Tissue-type	-	t-PA
Trichloroacetic acid	-	TCA
Trisaminomethane	-	Tris
Ultra-high temperature	-	UHT
Ultraviolet	-	UV
University of the Free State	-	UFS
Urokinase-type	-	u-PA

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NOTE TO THE READER

In this Thesis, each Chapter was written as an individual entity, therefore, repetition between the Chapters had been unavoidable.

CHAPTER 1

Introduction

Milk is a popular liquid to consumers due to a high nutritional value, however, it is a very complex medium since it is comprised of numerous components. This medium can be described as a colloidal suspension due to the presence of three phases which are fat globules, casein micelles and a serum part compiled of liquefied components which include; lactose, whey proteins, vitamins, and minerals thus it is a complete food for consumers. It is regarded as being sensitive and unstable since the properties can be transformed by varying factors which include; enzymatic reactions and heat treatments (Gaucher *et al*., 2008; Hallén, 2008; Raikos, 2010; Vaghela *et al.,* 2017).

Raw milk does not undergo any pasteurisation thus can be contaminated with harmful bacteria hence the importance of the application of heat treatments to milk. There are various applications of heat treatments to milk to deactivate bacteria thus enhancing the storing duration of milk such as pasteurisation, sterilisation, and ultra-high temperature (UHT) treatment. Although heat treatments inactivate bacteria, their heat resistant proteases survive and lead to problems within milk after processing and during storage. Heat treatments are beneficial, however, it can lead to the progress of off-tastes and the main destabilisation procedures which are coagulation, flocculation and age gelation. These complications diminish the shelf stability of milk (Hassan *et al*., 2009).

The awareness towards food consumption is rising along with the demand for aseptically packaged (UHT milk) products, however, the quality of this type of milk is in jeopardy due to the occurrence of chemical and physical changes during storage. Age gelation is considered as a huge concern for dairy manufacturing due to milk quality being negatively affected and consumers reject this type of milk due to it being undesirable. This phenomenon includes changes in milk that arise due to exposure to extreme destabilisation conditions by either chemical or enzymatic actions. These changes are characterised by reduced fluids, improved thickness and lastly the presence of a formed protein gel that is three-dimensional (Datta & Deeth, 2001; Vaghela *et al.,* 2017; Anema, 2019).

Detection techniques for age gelation are essential to find the root and possibly fight this problem. Previous research work (Hattingh, 2017) mainly focused on testing and establishing rapid and sensitive detection techniques for age gelation. It was established that all the techniques successfully identified risky milk likely to undergo age gelation and some of the techniques were successful to make a distinction among the protease activity of indigenous plasmin and microbial proteases.

It is already known through previous research work (Hattingh, 2017) that proteases cultivated by *Pseudomonas fluorescens* and *Bacillus licheniformis* have a major impact on age gelation, however, it is essential to establish peptide profiles for a wider range of thermo-tolerant bacteria (previously

known as psychrotrophic bacteria). Plasminogen activation is also a complex process and only the activators found in the literature are known, therefore it is vital to investigate various constituents to maybe find more plasminogen activators. The milk agar plate technique will be optimised since it was established in previous research work (Hattingh, 2017) that it is a very accurate and cheap proteolytic detection method thus optimisation is beneficial. The focus of this study is also to further investigate proteolytic assays, specifically plasmin assays and hopefully find a trustworthy, cost-effective assay. The reverse-phase high-performance liquid chromatography (RP-HPLC) detection technique will also be further optimised by relative area integration versus enzyme activity which will be helpful when an unknown sample is analysed for possibly the presence of age gelation.

CHAPTER 2

Literature review

Objectives

The objectives were to do a comprehensive literature review which included the methods for detecting and differentiating between microbial proteases and indigenous milk plasmin that has an effect on age gelation.

2.1 Instability of milk

Milk is regarded as an unstable and perishable medium due to numerous components that are present which include proteins, fat, microorganisms, and enzymes which make milk susceptible to microbial and chemical degradation processes. Milk is also available in various forms such as raw, pasteurised and ultra-high temperature (UHT) of which raw milk is the most perishable hence the importance of heat treatments during milk production. Heat treatments are advantageous in the sense that it results in milk without the presence of microorganisms, however, it may affect the overall stability of milk since milk is not stable upon heating.

2.1.1 Raw milk

Raw milk is a fragile medium due to its shelf stability lasting for a maximum of 3-5 days. This type of milk also contains factors that are ideal conditions for microorganism growth including; numerous nutrients and a neutral pH. This type of milk does not undergo any pasteurisation thus cooled storing is vital to avoid spoilage. The varying aspects affecting the shelf stability are milk collection, handling techniques, the hygiene of the milking surroundings, the temperature of storing, somatic cell count (SCC) and existent bacterial amount (Vijayakumar, 2012; Von Neubeck *et al.,* 2015; Vithanage *et al.,* 2016).

Both Gram-positive and Gram-negative bacteria dictate the microbial population within raw milk. Lengthy storing of raw milk at a temperature range between 2-6°C has an enormous effect on the indigenous microbial population conformation as well as supports the growth of thermo-tolerant bacteria (previously known as psychrotrophic bacteria). It is ordinary that the primarily leading Gram-positive organisms are substituted by Gram-negative and Gram-positive thermo-tolerant organisms. Therefore, 90% of thermo-tolerant bacteria are existent within cooled raw milk thus making them the dominant species (Samaržija *et al*., 2012; Baur *et al.,* 2015; Von Neubeck *et al.,* 2015; Vithanage *et al.,* 2016; Xin *et al.,* 2017).

The presence of Gram-positive spore-forming bacteria is of lesser amount than Gram-negative bacteria due to extended generation time (8.5 hours) and elongated lag phase at 2-7°C. It is

essential that the total count of mesophiles within raw milk is less than 30 000 colony-forming units (CFU/mL^{-1}) and the total count for thermo-tolerant bacteria must be lesser than 5000 CFU/mL^{-1}. The most common spoilage bacteria within raw milk are species (spp.) from *Pseudomonas* and *Bacillus* (Samaržija *et al.*, 2012).

It is vital to use raw milk with superior quality to attain UHT milk with an improved shelf life. Contamination of raw milk by thermo-tolerant bacteria is inevitable therefore rapid cooling is essential. Minimum thermo-tolerant growth and reduced amounts of extracellular microbial proteases can be obtained with storing at 4°C for not longer than 48 hours, however, the storing of raw milk at 2°C is expected to be more efficient than storage temperatures that range between 4-7°C. Keeping raw milk at a temperature of 2°C lead to positive effects in UHT milk quality since this temperature guarantees UHT milk with a longer lifespan (Tamime, 2008; Xin *et al.,* 2017). Heat treatments are an essential step to be taken and must be applied to milk since raw milk is regarded as being perishable (Ye *et al.*, 2011; Chove *et al.*, 2013).

2.1.2 Heat-treated milk

2.1.2.1 Importance of heat treatment

Milk that is regarded as safe, high in nutrients and of substantial quality is vital for consumers hence the importance of thermal processing during milk production of which the aim is to lengthen the shelf stability of milk by improving the overall quality (Raikos, 2010; Richards *et al.*, 2014). Heat application is important since it leads to the inactivation of the microorganisms present within milk thus guaranteeing milk with high shelf stability and safe for ingestion (Gaucher *et al.*, 2008; Chavan *et al.*, 2011).

2.1.2.2 Heat stability of milk

The heat stability of milk is the capability to undergo heat treatments without the occurrence of coagulation or gelation. Variations that arise in milk due to heat treatments can be changeable or irretrievable depending on the heat treatment conditions. Variations within milk that follow for the period of the heating process are decreased pH, inactivation of enzymes, calcium (Ca) phosphate precipitation, lactose isomerisation, Maillard browning whey protein destruction, interfaces of whey proteins with caseins and micelle modifications. These heat-induced alterations have a substantial influence on the chemical aspect of milk and thus are a huge role player in milk stability. The overall consequence of heat treatments is altering the sensory features of milk such as appearance, colour, taste, texture and nutritional value. Heat treatments can lead to the three main destabilisation processes in milk which are coagulation, flocculation and age gelation (Thompson *et al.*, 2008; Tamime, 2008; Chavan *et al.*, 2011).

2.1.2.3 Different heat treatments

There are various types of heat treatments that are applied and vary depending on the end usage of the milk. Homogenisation is a treatment applied to delay the fat separation within milk thus increasing the physical shelf life of which 100-200 bar pressure is generally applied to lead to a decrease in fat globule size (Islam *et al.,* 2017). Raw milk undergoes initial heat treatment, thermisation, which is used to deactivate and diminish the growth of thermo-tolerant bacteria. Thermisation is heat-treating milk at 57-68°C for 10-20 seconds straight after arrival at the processing plant prior to storing in the silo. It is regarded as the mildest heat treatment with the purpose of enhancing the shelf life by decreasing the time for thermo-tolerant bacteria to multiply and produce heat resistant microbial proteases thus postponing the spoilage of milk during storage prior to pasteurisation (Tamime, 2008).

Thermisation is not regarded as effective in the destruction of all the pathogenic microorganisms in raw milk hence the application of pasteurisation, which is highly effective in destroying most of these organisms, therefore pasteurised milk contains a reduced number of pathogenic microorganisms. Pasteurisation consist of the application of a heating temperature (63°C for 30 minutes) which is referred to as "low-temperature long-time batch pasteurisation" or 72°C for 15 seconds which is called "high-temperature short time-continuous flow pasteurisation" (Tamime, 2008). The type of pasteurisation method used depends on the final milk product since lower temperatures are mainly applied for refrigerated milk and higher temperatures are applied for milk stored at room temperatures (Samet-Bali *et al.*, 2013).

Pasteurisation destroys most spoilage bacteria, however, it does not fully inactivate indigenous enzymes and microbial proteases since the proteases are heat resistant (Chavan *et al.*, 2011; Holland *et al.*, 2011; Vijayakumar, 2012). This heat treatment also does not cause colour changes, changes in tastes and changes in milk appearance. Post-pasteurisation contamination of milk arises when milk is exposed to dust, dirty udders, contaminated equipment, tanks, and pipelines. Other sources of post-pasteurisation contamination are the air and packing material (Tamime, 2008).

Sterilisation is another heat treatment applied to milk which consists of a greater effect than pasteurisation since it is regarded as a severe heat treatment that elongates the duration of milk storing thus enables milk to be kept at room temperatures for numerous months without quality decline. Sterilisation is generally common to produce long-life milk since milk is subjected to very high temperatures which inactivate pathogenic microorganisms and proteolytic enzymes, however, this treatment does not result in complete sterility (Tamime, 2008; Hassan *et al.*, 2009; Chavan *et al.*, 2011). This treatment has certain disadvantages such as the development of cooked tastes and a noticeable brown colour (Tamime, 2008). Sterilisation involves heating of milk within containers at 115-120°C for 20 minutes. Sterilisation temperatures can be increased from 142-152°C in order to postpone the onset of age gelation since this will enable milk to be warehoused for a lengthier

duration without the occurrence of age gelation. A sterilisation temperature of 150°C prolongs the duration at which milk can be stored since it diminishes the incidence of proteolysis, gelation, and bitterness (Chavan *et al.*, 2011).

Another heat treatment applied to milk is UHT treatment which is applied for the production of long-life milk that can be warehoused for numerous months (room temperature) since this treatment is used along with aseptic packaging (Hassan *et al.*, 2009; Holland *et al.*, 2011). UHT treatment of milk is beneficial since it prolongs shelf life, however, it results in quality deterioration and chemical and physical changes due to extreme heat temperatures which in turn affect the properties of milk in a negative way (Vijayakumar, 2012; Pulkkinen, 2014). This type of milk can normally be warehoused for numerous months without deterioration, however, it is also considered to be the most problematic type of milk since age gelation mainly occurs in UHT milk hence the focus of this type of milk in this study (Zhang *et al.,* 2018).

2.1.2.3.1 UHT milk

2.1.2.3.1.1 Definition and conditions of UHT milk

UHT treatment of milk is a popular heat treatment and is regarded as a well-established technology. This milk is especially prevalent in developing countries since it can be stored in the absence of refrigerated conditions for approximately 4-6 months. UHT treatment of milk comprises of heat treatment at high temperatures for short periods. This treatment, therefore, has the capability to elongate milk shelf stability thus milk can be considered to be commercially sterile and spoilage resistant after the application of UHT treatment thus can be warehoused at room temperatures for numerous months without a decline in quality under normal circumstances. However, it is not guaranteed that this type of milk will remain microbiologically stable for the whole storage period (Chavan *et al.*, 2011; Chove *et al.*, 2013; Rauh *et al.*, 2014, Vaghela *et al.,* 2017; Zhang *et al.,* 2018; Anema, 2019).

The stages of the UHT process include; pre-heating with heat restoration, holding at pre-heat temperature, sterilising and holding at this temperature, chilling, and sterilised packaging. The heating conditions applied to milk during UHT treatment are at 140-142°C for 4 seconds or at 138°C for 2-5 seconds. The two main UHT treatment types are the direct UHT treatment at 142°C for 5 seconds and indirect UHT treatment at 145°C for 3 seconds. The direct UHT treatment entails steam inoculation or mixture with vacuum-flash refrigeration which comprises a mixture of superheated steam with milk. The indirect UHT treatment system uses high-performance heat exchangers that transfer heat crosswise a divider among the steam (heat medium) and the milk. The most recent UHT treatment used in some countries is a combination of the direct and indirect UHT treatments of which the benefits include; heat regeneration at higher rates and less chemical damage such as changes in the taste. UHT treatment of milk has an extensive consequence on the steadiness of

caseins, cooperate with whey proteins and eventually proteolysis (Tamime, 2008; Chove *et al*., 2013; Rauh *et al*., 2014; Stoeckel *et al.,* 2016; Vaghela *et al.,* 2017; Raynes *et al.,* 2018).

2.1.2.3.1.2 Changes in UHT milk during processing and storage

The handling and packaging conditions for UHT milk occurs under sterile conditions thus continuing freshness for numerous months (Oupadissakoon, 2007). UHT treatment yields commercially sterile milk hence it should be free from bacteria that is prevalent throughout storing since it should remain sterile when stored at room temperature for 4-6 months (Tamime, 2008; Gaucher *et al*., 2008; Raynes *et al.,* 2018).

UHT handling is regarded as a severe application thus causes some physical and chemical alterations to milk (Gaucher *et al*., 2008). Changes that occur during UHT processing include the destruction of whey proteins, protein-protein interfaces, lactose-protein interfaces, isomerisation of lactose, Maillard browning, compound development, the development of flavoursome compounds, decreased pH and the development of insoluble constituents (Datta *et al*., 2002). Alterations in the chemical part of UHT milk have negative consequences on the taste, nutrients and physical stability (Tran *et al*., 2008). Some of these changes to UHT milk during processing can either be desired or unwanted (Enright *et al*., 1999; Chavan *et al*., 2011). Off-tastes are linked with tyrosine discharge whereas thickness alterations are linked with casein hydrolysis (Richards *et al*., 2014). Changes in macro and micronutrients are also common throughout the UHT process of which the level of these changes is subjected to the type of UHT processing and temperature used (Valero *et al*., 2001; Datta *et al*., 2002; Rehman & Salariya, 2005).

The key variations that arise in UHT milk throughout storing are due to proteolytic, and oxidative actions (Richards *et al*., 2014). Chemical and biochemical actions may also yield during UHT milk storing thus leading to modified constituents (Dupont *et al*., 2007). Variations that befall during UHT milk storing are influenced by storage temperature, light contact, and oxygen (Hassan *et al*., 2009).

Unwanted alterations in the sensory department of UHT milk occur due to the decay of milk fat and protein that arise during storing as a consequence of proteolysis (Rehman & Salariya, 2005). Other changes that occur during UHT milk storing include changes in colour, odour, texture, taste, pH, and sediment. Extended storing is responsible for off-tastes such as bitter, stale and lipolysed tastes due to active heat resistant enzymes (Celestino *et al*., 1997; Stoeckel *et al.,* 2016; Raynes *et al.,* 2018; Anema, 2019). Lipolysed tastes are characterised as rancid, butyric and bitter (Oupadissakoon, 2007). A decline in the sweetness of UHT milk is also common during storing (Hassan *et al*., 2009).

These unwanted alterations may cause the UHT milk to destabilise and to develop a gel throughout storing thus diminishing the quality and disturbing the shelf stability of UHT milk negatively (Datta *et al*., 2002; Gaucher *et al*., 2008; Zhang *et al.,* 2018). Examples for UHT milk with the presence of age gelation include; a formed three-dimensional protein network after several months of storage, a

sediment rich in proteins at the bottom of the container or an accumulated fat layer at the top (Anema, 2019).

Shelf life states the time at which UHT milk can be stored before a decline in quality occurs (Chavan *et al.*, 2011). The shelf stability of UHT milk can vary (3-9 months) when warehoused at 20-30°C (Celestino *et al.*, 1997; Richards *et al.*, 2014). The shelf stability of UHT milk also hinges on the progress of a variety of physicochemical and biochemical variations afterward handling. The sensory properties of UHT milk regulate the duration at which it can be warehoused as well as consumer satisfactoriness (Richards *et al.*, 2014).

UHT milk is a successful type of milk, however, commercial recognition hinges on contamination after processes, consumer approval, chemical and physical variations due to heat treatment and lengthy storing (Chavan *et al.*, 2011).

2.1.2.3.1.3 Direct and indirect processed UHT milk

The susceptibility towards age gelation of both direct and indirectly processed UHT milk differs. Generally, indirectly processed UHT milk is regarded as being steadier than UHT milk produced by the direct heat treatment method thus age gelation normally does not occur in UHT milk that is administered by the indirect heat treatment method (McMahon, 1996; Celestino *et al.*, 1997). Indirect heat-treated UHT milk undergoes a greater heat intensity thus deactivating the proteases to a greater extent (Datta & Deeth, 2001; Cilliers, 2007).

Therefore, UHT milk processed by the direct heat treatment method are regarded as being less steady hence more prone to age gelation due to more severe heat treatment, reduced heat load, speedy heating and cooling when equalled to UHT milk processed by the indirect heat treatment method (Kohlmann & Nielsen, 1988; Rauh *et al.*, 2014). Directly processed UHT milk also consists of higher plasminogen and plasmin activity than indirectly processed UHT milk (Datta & Deeth, 2001).

The alteration in proneness towards age gelation for direct and indirect processed UHT milk is that throughout indirect UHT processing, larger quantities of enzyme and beta-lactoglobulin (β-LG) are denatured which hinders proteolytic action within indirectly processed UHT milk (Kohlmann & Nielsen, 1988). It is therefore clear that indirectly processed UHT milk is less vulnerable to age gelation than directly processed UHT milk. The indirect UHT treatment method is thus a preferred and practical processing method in order to delay age gelation and also UHT milk free from cooked tastes (Datta & Deeth, 2001; Chavan *et al.*, 2011).

2.2 Main destabilisation processes in milk

2.2.1 Coagulation

The context in which the term coagulation is used must be wisely judged in order to understand the precise meaning. Generally, coagulation of milk is considered to be an intricate procedure due to the various constituents present in milk which affect the stability such as the proteins, fat content, and enzymes. According to the dictionary, coagulation is the procedure where liquids transform into semi-solid masses (Allen, 1991). In science, coagulation is used when protein denaturation takes place due to insoluble proteins that endure suspension or precipitates (Isaacs & Uvarov, 1979). In food science, coagulation is random aggregation along with denaturation where protein-protein interfaces rule over protein-solvent interfaces which eventually lead to the development of a course coagulum (Cheftel *et al.,* 1985).

In dairy manufacturing, coagulation does not automatically refer to protein denaturation of which other terms may include precipitation, flocculation, and gelation. Distinct variation among aggregation, flocculation, and coagulation is provided by Walstra, (2003). According to this source, aggregation occurs when two units stay organised for an extended period than the normal duration. Flocculation is alterable aggregation whereas coagulation is aggregation that is irretrievable.

The term coagulation is used in dairy science to include a range of events that occur during experimental procedures where protein aggregation leads to the development of a coagulum, precipitate or gel. Milk destabilisation can take place in several ways (refer to Table 1 and Figure 1). Other destabilisation processes include flocculation and age gelation.

Table 1. The destabilisation of milk by various agents (Federation, 2007).

Destabilisation agent	Proteins affected	Destabilisation mechanism	Chemical forces involved
Heat	Casein + whey	Protein unfolding, SH exchange, whey + micelle interface, steric alteration, casein micelle stabilisation	Covalent, hydrophobic, ionic and Ca-mediated connection. Disulphide bond development
Proteolytic enzymes	Casein	Steric and electrostatic stabilisation losses of micelles due to enzymatic κ-casein hydrolysis	Hydrophobic, ionic and Ca-mediated attachment
Acid	Casein	Electrostatic stabilisation losses due to charge neutralisation. Colloidal Ca phosphate demineralisation	Hydrophobic, ionic and Ca-mediated attachment
Ethanol	Casein	Steric firmness losses due to moisture deficiency, Ca-mediated interfaces	Hydrophobic, ionic and Ca-mediated attachment
Charged polysaccharide	Casein + whey	Specific interfaces, thermodynamic mismatch	Specific polysaccharide/protein interfaces, hydrophobic, ionic and Ca-mediated attachment
Low temperature	Casein + whey	Steric stabilisation losses due to moisture deficiency	Hydrophobic, ionic and Ca-mediated attachment

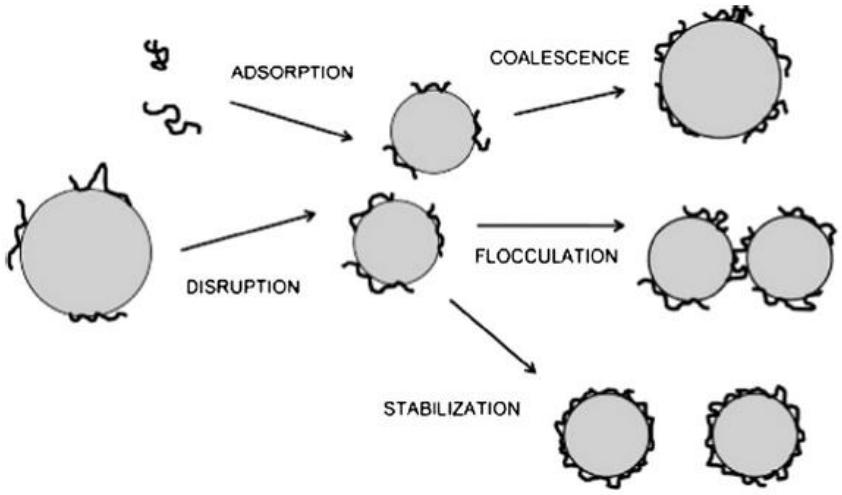

Figure 1. Illustration of milk destabilisation processes (Raikos, 2010).

2.2.2 Flocculation

The association that occurs between a number of drops due to disturbed repulsive and attractive forces can be defined as milk flocculation (Reiffers-Magnani *et al.*, 2000). Milk is considered to be flocculated when drops are not accidentally distributed within the solution (Dickinson, 2010).

Flocculation results in milk that is unstable. The quality of milk is also drastically reduced since flocculation results in enhanced emulsification, an increase in viscosity and lastly sedimentation (Dickinson, 2010). The emulsifying capabilities of the proteins within milk are sensitive towards the state of casein aggregation, the pre-heat treatment of proteins as well as the quantity of Ca (Liang *et al.*, 2013).

Micelles play a vital part during the manufacturing of flocculated milk products, for example, yogurt and cheese (Tuinier & De Kruif, 2002). These micelles exist as colloidal units within milk and kappa (K)-casein has the capability to lead to the stabilisation of micelles against flocculation (Payens, 1982). Characteristics of milk flocculation are that this process tends to be reliant on temperature and can be mostly rescindable (Dickinson, 2010). The process of milk flocculation takes place in three phases namely the deprivation of K-casein by enzymes, casein micelle flocculation and lastly formation of a gel. A different pattern is distinctive for each of the three phases. The pattern of the second phase (flocculation of the micelle) is greatly subjective to the supportive nature of micelle flocculation. The type of proteolytic action, the kind of milk and the pattern of casein proteolysis greatly affect the characteristics of the developed gel. A variety of aspects including pH and temperature has a big impact on the overall flocculation process within milk (Ageitos *et al.*, 2006). Flocculation occurs after 75% of K-casein hydrolysis took place (Carlson *et al.*, 1987).

A pH reduction (4.7-4.8) within cow's milk leads to casein micelle flocculation due to destruction in the steric stabilisation of the micelles (De Kruif & Roefs, 1996; Tuinier & De Kruif, 2002). The subjection of milk to heat treatment along with an amplified pH 6.5 to 7.0 leads to a diminished occurrence of flocculation (Vasbinder & De Kruif, 2003). Casein micelle flocculation that occurs in heat-treated milk can be ascribed to the whey protein degradation, however, whey protein denaturation cannot induce flocculation (Vasbinder *et al.*, 2003).

Unstable caseins can result in milk flocculation when milk is stored under cold conditions. However, storage of milk at lower temperatures can lead to longer storing of milk without loss in quality and lower storage temperature can cause milk to be stored for a lengthier duration. In general, flocculation will arise inevitably after storing for 4 months throughout cooling (Nakanishi & Itoh, 1970).

2.2.3 Age gelation

A common shortcoming that arises in milk after a long duration of storing can be referred to as age gelation. This phenomenon can be described as variations in the physical and chemical part in milk and consist of certain characteristics such as a decrease in fluidity as well as a rise in thickness due to the development of a network (proteins) that is characteristically three-dimensional (Datta & Deeth, 2001). A common appearance that can be visually observed during age gelation is extreme sedimentation which is rich in proteins at the bottom of the milk container along with the accumulation of fat at the top (Kelly & Foley, 1997; Anema, 2019).

This occurrence is considered to be very problematic for the dairy industry due to milk having a shorter shelf life and age gelation also negatively affects the market potential for milk (Datta & Deeth, 2001). UHT milk is considered to be stable against microorganisms under normal circumstances, however, it cannot sustain shelf stability for a long duration of time due to physical and chemical changes that lead to a formed gel. Raw and pasteurised milk are also at risk to undergo age gelation (Kohlmann & Nielsen, 1988; Anema, 2019).

Several factors have an influence on the age gelation mechanism thus it is considered as very complex (Crudden *et al.*, 2005). The numerous issues affecting the commencement of age gelation are heat processes, homogenisation, the arrangement of handling steps, milk solids, milk conformation, quality of milk and storing temperature (Cilliers, 2007). The properties of age gelation are also greatly influenced by fluctuations in the pH of milk (Vasbinder & De Kruif, 2003).

The temperature range at which age gelation occurs is between 25-30°C, however, the process of gelation can be delayed by subjecting milk to either higher or lower temperatures (Holland *et al.*, 2011). Age gelation generally occurs due to the intense heat treatment used for UHT processing (Celestino *et al.*, 1997).

The starting point of gelation, as well as interfaces among the micelles, can be accelerated by the variations that occur on the external part of casein micelles which is when the micelles undergo a drop in colloidal stability and then cause the rise of a gel that is three-dimensional. Gels formed during age gelation can either form via pH reduction (pH-mediated gels) or proteolytic activity. pH-mediated gels are characterised as the gels that form during milk spoilage (raw and pasteurised milk) and the production of yogurts and cheese where acidification (direct or proteolytic activity) neutralises the casein micelle surface leading to a reduction in repulsion which keeps the micelles apart. The gels formed by proteolytic activity in UHT milk differ substantially from that of pH-mediated gels (Cilliers, 2007).

Protein denaturation and breakdown of caseins within milk can be enhanced by an increase in proteolytic activity. Gelation configuration is linked to the development of gamma (y)-casein thus it is

associated with the activity of plasmin present within the milk. Age gelation causes the occurrence of various changes within milk during the duration of storing (Cilliers, 2007). These fluctuations in milk are generally instigated by casein hydrolysis which issues the beta-(β) K-complex and develops throughout heat treatments. The free βK-complex forms aggregates which in turn result in the development of a network that is characteristically three-dimensional consisting of cross-linked proteins. This eventually leads to the development of a gel (McMahon, 1996). Proteolysis of caseins is regarded to be a culprit process that causes age gelation (Datta & Deeth, 2001).

An escalation in viscosity is an alteration in milk physicality to advanced protein destruction and unfolding which is also linked to aggregation of the casein micelle and ultimately leads to coagulum development (Celestino *et al.*, 1997). Variations in viscosity can be distributed into four phases. The first phase is characterised with a short duration where produce diminishing takes place whereas the second phase consists of a longer duration and small changes in viscosity can be observed. Viscosity changes occur suddenly during the third phase and gel formation occurs eventually. During the final phase, a reduction in thickness is observed due to a fragmented gel which eventually results in a serum layer with protein flakes (Datta & Deeth, 2001). Viscosity changes in UHT milk can be unchanging for up to 30 days storing which escalates after 60 days. Gelation generally occurs after 90 days of storing (Fernandez *et al.*, 2008).

Structural alterations that occur within micelles, such as the association of micelle surface proteins have a greater influence on age gelation than proteolysis (Celestino *et al.*, 1997; Cilliers, 2007). A gel matrix cannot be formed by proteins that are extensively degraded (Chavan *et al.*, 2011). Therefore, any type of speeding up caused by handling or storing circumstances and result in the discharge of the βK-complex from the micelle will cause enhancement of age gelation or delay the process (Datta & Deeth, 2003).

Three processes are involved to ultimately cause age gelation and these processes also has an influence on the ease at which age gelation occurs namely, the interface among beta-lactoglobulin (β-LG) and K-casein, the discharge of the βK-complex from caseins and the cross-linking of the βK-complex and related proteins (Datta & Deeth, 2001; Chavan *et al.*, 2011), refer to Figure 2.

Age gelation is regarded as an irreversible process and is indicative of expired milk since it causes the termination of shelf stability for this type of milk and unfortunately, no warning signs are given during the occurrence of age gelation since no known physical and chemical indicators are present during the production process as well as during storage. Therefore, it is difficult to indicate whether a specific milk sample will remain stable and not prematurely gel throughout the duration of storage (Anema, 2019).

Consumers generally reject milk that had undergone age gelation due to variations that befall throughout storing such as the bitter tastes, amplified thickness, and acidity, reduced pH, milk becomes transparent and the formed residue (Newstead *et al.*, 2006; Hassan *et al.*, 2009).

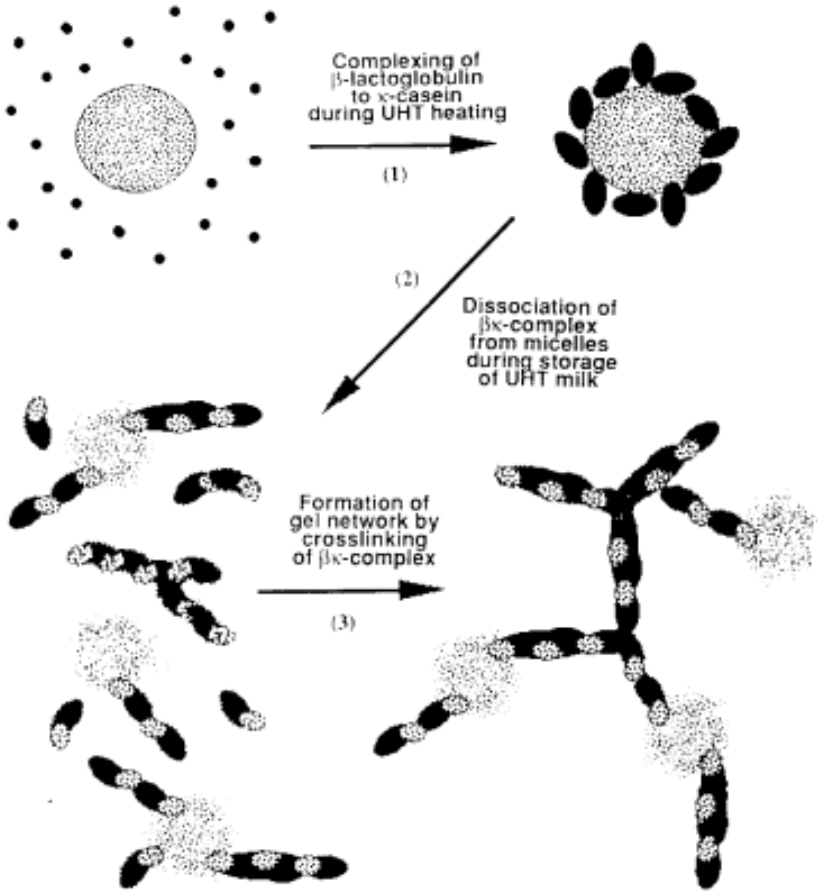

Figure 2. Age gelation process in UHT milk where number 1 shows the development of the βK-complex, number 2 shows its separation from micelles throughout storing and number 3 shows the consequent gelation through cross-linking of the βK-complex (Datta & Deeth, 2001).

2.3 The two mechanisms of age gelation

The age gelation mechanism consists of two stages and is regarded as being complex (Pulkkinen, 2014). The first stage is characterised with proteolytic degradation of proteins which leads to organisational alterations whereas the second stage is comprised of chemical responses that lead to diminished steadiness thus resulting in gel development (Datta & Deeth, 2001). The first stage involves the discharge of the micelle from the βK-complex which is formed amongst degraded β-LG and K-casein for the duration of the UHT-sterilisation of milk. Heat resistant enzymes are normally active throughout milk storing thus their proteolytic activity is considered to be accountable for the first stage (Gaucher *et al*., 2008; Zhang *et al.,* 2018; Anema, 2019).

The second stage is characterised by non-enzymatic/chemical alterations such as the parting of proteins from micelles along with the connotation of proteins to the exterior part of the micelle. This generally results in the development of a gel which is three-dimensional (Gaucher *et al*., 2008). The disturbance of K-casein can be encouraged by enzymatic or non-enzymatic action thus both actions are considered to be the two age gelation mechanisms (Datta & Deeth, 2001; Pulkkinen, 2014; Raynes *et al.,* 2018).

The possibility of the occurrence of age gelation by a combination of both enzymatic and chemical mechanisms most likely exists. The proposed process involves proteolytic enzymes which destabilise milk and enhancement of the age gelation process by the non-enzymatic mechanism, however, it has not yet been established (Anema, 2019).

2.3.1 Enzymatic mechanism

This mechanism proposes that heat resistant proteolytic enzymes cause the destabilisation of milk proteins which ultimately lead to gel formation and proteolytic enzymes involved to include; indigenous plasmin or proteases from microbial origin. The proteases release the βK-complex which develops a network made up of protein and ultimately leads to a developed gel. The mode of action for proteases is that they split the peptide bonds which anchor the K-casein and casein micelle to one another and thus assist with the discharge of the βK-complex. This mechanism can be separated into two stages whereas the parting of βK-complexes by proteases is the first stage. The second stage is characterised by the accumulation of βK-complexes and also the development of a protein network which is three-dimensional. Gel formation occurs when cross-linked βK-complexes and entrained proteins are subsequent which is generally within the period of which UHT milk is shelf-stable or shortly after the manufacturing process (Datta & Deeth, 2001; Chavan *et al*., 2011; Anema, 2019).

2.3.2 Non-enzymatic/Chemical mechanism

Age gelation can occasionally be a non-enzymatic process which is comprised of physicochemical processes due to a limited connection that generally occurs between gelation time and proteolytic

activity. During this process of age gelation, the milk proteins are not tarnished or hydrolysed by proteolytic enzymes. Factors influencing the chemical mechanism for age gelation include; milk/protein ratio, heat load during processes and the composition of the milk (Anema, 2019).

The onset of the chemical age gelation mechanism process occurs during the duration of milk storage when a three-dimensional protein network arises when β-LG and K-casein co-operate in the micelle throughout the application of treatments (heat) and this ultimately results in gel formation. Changes at the external part of the casein micelle can be attributed to the occurrence of age gelation. The hypothesis for the chemical age gelation mechanisms is that UHT treatment of milk leads to the development of exhausted K-casein micelles which are generally steady and sediments form fast in the presence of high ionic Ca levels and low pH levels. The exhausted K-casein micelles distillate to the end of the UHT milk container throughout milk storing. The pH of milk also naturally decline when milk is stored for a prolonged duration. The decline in pH along with the ionic Ca levels and severe K-casein micelle depletion finally lead to the occurrence of age gelation (Datta & Deeth, 2001; Chavan *et al.*, 2011; Anema, 2019).

Variations within the unrestricted energy of micelles can be ascribed to the dropped exterior potential of the casein micelles which can also result in the occurrence of non-enzymatic age gelation (Pulkkinen, 2014). Variances in latent energy lead to accumulation of casein micelles of which the level is determined by the likelihood of contact as well as the quantity of low-potential micelles. Micelle aggregation results in increased viscosity within UHT milk. The chemical process of age gelation within UHT milk generally occurs after several months (approximately 12 months) of storing and beyond the projected warehouse duration thus age gelation by the chemical mechanism occurs slower than the enzymatic mechanism (Datta & Deeth, 2001; Chavan *et al.*, 2011; Zhang *et al.,* 2018; Anema, 2019).

2.4 Milk components that play a role in age gelation

The components present within milk are regarded as fragile since proteins are sensitive towards heat and proteolytic attack, various processes influence the fat composition and proteolytic enzymes present in milk can lead to destabilisation such as age gelation.

2.4.1 Milk proteins

Proteins present in milk perform as enzymes, antimicrobial/antioxidant representatives, metal/vitamin binders and are biologically active. Their structure, function, and stability are therefore studied (Pellegrino *et al.*, 2011).

Proteins consist of amino acid sequences. Denaturation refers to proteins being changed from their natural shape which is a globular or native chain (Milk Facts). Decreased digestibility and the nutrient value of milk are caused by protein degradation (Pellegrino *et al.*, 2011). Milk consists of 3.3% protein and the important amino acids (Milk Facts).

The two chief proteins existent are casein and whey which consist of different chemical composition and physical properties (Milk Facts). The caseins embody 80% of the overall milk proteins. The residual 20% is the whey proteins (Oupadissakoon, 2007; Vaghela *et al.,* 2017). The quantity and quality of proteins highly affect the final milk quality (Forsbäck, 2010).

Proteases in milk cause protein degradation. Proteases originate from bacterial contamination, somatic cells in milk and through bacteria which are added during fermentation. Undesirable degradation results in milk with off-tastes and poor nutritional quality (Milk Facts).

2.4.1.1 Casein

The proteins in cow's milk contain 82% of casein. Casein contains phosphorus and coagulates at pH 4.6. There are different types of casein which differ according to the arrangement of amino acids, genetic dissimilarities, and functional properties. Casein consists of high proline content. The way casein is suspended in the aquatic segment of milk refers to a casein micelle. There are four kinds of caseins which are alpha-s_1 (αs_1), alpha-s_2 (αs_2), beta (β)-casein and Κ-casein (Yazdi *et al.,* 2014; Vaghela *et al.,* 2017; Perinelli *et al.,* 2019).

The casein part, particularly the casein micelle, is of pronounced worth for milk stability. These micelles have a characteristic spherical shape and are comprised of casein proteins, Ca phosphate and submicelles with Κ-casein on the external part, (Figure 3) (Nielsen, 2002). The assembly of casein micelles is administered by self-association among caseins, an equilibrium of hydrophobic and repulsive electrostatic forces and Ca-mediated interfaces (Federation, 2007).

Casein micelles entail the presence of numerous small nano-clusters which serve as the construction blocks for the micelle which generally self-assemble. The hydrophobic micelle is characterised by the existence of K-caseins at the external part and 169 amino acids (Tuinier & De Kruif, 2002; Yazdi *et al.,* 2014).

Enzymes are capable to destabilise casein micelles even if it is considered to be heat stable structures. Acid also has the ability to cause the destabilisation of micelles upon the dissolution of Ca phosphate (Nielsen, 2002, Vaghela *et al.,* 2017). The micelle steadiness in milk is preserved by external K-casein, colloidal Ca phosphate, surface potential (also zeta-potential) and steric stabilisation (Datta & Deeth, 2001; Tuinier & De Kruif, 2002; Yazdi *et al.,* 2014). The Ca phosphate serves as a barrier against aggregation. Interactions between casein micelles are stimulated and accumulated through the occurrence of any alteration at the external part of the micelle throughout UHT treating and storing which can be observed by a rise in the thickness of the UHT milk. Generally, these changes tend to arise slowly throughout the first phase of storing, however, after exterior changes, it is common for aggregation to occur rapidly which leads to the development of a gel (Datta & Deeth, 2001).

The formation of the βK-complex changes the K-casein network within the casein micelle which results in weak associations between this complex and other caseins such as αs_1-casein. The βK-complex can be perceived as protrusions and stems on the micelle exterior (Datta & Deeth, 2001).

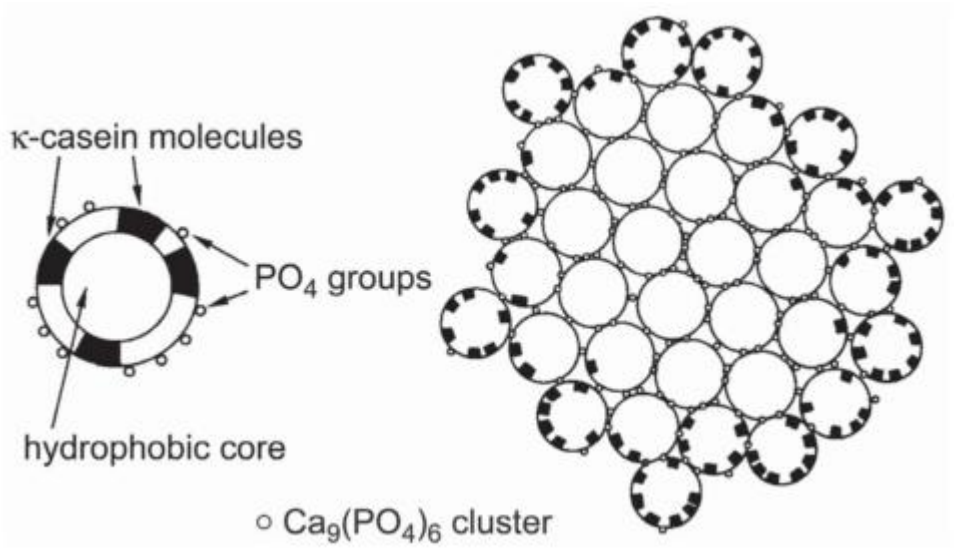

Figure 3. The casein micelle model, also protein structures and exterior arrangement of Κ-casein (Qi, 2007).

2.4.1.1.1 Proteolysis: The role of caseins in age gelation

Proteolysis can be defined as a process where proteins are degraded into reduced amino acids and peptides due to the breaking of peptide bonds by proteases which also generate a diversity of peptides (Vaghela *et al.,* 2017). The process of proteolysis can affect milk quality positively or negatively (Prado *et al*., 2006). The positive aspect of proteolysis is in the case of cheese, however, it affects UHT milk in a negative manner thus higher proteolysis leads to poorer milk quality (Vaghela *et al.,* 2017). Evidence suggests that proteolysis of caseins ultimately leads to age gelation when UHT milk is under storage (Datta & Deeth, 2001).

The occurrence of proteolysis during refrigerated storage can be ascribed to both plasmin as well as proteases cultivated by thermo-tolerant bacteria (Celestino *et al*., 1997; Datta & Deeth, 2001; Nielsen, 2002). Proteolysis induced by plasmin is important due to its intricacy and impacts on milk quality. Proteolysis in milk can be stimulated or prevented by the components of the plasmin system. Plasmin-induced proteolysis can enrich cheese quality through desirable flavour developments and textural changes that occur during the ripening period, however a delay in the ripening period can be ascribed to losses in plasmin from the casein micelle. It is of high importance that proteolysis must be controlled due to quality deterioration that occurs within milk and milk products when it is left uncontrolled (Ismail & Nielsen, 2010).

The action of enzymatic proteolysis occurs as three main reactions including; the formation of a complex among the original peptide (substrate) and the enzyme, peptide bond cleavage leading to the liberation of one/more resulting peptides and a nucleophilic attack on the remainder complex to split off the other peptide and reconstitute the free enzyme (Vaghela *et al.,* 2017).

It is known that casein micelles can be disaggregated by proteolysis (Fernandez *et al.*, 2008). The collapse of β-casein occurs quicker than that of αs_1-casein when milk is stored at 5°C and this can be ascribed to the existence of plasmin action (Datta & Deeth, 2001). Proteolysis, therefore, causes a reduction in the casein content (αs_1-casein, β-casein, and K-casein) within UHT milk throughout storing at 25°C (Bavarian *et al.*, 2010).

The milk properties negatively affected by proteolysis are organoleptic properties such as texture, consistency, and taste (Cilliers, 2007). Proteolysis has numerous negative effects on UHT milk such as the creation of bitter off-tastes, rise in thickness, fat separation, sedimentation and age gelation which in turn limit the lifespan (Datta & Deeth, 2003; Hassan *et al.*, 2009).

The temperature at which milk is stored is a role player in the occurrence of proteolysis since it is known that storage temperatures between 20-30°C lead to the onset of proteolysis, however proteolysis occurs at higher rates at 40°C rather than within milk that is warehoused at 20°C (Gaucher *et al.*, 2008).

Enzyme activity responsible for the occurrence of proteolysis during storage can be reduced by a homogenisation step prior to UHT treatment. The existence of the βK-complex at the exterior part of micelles has the capability to diminish proteolysis as well as age gelation due to inhibition of the admittance of proteases to caseins (Chove *et al.*, 2013). The process of proteolysis can be investigated by two methods which are the quantification of proteolytic variations and also by the determination of proteolytic activity (Němečková *et al.,* 2009).

2.4.1.2 Whey

The proteins in cow's milk contain 18% of whey and normally whey proteins do not coagulate. The whey protein content of milk comprises of β-LG, alpha (α)-lactalbumin and serum albumin (Oupadissakoon, 2007). Whey proteins consist of sulphur-containing amino acids that form disulphide bonds and therefore can result in denaturing due to broken disulphide bonds. Whey proteins play several different roles in milk. β-LG is a carrier of vitamin A and lactose is produced in the mammary gland from α-lactalbumin. Whey proteins are dissolved as individual units in milk's water phase (Milk Facts).

2.4.1.2.1 Background information on β-LG

β-LG is a spherical protein and has numerous influences on milk quality (Figure 4). This protein consists of a neutral pH and consists of a bushier and thinner monolayer than the casein proteins. Milk with the application of heat treatments undergoes degradation, unfolding, and aggregation of β-LG since it is regarded as a heat-labile protein. Temperatures that range between 65-80°C results in this protein losing its tertiary globular structure. The unfolding process of β-LG results in the creation of a layer of gel, which is two-dimensional, and followed by the firming of a non-bonded physical intermolecular interface and slows covalent cross-linking. The heat application to milk also results in

β-LG being more vulnerable towards enzymatic cleavage due to heat-induced change. Therefore, β-LG is sensitive to heat processes applied to milk (Dickinson, 2010; Vaghela *et al.,* 2017). The pH of milk is a vital parameter since it has a substantial influence on the interfaces among degraded β-LG and micelles. The release of destructed β-LG along with K-casein from the micelles generally takes place at a pH greater than 6.8 (Thompson *et al.*, 2008).

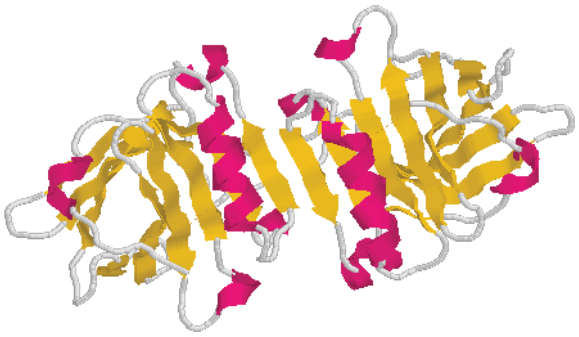

Figure 4. Three-dimensional structure of β-LG (Rytkönen, 2006).

2.4.1.2.2 The role of β-LG in age gelation

The development of disulphide bonds and aggregates among β-LG molecules results in gelation (Dickinson, 2010). The properties and steadiness of milk are greatly impacted by the interface among β-LG and K-casein (Crudden *et al.*, 2005). Throughout milk heating, a βK-complex is developed at pH 6.5 and 70°C due to the destruction of β-LG and aggregation of K-casein during proteolysis (McMahon, 1996; Pulkkinen, 2014). The discharge of the βK-complex from the micelle is a recognised role player in age gelation (Richards *et al.*, 2014; Vaghela *et al.,* 2017).

The other casein proteins such as αs_1 and αs_2-casein do not interact with β-LG while milk is exposed to heat processing (Thompson *et al.*, 2008). The denaturation of β-LG has a serious impact on the occurrence of age gelation. Subjecting milk to heat treatments above 65°C leads to amplified β-LG denaturation thus the existence of additional reactive β-LG molecules, therefore milk more prone towards gelation (Dickinson, 2010).

2.4.2 Milk fat

2.4.2.1 Background

Along with the other components within milk, there is approximately 3.4% total fat present of which it is well known that more than 400 fatty acids have been recognised to form part of the total percentage fat. The main fatty acids found in milk are saturated, mono-unsaturated, poly-unsaturated and trans-fatty acids (Milk Facts) of which the percentages are approximately 65% saturated, 30% mono-unsaturated 5% poly-unsaturated and 12.5% of glycerol. In the lipid segment of milk, triglycerides, phospholipids, cholesterol, free fatty acids, and diglycerides are also present (Pulkkinen, 2014).

Production of the saturated fatty acids arises in the mammalian gland whereas the mono-unsaturated fatty acids are also created in the mammalian gland or by the diet of the cow. The poly-unsaturated fatty acids are generally derived from the cow's diet (Milk Facts). Milk fat contributes to milk consisting of a unique appearance, taste, and texture. Larger fat globules are characteristically found in milk with higher fat loads. Oxidized and cardboard taste in milk is related to oxidised phospholipids of milk which may lead to milk consisting of a brown colour along with unpleasant odour (Oupadissakoon, 2007).

A protein phospholipid membrane, whose main function is to stabilise globules within the serum phase, surrounds the triglycerides existent in the arrangement of globules within the milk. The fat globule size range from 1µm to over 10µm and the process of homogenisation diminish the size of the large globules to less than 1µm and also result in a uniform distribution of fat globules (Milk Facts).

Processes such as lactation can lead to differences in fatty acid composition. During the initial lactation stage, energy is released for the cow and restricted fatty acids are accessible for fat synthesis to occur hence dietary mono-unsaturated and poly-unsaturated fatty acids are used for fat production. During late lactation, fatty acids are produced by the mammalian gland which causes higher levels of saturated fatty acids. These changes within the fatty acid composition have an effect on processing characteristics for milk products (Milk Facts).

Enzymatic action (mainly lipases), protein degradation and oxidation are the culprits causing milk fat deterioration. Protein degradation leads to the presence of off-tastes. The oxygen attacks the dual bonds of the fatty acids resulting in off-tastes that are described as painty, fishy or metallic (Milk Facts). The short-chain fatty acids are accountable for the presence of rancid tastes, the medium-chain fatty acids cause unclean (soapy) and bitter tastes whereas the long-chain fatty acids do not cause any off-tastes (Samaržija *et al.*, 2012).

Heat processes also affect milk fat, however, pasteurisation does not disturb the functional and nutritional properties of milk. In contrast, higher heat treatments encourage oxidation reactions and in turn cause fat degradation in the form of off-tastes. UHT treatments of milk have the capability of disrupting the milk fat globule membrane and in turn, leading to protein destabilisation within the globules and ultimately cause gelation (Milk Facts).

2.4.2.2 The role of milk fat in age gelation

The unsteadiness in milk fat when UHT milk is under storing is attributed to variations in the fat globule exterior. Homogenisation results in the modification of the fat globule membrane subsequent in losses in the compounds present in the membrane thus leading to the unsteadiness of fat globules when UHT milk is under storage (Celestino *et al.,* 1997). The proneness of milk towards age gelation fluctuates due to milk fat content such as low fat or full cream. Research shows that the storage duration of low fat milk is shorter than that of full cream milk. The different spoilage rates for both milk types may be attributed to varying growth rates of thermo-tolerant bacteria, different handling conditions, and varying storage surroundings. The spoilage configurations and influences of thermo-tolerant bacteria on milk with varying fat content differ. The culprit bacteria causing spoilage of both low fat and full cream milk also vary due to different milk compositions resulting in the selection of different bacteria. The culprit thermo-tolerant bacteria for both low fat and full cream milk are *Pseudomonas* spp. which produces proteases (Deeth *et al*., 2002).

The subjection of milk to homogenisation prior to UHT treatment may cause the presence of lower levels of enzymes which add to the occurrence of proteolysis when milk is stored. Proteins can become more prone towards proteolytic attack due to homogenisation since it leads to caseins and whey being attached to the fat globule membrane hence giving escalation to reduced micelle particles (Chove *et al*., 2013). Generally, low fat milk contains a higher occurrence of proteolysis thus leading to milk incapable of being stored for the same duration of full cream milk which can be attributed to advanced protease activity in low fat milk (Deeth *et al*., 2002). The explanation for lower proteolytic attack in full cream milk is that fat has a shielding effect against proteolytic attack by proteases (Valero *et al*., 2001).

Low fat milk also comprises of higher levels of protein degradation and micelle destabilisation due to advanced proteolytic activity (Deeth *et al*., 2002). Quality deterioration occurs faster within low fat UHT milk than UHT full cream milk and can be attributed to proteolysis and the development of bitter tastes (Valero *et al*., 2001). The chief variance among low fat and full cream milk is that low fat milk has advanced proteolytic activity present hence lead to elevated proteolysis thus causing this type of milk to have shorter storage duration than full cream milk. Low fat milk is thus extra vulnerable to age gelation (Deeth *et al*., 2002).

2.4.3 Proteolytic enzymes in milk

2.4.3.1 Introduction to proteolytic enzymes

Enzymes are considered as proteins that consist of specific biological roles. The origin of enzymes can be from sources which include; the milk itself (indigenous), blood, cytoplasm, milk fat globule membrane, somatic cells, and microbial contamination and this causes varying enzyme levels (Guinee & O'Brien, 2010; Vijayakumar, 2012).

Various enzymes are naturally present, of which over 70 indigenous enzymes have been categorised, thus milk is regarded as very biologically active (Chen *et al.*, 2003; Gazi *et al.*, 2014). Proteolytic enzymes within milk are characterised in two groups which are indigenous or produced by thermo-tolerant bacteria (extracellular, intracellular or periplasmatic), which is referred to as microbial/bacterial proteases, which defines characteristics and their inactivation mechanism (Chavan *et al.*, 2011; Vijayakumar, 2012; Chove *et al.*, 2013; Vaghela *et al.,* 2017).

The present enzymes affect the quality and shelf stability of milk, especially UHT milk since reactivation of heat resistant proteases occurs when warehoused at room temperature for longer (Chen *et al.*, 2003; Stoeckel *et al.,* 2016; Vaghela *et al.,* 2017). Indigenous enzymes serve a biologically important role after milk secretion. Proteolytic enzymes can serve as indicators of the health of cows (indices for mastitis) as well as the history of heat applications towards the milk (Vijayakumar, 2012).

Plasmin produces greater and supplementary hydrophobic peptides than thermo-tolerant proteases and dissimilar attractions for the various caseins exist thus this may be used for distinction among these proteases (Datta & Deeth, 2001; Le *et al.*, 2006).

2.4.3.2 Microbial proteolytic enzymes

2.4.3.2.1 Introduction to thermo-tolerant bacteria

Thermo-tolerant bacteria are regarded as the dominant bacteria within milk since it comprises 90% of the entire microbial inhabitants (Samaržija *et al.*, 2012). Categorisation for thermo-tolerant bacteria existing in milk can be either pathogenic or spoilage organisms (Tamime, 2008). Thermo-tolerant bacteria are generally not present in the udder thus their presence is due to contamination after milking which include; the internal part of the udder, during milking/teats, milk handling, the environment and milking, storing equipment, poor hygiene and cleaning practices, lengthy storing of milk under 4°C and post-pasteurisation pollution (Chen *et al.*, 2003; Cilliers, 2007; Vijayakumar, 2012).

Thermo-tolerant bacteria are the most prevalent towards dairy foodstuffs which can be attributed to them affecting milk quality negatively (Pulkkinen, 2014). Storing length and temperature conditions

have a remarkable impact on the activity of thermo-tolerant bacteria of which the optimum activity of thermo-tolerant bacteria occurs at 20-30°C (Celestino *et al*., 1997; Samaržija *et al*., 2012; D'Incecco *et al.,* 2019). These bacteria generally cultivate during milk refrigeration since they have the capability to cultivate at 7°C and lower thus thermo-tolerant bacteria normally dominate the bacterial population in refrigerated milk, therefore, causing quality problems since they produce heat-stable metalloproteases (Nielsen, 2002; Pulkkinen, 2014).

The thermo-tolerant bacteria present within refrigerated milk belong into two groups which are the Gram-negative and Gram-positive groups. The gram-negative bacterial strains within milk are *Pseudomonas, Aeromonas, Serratia, Acinetobacter, Alcaligenes, Achromobacter,* Enterobacter and *Flavobacterium* (Samaržija *et al*., 2012; D'Incecco *et al.,* 2019).

The thermo-tolerant bacteria dominating milk are the *Pseudomonas* spp. since they comprise more than 50% of the overall bacterial population thus are a chief cause of microbial pollution in milk. The *Pseudomonas* spp. is considered to be motile, catalase-positive, strict aerobes produce extracellular and produce heat resistant metallo-proteases and the optimum pH of these proteases is 7-9. The ideal cultivation temperature for *Pseudomonas* is 25-30°C however this species consists of the ability to survive at above freezing temperatures (Fajardo-Lira, 1999; Cilliers, 2007; Samaržija *et al*., 2012; D'Incecco *et al.,* 2019).

The presence of gram-positive bacteria is in lower quantities than the gram-negative bacteria (Champagne *et al*., 1994). The gram-positive bacterial strains within milk include *Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus, Arthobacter, Streptococcus, Staphylococcus* and *Lactobacillus* (Samaržija *et al*., 2012).

The *Bacillus* spp. is broadly dispersed in the surroundings and contaminates milk throughout manufacture, handling, and processing (Chen *et al*., 2003). The *Bacillus* spp. is spore formers and thermoduric thus able to survive heat treatments hence proteases produced by these species can be considered to be unaffected by heat (Champagne *et al*., 1994). The *Bacillus* spp. consists of varied nutritional requirements and grows at a broad temperature and pH range. The *Bacillus* spp. generally produces a wider range of heat resistant proteases in comparison with the *Pseudomonas* spp. The gram-positive bacteria consist of extended generation time (8.5 hours) and a lengthier lag phase at 2-7°C. The *Bacillus* spp. has the capability to cultivate at a temperature as low as 2°C hence they are well-thought-out to be one of the core microbial sources for the spoiling of milk (Samaržija *et al*., 2012).

Thermo-tolerant organisms consist of the capability to stick to solid surfaces thus leading to the formation of biofilms on dairy equipment, of which removal is generally difficult. Biofilms are considered to be complex structures since it differs in genetics. The nature of biofilms is influenced by growth conditions, the ratio of proliferation as well as the interaction between cells. Bacterial

species such as *Bacillus subtilis, Bacillus cereus,* and *Pseudomonas fluorescens* are capable of forming biofilms cooperatively. Bacteria within biofilms have the ability to develop different physiological characteristics than their planktonic cells, they develop greater cell biomass and they can temporarily separate from the biofilm. The cells within biofilms are generally more resistant towards antibiotics and disinfectants than planktonic cells since these biofilm cells consist of diverse properties and alterations in the bacterial physiology when compared to planktonic cells. Therefore, biofilms are regarded as difficult to resolve and are stubborn sources of permanent thermo-tolerant contamination (Samaržija *et al*., 2012). The chief causes of thermo-tolerant contamination to milk are therefore the growth of thermo-tolerant bacteria prior to pasteurisation, biofilm formation and post pasteurisation contamination such as the activity of heat resistant proteases (Champagne *et al*., 1994). Therefore, it is distinctive that the attendance of thermo-tolerant organisms affects milk quality negatively which can be attributed to their capability of growing and multiplying quickly (Samaržija *et al*., 2012).

2.4.3.2.2 Effect of refrigerated storage on the growth of thermo-tolerant bacteria

Milk storing at cooling temperatures accelerates the growth of thermo-tolerant bacteria which is problematic for dairy manufacturing since storing under these conditions is essential. Bacteria that are most dominant in the refrigerated storing of milk are *Bacillus* and *Pseudomonas* (Chen *et al*., 2003). It is clear that refrigerated storage stimulates the cultivation of thermo-tolerant bacteria, however storing at 4-6°C can slow their cultivation and the action of their heat resistant proteases (Tamime, 2008; O'Brien & Guinee, 2011; Samaržija *et al*., 2012; Samet-Bali *et al*., 2013). The heat resistant microbial proteases produced by thermo-tolerant bacteria during cooled storing have a negative, deteriorative effect on milk (Montanhini *et al*., 2013).

2.4.3.2.3 Enzymes from the thermo-tolerant origin (Microbial proteases)

Microbial proteases produced by thermo-tolerant bacteria are categorised as metalloproteases since metal ions such as Ca is essential and required for optimum activity (Nielsen, 2002; Němečková *et al.,* 2009). Microbial proteases are categorised into four classes grounded on their action mechanism which include; an acid, a cysteine, a neutral or an alkaline protease (Chen *et al*., 2003).

These enzymes are cultivated by bacteria, fungi, and yeasts. The bacteria producing microbial proteases include; *Pseudomonas, Bacillus, Clostridium, Proteus, Escherichia, Micrococcus, Microbacterium, Flavobacterium* and *Chryseobacterium* of which the first two are most important affecting the quality of milk (Němečková *et al.,* 2009; Radha *et al*., 2011; Vaghela *et al.,* 2017; D'Incecco *et al.,* 2019). Microbial proteases from the thermo-tolerant origin are generally produced at the completion of the exponential phase and also during the stationary phase (Němečková *et al.,* 2009; Anema, 2019).

Pseudomonas fluorescens produces heat resistant proteases that are of attention to dairy manufacturing and this organism consists of great multiplication ability (Keogh & Pettingill, 1982; Samaržija *et al*., 2012; Xin *et al.,* 2017; Zhang *et al.,* 2018; D'Incecco *et al.,* 2019). *Pseudomonas* produces only one type of protease, generally at the completion of the exponential phase and throughout the stationary growth phase and these proteases have an effect on casein micelles, K-casein, and Ca phosphate. *Pseudomonas* proteases are characterised with optimum temperatures between 17.5-30°C, an optimum pH of 6.5-7.0 and consist of molecular masses that range between 40-50 kilo Dalton (kDa) (Němečková *et al.,* 2009; Vaghela *et al.,* 2017). More specifically, the proteases cultivated by *Pseudomonas fluorescens* is characterised by optimum pH 6-8, ideal temperatures at 37-45°C and are typically delicate towards ethylenediaminetetra-acetic acid (EDTA) (Fajardo-Lira, 1999). The heat resistant proteases produced by *Pseudomonas* spp. are the chief source of quality imperfections in milk (Champagne *et al*., 1994; Andreani *et al.,* 2016; Xin *et al.,* 2017).

Proteases cultivated by *Bacillus* spp. are also important to the dairy industry and have an optimal pH 8, an ideal temperature between 30-37°C and molecular masses that range between 40-50 kDa (Chen *et al*., 2003; Vaghela *et al.,* 2017). *Bacillus* spp. is responsible for the production of a broad spectrum of proteases which may differ in quantity and ratio between the various strains (Němečková *et al.,* 2009). Certain *Bacillus* spp. has the ability to cultivate additional proteases concurrently which is comparable to the proteases produced by *Pseudomonas fluorescens* (Champagne *et al*., 1994). The heat resistant proteases of *Bacillus subtilis* are known to hydrolyse casein and in turn destabilising the casein micelles and causing flocculation, proteases from *Bacillus cereus* result in unclean, bitter, fruity, rancid and yeasty tastes and proteases from *Bacillus licheniformis* cause cheese-like flavours within the milk. The heat resistant proteases produced by *Bacillus* spp. and *Pseudomonas* spp. are the distinctive microbial spoiling of milk and also the chief source for financial losses in dairy manufacturing (Samaržija *et al*., 2012; Vaghela *et al.,* 2017).

Microbial proteases, therefore, play an important role in milk quality since they normally cause deterioration and shorten the shelf life. Due to these enzymes being heat resistant and surviving heat treatments, defects in UHT milk can be observed such as off-tastes formation, increased non-protein nitrogen concentration, the formation of parra-K-casein, destabilisation of casein micelles thickening, coagulation, flocculation and age gelation. *Pseudomonas* proteases are the most common post-pasteurisation contaminant whereas proteases from *Bacillus* limit the shelf stability of pasteurised milk (Němečková *et al.,* 2009; Andreani *et al.,* 2016; Anema, 2019).

2.4.3.2.4 Effect of heat treatment on thermo-tolerant bacteria and heat resistant proteases

The presence of thermo-tolerant bacteria within milk leads to negative effects on milk quality, therefore, heat treatments are essential and although proper heat treatments might destroy thermo-tolerant bacteria, their heat resistant proteases survive (Samaržija *et al*., 2012). Heat resistant proteases preserve their activity after the application of heat processes since they have the capability to endure pasteurisation, and UHT treatments (Fajardo-Lira, 1999). Therefore, the spoilage that occurs in pasteurised and UHT milk can be ascribed to contamination by heat resistant proteases after heat treatments and also during storage (Samaržija *et al*., 2012).

Gram-positive along with gram-negative bacteria are regarded to be unaffected by heat since they can survive heat treatments thus causing lower shelf stability of milk with heat applications since these bacteria continue their growth in refrigerated heat-treated milk (Samaržija *et al*., 2012). Proteases originating from these bacteria are also heat resistant therefore will generally not be inactivated during heat treatments thus protease activity is affected by heat treatments (Champagne *et al*., 1994). Heat processes can lead to decreased proteolytic activity since it results in the unfolding of the proteases through denaturation, self-digestion, auto-proteolysis and irretrievable covalent alteration (non-enzymatic). The activity of proteases present within milk cannot be totally inactivated whether by low temperatures or severe heat treatments thus proteases continue to be active within heat-treated milk during storage hence leading to quality defects in milk since (Chen *et al*., 2003).

The process of pasteurisation extinguishes all the heat-labile thermo-tolerant organisms within milk, however, some may survive this process. The bacteria that survive the UHT treatment process are *Bacillus subtilis*, *Bacillus cereus, Bacillus licheniformis, Bacillus sporothermodurans* and *Paenibacillus lactis* (Tamime, 2008). Heat resistant proteases are produced by the majority of thermo-tolerant bacteria and these enzymes have the capability to reserve 30-100% activity afterward heat application. The heat resistant proteases cultivated by *Pseudomonas* spp. and *Bacillus* spp. have the utmost substantial significance in dairy manufacturing in terms of milk quality and economics (Samaržija *et al*., 2012). Heat resistant proteases cultivated by *Pseudomonas* spp. have the ability to maintain 60-70% activity after heat application and are stable even after severe heat treatments at 100°C (Valero *et al*., 2001; Topçu *et al*., 2006; Richards *et al*., 2014).

The level of activity of heat resistant proteases that survive UHT treatments may be used as pointer for quality of UHT milk (Chen *et al*., 2003) since their proteolytic and lipolytic action cause bitter, stale and oxidised tastes, viscosity changes and serious defects therefore ultimately lead to age gelation (Celestino *et al*., 1997; Newstead *et al*., 2006; Chavan *et al*., 2011; Richards *et al*., 2014; Andreani *et al.,* 2016).

2.4.3.2.5 Contamination of milk by microbial proteases after heat treatments

Microbial proteases are known to be heat resistant hence their presence in milk during storage after the application of heat treatments (Chen *et al*., 2003). Other influences on shelf stability of pasteurised and UHT milk are contamination by thermo-tolerant bacteria (both *Bacillus* and *Pseudomonas* spp.), storage temperature, and duration of storage (Samaržija *et al*., 2012).

2.4.3.2.6 Negative effects of microbial proteases on milk quality

Microbial proteases are harmful to the quality and shelf stability of milk (Larsen *et al*., 2006; O'Brien & Guinee, 2011). These proteases lead to certain problems such as technical problems and various defects within milk which include; sensory, rheological and functional defects such as spoilage of milk during storage, the development of off-tastes, stiffening and ultimately age gelation (Němečková *et al*., 2009; Andreani *et al.,* 2016; D'Incecco *et al.,* 2019). Biochemical destruction of milk such as the breakdown of major milk components as well as serious defects within UHT milk occurs due to on-going proteolytic activity by heat resistant microbial proteases (Gaucher *et al*., 2011; Samaržija *et al*., 2012). Other negative occurrences include protein and lipid breakdown (Chen *et al*., 2003).

The microbial proteases cultivated by *Pseudomonas* spp. and *Bacillus* spp. are most significant to the dairy industry since it results in quality deterioration, undesirable sensory qualities, proteolysis and age gelation, therefore, straining milk shelf stability dramatically (Samaržija *et al*., 2012; Montanhini *et al*., 2013).

2.4.3.2.7 Microbial protease attack on milk proteins

Microbial proteases favour attack towards the caseins above the whey proteins (Andreani *et al.,* 2016). The sensitivity of proteins towards proteolytic attack also differs according to their structure hence whey proteins are less vulnerable towards microbial proteases since they are spherical in comparison to the unsystematic construction of caseins. The reason for higher proneness to proteolytic attack for caseins is due to their open structures thus their peptides are more unprotected (Fajardo-Lira, 1999). The susceptibility of caseins differs among the various types since β-casein, Κ-casein and α-casein are regarded to be more susceptible to proteolytic attack. The destruction of β-casein causes the development of γ-caseins and proteose-peptones (Chen *et al*., 2003).

The various bacterial species also prefers to attack certain caseins. Microbial proteases from *Pseudomonas* spp. generally, destabilise the casein micelles initiating the destruction of Κ-casein (forming parra-Κ-casein), αs_1-casein and β-casein leading to age gelation (Němečková *et al*., 2009). The microbial proteases produced by *Bacillus* spp. can also result in Κ-casein degradation whereas the interruption of α and β-casein is fewer. Since Κ-casein is positioned on the micelle exterior, it is more accessible for preliminary destruction thus the preferred attack on this type of casein (Samaržija *et al*., 2012).

Microbial proteolytic breakdown of caseins alters milk properties, such as a decline in proteins, which affect milk quality negatively due to the development of bitter and unpleasant tastes (Datta & Deeth, 2001; Cilliers, 2007; Vijayakumar, 2012; Andreani *et al.,* 2016; Ribeiro Júnior *et al.,* 2017).

2.4.3.2.8 The role of microbial proteases in age gelation

Microbial proteases are enormous role players in age gelation due to their ability to weaken casein micelles by hydrolysing the Κ-casein, therefore, induce proteolysis which accelerates the occurrence of age gelation (Champagne *et al*., 1994; Pulkkinen, 2014; Anema, 2019). These gels characteristically have additional unbroken micelles. The level of microbial proteolytic activity strongly influences the time when age gelation occurs in milk (Datta & Deeth, 2001). Microbial proteases are role players in age gelation throughout the storing of pasteurised and UHT milk (Cilliers, 2007).

2.4.3.3 Indigenous proteolytic enzymes

2.4.3.3.1 The plasmin system

The plasmin system is regarded to be intricate of nature due to it being multipart. The components of this system include; plasmin, plasminogen (inactive), plasminogen activators, plasmin inhibitors and inhibitors of plasminogen activators, refer to Figure 5 for the system illustration (Hamed *et al*., 2012; Richards *et al*., 2014; Gazi *et al*., 2014; Stoeckel *et al.,* 2016; Vaghela *et al.,* 2017; Anema, 2019). There are various factors that affect this system thus contributing to its complexity. The elements affecting this system in several ways include; handling circumstances such as heat treatments, pH variations, storing duration and temperature since refrigerated storage results in plasmin autolysis thus increased plasminogen activation is common (Prado *et al*., 2007; Schroeder *et al*., 2008).

Plasmin, plasminogen, and plasminogen activators are allied with the micelles and is greatly affected by pH since drops in pH leads to discharge, whereas all the inhibitors are soluble in the milk serum (Němečková *et al*., 2009; Vijayakumar, 2012; Gazi *et al*., 2014; Stoeckel *et al.,* 2016; Vaghela *et al.,* 2017; Anema, 2019). The inhibitors form complexes with proteins thus they might appear in various forms within milk (Chavan *et al*., 2011).

The plasmin system has dissimilarities towards steadiness against heat. The heat resistant components include; plasmin, plasminogen and plasminogen activators though the plasmin inhibitor is heat-labile (Guinee & O'Brien, 2010; Anema, 2019). Changes that arise within the plasmin system throughout heating are the deactivation of the plasminogen activator inhibitor and the plasmin inhibitor (Gazi *et al*., 2014). Elevated plasminogen activation arises due to the deactivation of plasminogen activator inhibitor and this results in increased plasmin levels in milk after pasteurisation (Prado *et al*., 2007). The regular equilibrium among the activators and inhibitors is transformed during the heat application of milk which may cause speeded up proteolysis in heat-treated milk (Chavan *et al*., 2011).

Due to interactions within the plasmin system with one another and with the components within milk, milk quality may be negatively affected since the occurrence of proteolysis is stimulated (Prado *et al.*, 2007; Pulkkinen, 2014). The ultimate plasmin level in milk is significantly subjected to the level of plasminogen, plasminogen activators and plasminogen activators inhibitors (Chavan *et al.*, 2011).

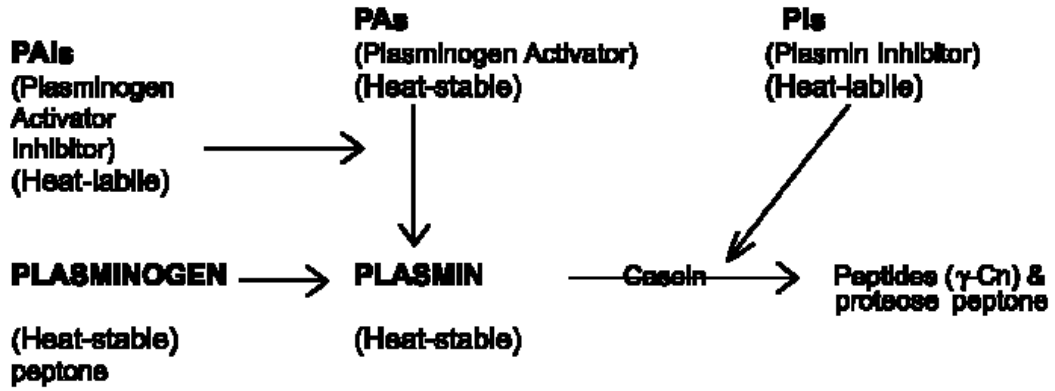

Figure 5. The plasmin-plasminogen system (Datta & Deeth, 2001).

2.4.3.3.2 Plasminogen: The inactive form of plasmin

2.4.3.3.2.1 Background information

Plasminogen is the inactive part of plasmin within milk, consists of higher heat stability than its active form and the various activators are capable of activating plasminogen. Plasminogen has a vital role which is the dissolution of fibrin blood clots for the prevention of thrombosis (Datta & Deeth, 2001; Richards *et al.*, 2014). The characteristics of plasminogen include; 786 amino acid remainders, a molecular mass of 88 kDa and an ideal activation temperature of 37°C thus little activation befalls at 4°C (Crudden *et al.*, 2005; Cilliers, 2007; Pulkkinen, 2014). Plasminogen originates from the liver tissue rather than from within the mammary gland (Berglund *et al.,* 1995; Bastian & Brown, 1996). Plasminogen is regarded to be heat resistant, however, the application of heat exposure (72°C for 15 seconds) can result in a 10% decrease in plasminogen (Fajardo-Lira, 1999).

The components of the plasminogen molecule include; an amino-terminal pre-activation peptide (PAP), five triple loop structures (kringles), cysteine residues, serine protease area that is comprised of catalytic triad and stabilisation is provided by three disulphide bonds (Benfeldt *et al.,* 1994). Plasminogen consists of an attraction towards lysine and *(t)*-amino carboxylic acids such as ε-aminocaproic acid (EACA). Attachment of plasminogen, plasmin and plasminogen activators to the micelle are provided by lysine (Ismail & Nielsen, 2010). The association of plasminogen with the micelles within milk occurs in the same manner as the binding to fibrin in the blood due to the presence of EACA influencing the interaction with casein. Lysine also has the ability to separate plasmin and plasminogen from the micelles. EACA has the capability to eliminate lysine association to casein thus causing the discharge of plasminogen from the micelles (Benfeldt *et al.,* 1994).

A lysine binding site is also located in the kringles with one position having a high attraction for lysine whereas the additional four positions consist of the inferior attraction of which the lysine binding sites are regarded as crucial for plasminogen activation regulation (Bastian & Brown, 1996). Contradictory to the latter, lysine can also hinder plasminogen activation since it strives for the lysine association positions (Datta & Deeth, 2001).

2.4.3.3.2.2 The process of plasminogen activation

The process of plasminogen activation by activators can arise when milk is still present in the udder prior to milking as well as throughout storing (Ismail & Nielsen, 2010). Plasminogen contains an N-terminal aspartic acid which is transformed by plasmin to an N-terminal arginine by eliminating the first 77 residues, referred to as PAP, and the release of this terminal does not result in plasminogen activation but rather leads to an alteration in plasminogen (Bastian & Brown, 1996).

The process of plasminogen activation is the consequence of the flow of various responses (Cilliers, 2007). Plasminogen activation arises the moment when plasmin discharges the PAP, leaving a structurally altered and inactive protein. Certain plasminogen activators cleave the Arg^{557}-Ile^{558} bond in order to give rise to an energetic molecule with two chains made of a heavyweight chain which is linked to a lightweight chain with a solitary disulphide bond. Thereafter, an autolytic cleavage of the Lys^{77}-Arg^{78} bond follows which results in the ultimate discharge of the PAP which results in a conformational change within plasminogen (Benfeldt *et al.,* 1994; Ismail & Nielsen, 2010). A salt bridge formation that arises between Val^{562} and Asp^{740} plays a substantial role in this alteration within plasminogen. Plasminogen can also be activated by the replacement of the Ile^{558} amino group with Val^{562} (Cilliers, 2007). The active site for plasmin is considered to be the lightweight chain which comprises His^{598}, $Asp^{641,}$ and Ser^{736}. The Arg^{557}-Ile^{558} cleavage transforms plasminogen to plasmin (Vijayakumar, 2012).

Casein may lead to elevated plasminogen activation due to K-casein and αs_2-casein being considered to be binding components for certain activators thus casein degradation is normal practice during the activation procedure (Bastian & Brown, 1996).

Plasminogen activators

Plasminogen activators are of high importance since plasminogen activation to plasmin cannot take place without them (Gazi *et al*., 2014). Plasminogen activators are considered to be serine proteases consisting of the responsibility to convert inactive plasminogen to active plasmin (Crudden *et al*., 2005). Plasminogen activators are resistant to heat since processes such as pasteurisation do not affect them (Datta & Deeth, 2001). The activators consist of higher heat stability than plasminogen and plasmin, however, they are somewhat deactivated throughout the UHT process which can be attributed to the extreme temperatures (Datta & Deeth, 2003; Ismail & Nielsen, 2010).

The two chief plasminogen activators are tissue-type (t-PA) and urokinase-type (u-PA) (Aslam & Hurley, 1997; Enright *et al*., 1999; Ismail & Nielsen, 2010). Association differs among the two activators since the t-PA is allied with the casein micelles due to static powers while u-PA is mainly allied with the mammary epithelial cells and the somatic cells within milk due to the presence of casein leftovers in the cell extracts (Bastian & Brown, 1996; Crudden *et al*., 2005; Ismail & Nielsen, 2010; Hamed *et al*., 2012).

The roles played in plasminogen activation also differ among the two activators (Wang *et al*., 2007). Since u-PA is created by epithelial cells, the hormonal control is responsible for the construction and is influenced by factors which include; increased production by insulin and construction inhibition by glucocorticoids (Baldi *et al*., 1997). The activity levels mutually for t-PA and u-PA can be encouraged by casein, fibrin consists of the capability to expand t-PA activity and amiloride inhibit u-PA activity but have no influence on t-PA activity (Ismail & Nielsen, 2010).

Contradictory to Verdi & Barbano, (1991), plasminogen activation is substantially affected by microbial proteases cultivated by thermo-tolerant bacteria during milk storing (Schroeder *et al*., 2008). A connection is existent among microbial proteases and plasmin. *Pseudomonas* spp. has the capability to destruct the micelles and lead to variations in pH levels thus greatly affecting the stability of the micelles. It is therefore clear that a functional interaction exists between the plasmin system and metalloproteases (Fajardo-Lira, 1999; Ismail & Nielsen, 2010).

Plasminogen inhibitors

Inhibitors for plasminogen activation as well as plasmin inhibitors, referred to as α_2-antiplasmin, are also present in the serum phase within milk and regulate activities associated with plasmin which include; constrain the activity of both plasmin and plasminogen activators, delay the transformation of plasminogen to plasmin and retard proteolysis (Datta & Deeth, 2001; Nielsen, 2002; Chen *et al*., 2003; Prado *et al*., 2006; Wang *et al*., 2007). Factors such as fluctuations in pH and heat treatments impact the activity of the inhibitors (Ismail & Nielsen, 2010). The molecular weights of the plasminogen activator inhibitor (55 kDa) and the plasmin inhibitor (60 kDa) differ (Cilliers, 2007). The action of these inhibitors has a substantial influence on the final plasmin activity within milk (Fajardo-Lira, 1999).

Heat treatments consist of the ability to modify the natural balance between activators and inhibitors (Datta & Deeth, 2001). The inhibitors are considered to be unstable against heat treatments thus they are not regarded as heat resistant and the heat stabilities for both differ since resistance against heat for the plasminogen activator is less than the plasmin inhibitor (Newstead *et al*., 2006; Prado *et al*., 2006; Vijayakumar, 2012; Chove *et al*., 2013). Heat treatment, therefore, leads to the

inactivation of the inhibitors. Plasminogen activators thus increase plasmin activity in warehoused heat-treated milk (Fajardo-Lira, 1999; Datta & Deeth, 2003).

Negative effects of plasminogen activation

Plasminogen activation affects milk and its constituents resulting in protein collapse (casein), including β-casein and α-casein whereas Κ-casein is unaffected by plasminogen activation. The formation of peptides also occurs during activation which includes; γ-casein and proteose-peptones. The breakdown of proteins is regarded as a negative occurrence within milk since it leads to negatively disturbing the texture, physical and chemical properties of milk (Cilliers, 2007).

It is normal practice that the plasmin activity within UHT milk escalates throughout storing which can be attributed to plasminogen activation and also modifications in the normal symmetry among activators and inhibitors throughout heating. This can result in accelerated proteolysis thus it is clear that plasminogen activators are role players in age gelation (Datta & Deeth, 2001). Therefore, plasminogen activation affects milk negatively since it promotes age gelation (Kohlmann & Nielsen, 1988; Cilliers, 2007).

2.4.3.3.3 Plasmin: The active form of plasminogen

Plasmin is the chief protease that is naturally present within bovine blood and milk thus is characterised as indigenous and being part of the peptidase/trypsin family (Fajardo-Lira, 1999; Prado *et al*., 2006; Gaucher *et al*., 2008). Secretion of plasmin into milk occurs from the blood through the mammalian cell wall liner hence plasmin arises from the blood plasma, mammary tissue cells or leucocytes (Ismail & Nielsen, 2010; Vijayakumar, 2012; Vaghela *et al.,* 2017). Characteristics of plasmin include; being an alkaline serine protease with an amino acid serine at the active site with trypsin-like activity, heat resistant, ideal temperature (37°C), prime pH 7.5-8.0 and a molecular mass of 58 kDa (Bastian & Brown, 1996; Nielsen, 2002; Chen *et al*., 2003; Cilliers, 2007; Ismail & Nielsen, 2010; Zhang *et al.,* 2018; Anema, 2019).

Connotation of plasmin with the casein micelles is more projected to occur than with the whey fraction, however, plasmin is also allied with the fat globule membrane (Chen *et al*., 2003; Datta & Deeth, 2003; Tamime, 2008). Plasmin generally hydrolyses all the caseins, however, it has a preference for β-casein, αs_1-casein, and αs_2-casein (Chen *et al*., 2003; Gaucher *et al*., 2008; Vaghela *et al.,* 2017; Anema, 2019). Plasmin hydrolyses proteins at L-Lys and L-Arg with a high liking for L-Lys (Nielsen, 2002; Ismail & Nielsen, 2010). Plasmin hydrolyses β-casein predominantly at spots Lys28-Lys29, Lys105-His106, and Lys107-Glu108 to contribute to the rise of three β-casein remains (γ_1-casein, γ_2-casein, and γ_3-casein) and proteose-peptones (Chen *et al*., 2003; Ismail & Nielsen, 2010; Vaghela *et al.,* 2017). Generally, Κ-casein is unaffected by plasmin hydrolysis due to the presence of attached carbohydrate moieties and its polymeric nature (Bastian & Brown, 1996; Vaghela *et al.,* 2017), however according to Groves *et al.* (1998), Κ-casein can be hydrolysed by

plasmin at Lys21 and Lys24 when carbohydrate is absent and hydrolysis of the other caseins can also lead to the disruption of K-casein (Gazi *et al.*, 2014). The pH of milk greatly impacts the association of plasmin with casein since losses in plasmin activity from micelles to occur at a pH range between 4.8-6.6 and few or no plasmin is present within the casein segment below pH 4.6 (Pulkkinen, 2014). Plasmin does affect the whey proteins including, α-lactalbumin, β-LG and bovine serum albumin (BSA) due to inhibitory effects (Datta & Deeth, 2001; Chen *et al.*, 2003; Tamime, 2008; Ismail & Nielsen, 2010).

2.4.3.3.3.1 The role of plasmin in age gelation

Plasmin may disturb milk quality negatively. UHT treatment of milk leads to an imbalance between the activators and inhibitors resulting in increased proteolysis. The presence of plasmin within UHT treated milk causes some negative changes which includes; formation of bitter and off-tastes, degradation of casein during storage, decreases in the viscosity of milk and ultimately age gelation during storage (Kelly & Foley, 1997; Saint Denis *et al.,* 2001; Le Roux *et al.*, 2003; Němečková *et al.,* 2009; Vaghela *et al.,* 2017).

The occurrence of age gelation by plasmin arises due to plasmin enduring the UHT procedure and remains active during storage. Plasmin undergoes unfolding at high heat treatments of milk, then refolding occurs when milk is cooled down and autolysis occurs during storage hence plasmin activity levels rise thus resulting in proteolysis which ultimately leads to age gelation (Chen *et al.*, 2003; Cilliers, 2007; Vaghela *et al.,* 2017; Anema, 2019). It is therefore clear that plasmin levels are an important stimulus on the occurrence of age gelation (Newstead *et al.*, 2006).

2.4.3.3.3.2 Factors that affect plasminogen activation/plasmin levels

There are many factors that either hinder or elevate plasminogen activation resulting in either lower or higher levels of plasmin. The interaction between the plasmin system and its components can be influenced by various conditions that are discussed below (Heegaard *et al.*, 1994; Cilliers, 2007).

External factors on the farm

- **Mastitis**

Mastitis is a disease that frequently occurs in cows, therefore, it is regarded as an important disease consisting of a pronounced effect on milk quality and quantity. During mastitis infection, inflammation in the udder occurs due to infection by microbes or deprived milking (Cilliers, 2007; Fernandez *et al.*, 2008; Forsbäck, 2010).

Mastitis infection results in an escalation in the SCC, therefore, these cells can be used for observing the udder wellbeing along with milk quality (Bavarian *et al.*, 2010). Milk is at high risk for pathogenic bacteria and antibiotic residues contamination due to the high SCC (Sharif & Muhammad, 2008).

Dairy products have a lower acceptance rate due to changes that arise as an outcome of high SCC (Fernandez *et al.*, 2008). A rise in somatic cells is, therefore, a characteristic of mastitis (Sharif & Muhammad, 2008; Forsbäck, 2010).

The components and properties within milk are changed due to an upsurge in the SCC as a consequence of mastitis infection (Sharif & Muhammad, 2008). The disadvantageous effects of mastitis are deterioration in milk quality such as lower shelf stability and reduced thickness, lower animal health, augmented antibiotics and augmented costs of labour (Fernandez *et al.*, 2008; Forsbäck, 2010). The composition of milk is also greatly subjective to the udder health status of cows (Sharif & Muhammad, 2008).

Plasmin is the proteolytic enzyme that plays a major role in milk during mastitis infection since plasmin tends to increase thus resulting in increased proteolysis since plasminogen activators are present within the somatic cells (Chen *et al.*, 2003; Forsbäck, 2010; Stoeckel *et al.,* 2016; Vaghela *et al.,* 2017). Therefore it is common for the activity of plasmin to be more pronounced in mastitis milk due to higher SCC than in normal milk that consists of lower SCC (Bastian & Brown, 1996). A direct association exists between higher SCC and increased proteolysis. Higher proteolysis arises in mastitis milk when warehoused at 20°C. Milk consisting of both high and low SCC with high plasminogen levels has a higher tendency for age gelation. It is clear that the existence of high levels of plasmin is greatly responsible for enhanced proteolysis in milk that contains increased amounts of somatic cells thus mastitis milk is therefore more at risk to endure age gelation (Datta & Deeth, 2001; Fernandez *et al.*, 2008; Ismail & Nielsen, 2010; Chavan *et al.*, 2011).

- **Colostrum**

Colostrum can be defined as the preliminary milk that is formed in the mammary gland within the first 5 to 7 days directly after calving and consists of variable composition and properties (Penchev Georgiev, 2008). Colostrum is crucial for the nutritive state of newborn calves since it stimulates the functioning of the gastrointestinal tract. The presence of carotenoids and red blood cells gives colostrum its distinct red-yellow colour. Mastitis infection can also be present within colostrum and is indicated by a red colour which is common since the SCC is higher within colostrum than in normal milk. Colostrum is generally considered to be unfit for the market since this type of milk results in problems during processes such as pasteurisation due to poor heat stability and the high protein content (McGrath *et al.,* 2016).

The components within colostrum of which the presence is generally higher than in normal milk include; fats, proteins, immunoglobulin, peptides, vitamins, minerals, hormones, growth factors, and enzymes. The only component that is present in lower quantity is lactose. The protein content also differs from normal milk. Colostrum consists of higher concentrations of casein (αs_1, αs_2, K-casein)

than normal milk and also larger casein micelles with diameters of 600mm. The whey proteins (β-LG and α-lactalbumin) are correspondingly four times advanced in colostrum than in normal milk. The pH of colostrum is generally lower than normal milk (pH 6.7) since it consists of a pH 6.0-6.2 (Penchev Georgiev, 2008; Zou *et al.,* 2015; McGrath *et al.,* 2016).

The existence of plasmin is 10x superior in colostrum when compared to normal milk and a reduction in plasmin activity throughout conversion from colostrum to normal milk is common. Ethanol stability refers to the lowest quantity of additional ethanol that results in the coalescence of milk and colostrum is regarded to consist of poor ethanol stability thus in combination with the high plasmin content, colostrum is therefore very prone to milk flocculation (McGrath *et al.,* 2016).

- **Age of cow**

Plasmin activity has the tendency to escalate when the age of the cow rises. During the lactation of the first lactation cows, plasmin activity normally remains constant, however, activity tends to increase in bovine milk obtained from older cows during late lactation. Milk obtained from mature cows has the tendency to undergo age gelation quicker in contrast with milk obtained from younger cows (Datta & Deeth, 2001; Ismail & Nielsen, 2010).

- **Stage of lactation/Involution**

Lactation can be defined as a physiological process that takes place within cows and this process is very demanding for both the cardiovascular and fluid regulatory systems. There exists an association between plasminogen activators and the decline phase of lactation. The declining phase of lactation is also strongly linked with amplified plasmin activity in milk. This can be prevented by treatment with bovine somatotropin which indicates that somatotropin hinders the transformation of plasminogen. The mechanism by which somatotropin suppresses involution is by overpowering the production of plasmin within the mammary gland. Therefore, injecting cows with somatotropin can sustain low plasmin levels during involution (Politis *et al.,* 1990; Silanikove *et al.,* 2006).

An undesirable link between β-casein and milk yield exists throughout the declining phase of lactation. β-casein is formed in the udder by plasmin when milk is in storage and this makes up 8% to 12% of the overall proteose–peptone section found in whey. Plasmin cannot further degrade β-casein and therefore β-casein is considered to be the perfect model to be used during milk secretion for the regulation of negative responses (Silanikove *et al.,* 2006).

The plasmin level within the mammalian gland generally rise and become advanced within 13 days when cows reach the involution stage, while still manufacturing lots of milk, of which termination of milking follows (Silanikove *et al.,* 2006). Plasminogen and plasmin activity is typically more elevated during late lactation than during early lactation (Baldi *et al.,* 1996).

Plasmin leads to the production of products that contain casein hydrolysates (CNH) which intensely increase the involution speed resulting in completion within 3 days. Plasmin activity naturally increases during late lactation which highlights these responses. Normal lactation can be observed in all glands treated with CNH (Silanikove *et al.,* 2006).

Age gelation is more pronounced in milk obtained from the first stage of lactation than in milk collected during late lactation. However, plasmin-induced proteolysis tends to be the opposite since it is higher in milk obtained during later lactation than in milk collected in the first stage of lactation (Datta & Deeth, 2001; Chavan *et al*., 2011).

- **Breed of cow**

The cow breed has a substantial stimulus on plasminogen activation since elevated plasmin activity is characteristic of milk obtained from Friesian breed than in milk from Jersey and Ayrshire breeds (Cilliers, 2007).

- **Milking frequency**

The composition of milk and milk yield are significantly subjective to the frequency of milking. Plasmin concentrations within milk can be reduced by once a day milking (Guinee & O'Brien, 2010). A decline in milking regularity is straight linked to elevated activity within the plasmin system. This outcome is partly due to limited contact with plasmin during a shortened duration of storage within the udder, but also due to improved preservation of reliability of the constricted epithelial junction as lactation progresses (Silanikove *et al.,* 2006).

Internal/Chemical factors

- **Stress**

Stress and the plasminogen–plasmin system are connected. Cows that undergo external stress exhibit the instigation of the hypothalamus-pituitary-adrenocortical axis which leads to the release of plasminogen activators within the epithelial cells present in the gland of the cow into the mammary cistern where plasminogen activation occurs. Therefore it can be settled that stress leads to amplified plasmin activity and also the creation of a plasmin-induced β-casein breakdown product. This β-casein breakdown product results in the decline of milk yield (Silanikove *et al.,* 2000; Silanikove *et al.,* 2006).

- **Growth hormones**

Plasminogen activation can be increased by the epidermal and an insulin-like growth factor which results in an amplified u-PA activity. The action of the two activators (u-PA and t-PA) is roused by

prostaglandin and serine proteases. Glucocorticoids have the ability to constrain the action of u-PA and t-PA (Le Roux *et al.*, 2003).

- **Storage temperature and refrigerated storage**

Plasmin activity is greatly impacted by the temperature at which milk is warehoused. Plasmin undergoes autolysis at a storage temperature of 37°C, therefore, increased levels of plasmin are common at this temperature (Crudden *et al.,* 2005). Plasminogen activation tends to be high when milk is stored at room temperatures (22-25°C) thus it is problematic when UHT milk is warehoused at this temperature (Ismail & Nielsen, 2010). Generally, the growth of thermo-tolerant organisms is accelerated by refrigerated storage as well as the plasmin system is also greatly affected (Crudden *et al.,* 2005). Plasmin can persist to stay active at 4°C (Gazi *et al.,* 2014). Storage at 5°C results in substantial autolysis for plasmin when compared to storage at 20 and 37°C, which is a problem since temperatures between 2-5°C are the established refrigeration temperatures within stores (Crudden *et al.,* 2005). Schroeder *et al.,* 2008, established that refrigerated storage at 4°C results in higher (almost double) plasmin activity than storage at 2°C. High levels of plasmin contribute to proteolysis, therefore, lower plasmin levels are desired (Schroeder *et al.,* 2008).

Refrigerated storage attribute to changes within the protein fraction of milk such as β-casein that becomes more soluble which results in plasmin being more accessible which ultimately leads to the occurrence of plasmin-induced proteolysis during refrigerated storage (Ismail & Nielsen, 2010). The storage of milk at refrigerated temperatures result in lower plasmin levels, however, it does not inhibit plasmin-induced proteolysis of caseins (Gazi *et al.,* 2014).

- **Ca ion concentration**

The presence of Ca in milk occurs as free ions or as Ca phosphate which can bind to casein and result in the formation of caseinate Ca or micelle Ca phosphate. Refrigerated storage leads to a rise in soluble Ca whereas reduction in the colloidal Ca phosphate is more common. Larger quantities of soluble Ca are directly linked to reduced plasminogen activation which may be attributed to the fact that Ca hinders plasminogen activation (Schroeder *et al.,* 2008).

- **Heat treatments/Thermal inactivation**

The inactivation of plasmin plays a vital role in general milk stability as well as shelf life stability (Van Asselt *et al.,* 2008). Characteristically plasminogen and plasmin are resistant against heat thus pasteurisation and UHT treatment of milk are not regarded as sufficient enough to inactivate this enzyme (Gazi *et al.,* 2014). The plasminogen activators also tend to stay unaffected during the pasteurisation and UHT process (Lu & Nielsen, 1993). Higher plasmin levels may even be present after pasteurisation due to the activator-inhibitors being inactivated by heat and also the unfolding of plasminogen which leads to increased accessibility of the plasminogen activation sites (Gazi *et al.,*

2014). Subjecting milk to heat applications at 50-60°C can lead to enhanced plasminogen activation due to heat treatments that result in the denaturation of plasminogen (Newstead *et al.*, 2006; Prado *et al.*, 2007; Ryan *et al.,* 2012).

There are some conditions that may result in the heat inactivation of plasmin. It is possible for plasmin to be inactivated by heat, however, inactivation is dependent on the specific heat treatments that are applied and also the behaviour of plasminogen activators and inhibitors due to the plasmin system being very complicated. Heat treatments may result in the deactivation of plasmin inhibitors and plasminogen activator inhibitors (Gazi *et al.,* 2014). The resistance against heat for plasmin tends to be higher when casein is present and β-LG is absent. The plasmin system is can withstand heat for up to 110°C since plasmin tends to have high conformational stability and high resistance against degradative chemical reactions (Ismail & Nielsen, 2010).

A study steered by Metwalli *et al.,* (1998), found that plasmin starts to unfold at 65°C. The tertiary arrangement of plasminogen unfolds at 50.1-61.6°C, however, it is still not inhibited at this temperature range. The inhibitors can be inhibited at this temperature range and the unfolding of plasminogen kringles also occurs which makes it more accessible to plasminogen action thus enhances plasminogen activation (Ismail & Nielsen, 2010). Heat treatments of milk at 75-90°C for 15-60 seconds generally do not result in the inactivation of plasmin. Extensive plasmin autolysis occurs when milk for UHT processing is subjected to additional heating at 80°C for 30 seconds whereas pre-heat treatment of milk at 55-90°C for 15 seconds causes enhanced plasmin activity. The total elimination of plasmin activity can be obtained by the subjection of additional heating at 90°C for 60/120 seconds preceding UHT processing (Stoeckel *et al.,* 2016; Anema, 2019).

The heat inactivation of plasmin is also greatly influenced by the SCC within milk since the stability of plasmin is normally higher at temperatures that range between 63-90°C in milk with higher SCC. The inactivation of plasmin during heating may be enhanced within mastitis milk which contains higher levels of thiol-groups and increased BSA (Kennedy & Kelly, 1997).

The presence of sulfhydryl compounds has a significant impact on heat inactivation since plasmin generally interacts with free sulfhydryl group (SH-groups), present within β-LG, upon the heating of milk (Kennedy & Kelly, 1997). Two stages are present during heat inactivation. During stage one, the unfolding of β-LG during heat treatment occurs which results in the release of the SH-groups whereas stage two arises when the free SH-groups lead to irretrievable denaturation of plasmin. A definite quantity of denatured β-LG is required for augmented deactivation of plasmin and plasminogen to occur therefore a specific heat treatment needs to be designed which will result in sufficient β-LG denaturation in order for plasmin inactivation to occur, however the applied heat must be low enough to avoid product degradation (Van Asselt *et al.,* 2008).

Irreversible inactivation of plasmin can occur when heat treatments above 80°C are applied in the absence of casein proteins or denatured caseins and unfolded β-LG since this whey protein leads to enhanced inactivation of plasminogen and plasmin (Metwalli *et al.,* 1998). Plasmin proteolysis can also be reserved during heat treatments through the degradation of whey proteins which binds to the surface of casein micelles (β-LG interactions with casein through disulphide bonding) and ultimately blocks plasmin from accessing the peptide bonds and interacting with the casein micelle (Enright *et al.,* 1999).

Plasminogen is regarded as a protein which contains high amounts of cysteine (Ryan *et al.,* 2012). Increased cysteine concentrations significantly accelerate the heat inactivation of plasmin at a temperature range between 80-140°C (Metwalli *et al.,* 1998) due to sulphydryl-disulphide exchange responses that occur between the disulphide bridges of β-LG and cysteine (Ryan *et al.,* 2012). Plasmin can also be inactivated with innovative steam injection (ISI) heat treatments that range between 80-180°C for short heating times (0.2 seconds) where a good quality UHT milk is still obtained without bitter tastes and minimal β-LG denaturation (Van Asselt *et al.,* 2008).

According to Gazi *et al.,* (2014), plasmin can only be entirely inactivated by a sterilisation process. The heating temperature of sterilisation has a significant influence on the deactivation of plasminogen and plasmin, however, deactivation hinges more on heating time than the heating temperature at heat treatments above 100°C (Saint Denis *et al.,* 2001). It is very essential for heat inactivation of plasmin to be successful since bitter tastes can arise within milk due to insufficient inactivation which results in milk being rejected by consumers. The onset of irreversible inactivation occurs when milk is subjected to heat treatment at a temperature range between 65-90°C (Metwalli *et al.,* 1998). Irretrievable inactivation of the inhibitors can also take place through intense heat subjection (UHT) which results in a decline in plasminogen activation (Enright *et al*., 1999).

- **β-LG**

β-LG is a key role player in the deactivation of plasmin by heat (Saint Denis *et al.,* 2001). The existence of β-LG results in heat liability of plasmin and plasminogen. Therefore, the degradation of β-LG is associated with the deactivation of plasmin (Newstead *et al*., 2006) since elevated β-LG denaturation leads to a decrease in plasmin levels (Rauh *et al*., 2014). This occurs due to interactions of the β-LG free thiol-disulphide group with thiol-rich structures of plasmin at high temperatures during the unfolding and denaturation processes (Enright *et al.,* 1999; Aaltonen & Ollikainen, 2011). Free SH-groups that are highly reactive become accessible during the degradation of β-LG since unfolding of β-LG occurs, therefore, these SH-groups can result in the irretrievable destruction of plasmin (Chavan *et al*., 2011).

- **Addition of potassium iodate (KIO_3)**

Milk treated with KIO_3 prior to UHT processing may enhance plasmin activity due to heat stability in the existence of KIO_3. The addition of KIO_3 protects plasmin from inactivation by complexation with β-LG. This result in the enhancement of plasminogen activation when milk is stored which in turn leads to extensive proteolysis and ultimately causes age gelation within UHT milk. The addition of KIO_3 adjusts the interaction that occurs between the whey and casein proteins in the sense that it decreases the connotation of β-LG with micelles (Enright *et al.,* 1999). KIO_3 is known to be an oxidising agent which has the ability to prevent the thermal inactivation of plasmin since the addition of this agent obstructs the irreversible thiol-disulphide exchange responses between plasmin and denatured β-LG (Kennedy & Kelly, 1997). Therefore, KIO_3 inhibit the release of SH-groups during β-LG denaturation which leads to accelerated plasminogen activation (Saint Denis *et al.,* 2001).

Other negative occurrences due to the addition of KIO_3 include milk being unstable during storage, UHT milk discolouration, bitterness in taste, decreased pH, micelle aggregation, casein degradation, accelerated proteolysis, sedimentation and phase separation (Enright *et al.,* 1999). However, other research findings suggest that the addition of KIO_3 can halt the process of proteolysis after a storage duration of 100 days (Andrews, 1982). This may be attributed to various factors namely the protection of αs_1-casein against plasmin attack (De Koning *et al.,* 1985); the amount of proteolysis that may lead to the separation of micelles (Kohlmann & Nielsen, 1988) and amplified whey protein degradation when KIO_3 is present which causes a lower occurrence of gelation (Manji & Kakuda, 1988). More reasons may be oxidation of SH-groups that occurs fast when KIO_3 is present which decreases the concentration of SH-groups, which is a role player in the mechanism of age gelation (Parnell-Clunies *et al.,* 1988) and also the prevention of the sulphydryl-mediated β-LG/K-casein interaction which would coat the micelle in protective K-casein (Auldist *et al.,* 1996).

Table 2. Comparisons between microbial protease and indigenous plasmin (Kaminogawa *et al.,* 1972; Nielsen, 2002; Němečková *et al*., 2009).

Factors	**Microbial protease**	**Indigenous plasmin**
Origin	Produced by thermo-tolerant bacteria	Activated by plasminogen
Heat resistant	Yes	Yes
Optimum pH	*Bacillus* proteases 8.0, *Pseudomonas* proteases 6.5-7.0	7.5-8.0
Stability range (pH)	6.0-8.0	6.0-8.0
Labile (pH)	Below 5.0, Above 9.0	Below 5.0, Above 9.0
Inactivation (Temperature)	80°C	80°C
Loss of activity	37°C	37°C
Inhibition in presence of β-LG	Yes	Yes
Molecular weight	48 000 Dalton (Da)	48 000 Da
Optimum temperature	*Bacillus* proteases 30-37°C, *Pseudomonas* proteases 17.5-30°C	37°C
Classification	Metallo protease	Serine protease
Casein attack	B-casein, α-casein, K-casein	B-casein, α-casein

2.5 Biochemical detection techniques for proteolytic enzymes in milk that play a role in age gelation

It is important to establish the root cause when confronted with milk samples that had undergone age gelation. This process is complex and involves determining whether the affected samples were handled sterile since this will affect whether spoilage and age gelation will occur rapidly. An analysis follows to determine whether age gelation was induced by proteolytic enzymes (indigenous plasmin or microbial proteases) or chemical reactions. Techniques are available to determine proteolysis. If no proteolysis is present within the sample, the likelihood of gelation is possibly due to chemical reactions (Anema, 2019). This study mainly focused on detection techniques for proteolysis caused by proteolytic enzymes.

Proteolytic enzymes present in milk are complex, normally present in low concentrations, consists of various modes of action and also different optimal conditions such as temperature and pH. Effective detection techniques are therefore desirable since proteolytic changes in milk are very intricate and recognition of these changes cannot be performed without special techniques (Němečková *et al.,* 2009).

Proteolysis can be investigated by two different methodologies namely, quantification of changes from a protease origin and determination of protease activity. Spectrophotometer assays, mostly applied to the determination of proteolytic activity, are simple and quick, however, consist of a low level of sensitivity (Němečková *et al.,* 2009).

The Alizarol test is popular and frequently used in the dairy industry. Techniques developed during previous research work (Hattingh, 2017) included a modified milk agar plate test, protease assays and peptide analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) and MILQC software.

Chromatographic methods such as RP-HPLC have been optimised by Datta & Deeth (2003), and by Hattingh (2017). This method can be used to evaluate whether plasmin or microbial proteases caused the defects in flocculated milk. This section includes a discussion of the various detection techniques for proteolysis caused by proteolytic enzymes.

2.5.1 The Alizarol test

The Alizarol test is easily performed and results are immediately available (Robertson, 2010). The Alizarol test is an established benchmark for milk quality determination and is usually applied during the initial quality screening (Kurwijila, 2006; Aquino, 2013; Robertson, 2010; Anema, 2019). Since interpretation relies on the human eye, complications arise which is regarded as a major weakness (Hattingh, 2017).

The chief operation is to identify an unacceptable acid/alkaline level in milk that is considered as being abnormal and this forms the basis of this test (Robertson, 2010). Alizarin indicates varying colour in milk samples which specifies abnormally sour milk (Milkman, 2010). The Alizarol test detects milk with a pH of 6.4 and lower. Therefore, abnormal milk will generally fail the test whereas good quality milk normally passes the test (Kurwijila, 2006). The Alizarol test is effective in either screening milk with exceptionally good quality or very poor quality. However, it is not a good test in determining whether milk will develop sediment or if it is suitable for further UHT processing. Thus this test is no longer practiced in most countries, however, South Africa still use the Alizarol test due to its simplicity, rapidness and low cost. Other detection techniques are considered as impractical and expensive (Anema, 2019). Refer to Table 3 and Figure 6 for the interpretation of the Alizarol test.

Table 3. Interpretation of the Alizarol test (Kurwijila, 2006).

Parameter	**Ordinary milk**	**Somewhat acidic milk**	**Intensely acidic milk**	**Alkaline milk**
pH	6.6-67	6.4-6.5	6.3/lower	6.8/higher
Colour	Red-brown	Yellow-brown	Yellow	Lilac
Appearance	No flocculation and flakes	No flocculation	Flocculation: yellow colour with the presence of small flakes/completely flocculated	Flocculation: lilac colour and the presence of flakes can be an indicator of mastitis milk

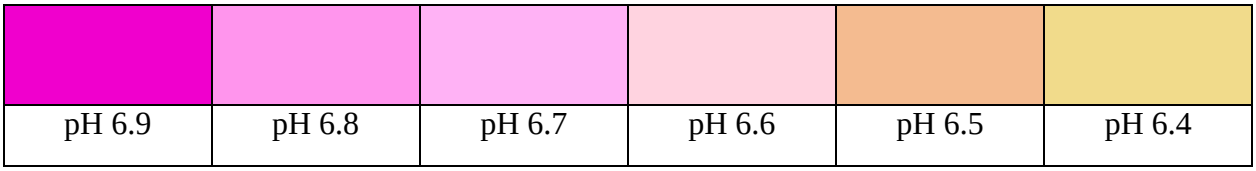

Figure 6. Colour chart range for the Alizarol test (Robertson, 2010).

2.5.2 Protease activity assay

Peptides bonds are broken by proteases, therefore, it is vital to measure and compare the levels of activity for various proteolytic enzymes. The protease activity assay is capable of measuring the total proteolytic activity (plasmin and microbial proteases) within milk samples.

The foundation for the protease activity assay is grounded on fluorescein thiocarbamoyl casein (FTC) hydrolysis in the relevant sample. The activity within the sample slices FTC into smaller, Trichloroacetic acid (TCA) soluble, FTC-peptides to discharge the yellow chromophore. TCA addition is necessary for precipitation of any residual FTC and to halt enzymatic actions. The chromophore is measured with a spectrophotometer at 492 nanometers (nm) (Twining, 1984; Wiesner & Troll, 1982).

2.5.3 Plasmin assay

Plasmin assays are essential for the establishment of plasmin activity within milk (Bastian & Brown, 1996). Plasmin activity determination by assays is recognised through the hydrolysis of a precise substrate which discharges a chromophore or fluorogenic product (Saint-Denis *et al.,* 2001).

Interfering effects are present and cause inconsistent results in chromophore or fluorogenic methods such as EACA, which binds to the lysine-binding spots resulting in the detaching of plasminogen/plasmin from the caseins thus causing higher measured plasmin activity (Korycka-Dahl *et al.,* 1983). The inhibition of the artificial substrate of casein can be lowered by the detachment of plasmin and caseins, therefore, affecting the interface of plasmin with plasmin inhibitors and whey proteins. Plasmin levels are also hindered by the caseins within milk which obstruct the chromophore substrate used in assays (Bastian *et al.*, 1991). The link between plasmin and caseins is also influenced by pH which is not reduced between pH 4.8 and 6.6, however, maximum plasmin activity is dissociated from micelles between pH 4.6-4.7 (Grufferty & Fox, 1988; Nielsen, 2002).

Plasmin activity can also be repressed by whey. B-LG and BSA consist of higher inhibitory effects than α-lactalbumin. Milk turbidity can also interfere with the measurement of fluorescence plasmin assays. Samples need to be highly diluted or additional clarification treatments are needed prior to measurement. Proteins such as azocasein and 14C-methylated casein can also result in magnification of plasmin activity (Zittle, 1965; Donnelly *et al.,* 1980).

It is also known that microbial proteases can interfere with the assay. It is therefore important to consider all the interferences and extensive sample preparation is needed during plasmin assay protocols in order for the assay to be accurate and effective (Kelly & Fox, 2006).

A variety of plasmin assays described in the literature is performed in similar ways however with some modifications. Rollema *et al.* (1983) used a chromophore substrate, H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide and plasmin cleaved the lysine-nitroanilide bond and released 4-nitroanilide and absorbance was determined at 405nm (Kelly & Fox, 2006). Another modified colorimetric assay in

literature measured plasmin activities in both the casein and whey fractions in a similar way as mentioned above (Fajardo-Lira *et al.,* 2000).

2.5.4 Milk agar plate technique

The milk agar plate technique for the detection of protease action is an alteration of the one generally used throughout microbe sorting (Hanan, 2012; Vijayaraghavan & Vincent, 2013; Himedia Laboratories, 2015). This technique is mainly described as a qualitative determination method for protease activity. Milk agar plates can be used for the detection of proteolysis of casein which ultimately leads to age gelation. Casein has the ability to form an opaque suspension which causes milk to have a white appearance. Milk no longer appears white when casein is hydrolysed by proteases which ultimately lead to the appearance of clear halos. The clear halos appearing on the milk agar medium are caused by proteolytic enzymes hydrolysing peptide bonds of proteins (casein). The clear halos are indicative of soluble nitrogenous compounds that are formed by proteolytic enzymes thus the clear halos, therefore, represent protease activity (Hattingh, 2017).

In this study, the milk agar plates will be used for the rapid discovery of protease activity. The goals will be to differentiate among indigenous plasmin and microbial proteases, to optimise the technique in terms of % agar and milk and also to hourly monitor halo formation. This technique is sensitive, rapid, cost-effective and simple. It is ideal for use on farms to establish whether milk test positive for protease activity and possibly which protease, indigenous or microbial, caused damage towards the milk sample by differentiating between the halo edges.

2.5.5 RP-HPLC for proteolytic peptide profiles

High-performance liquid chromatography (HPLC) is successful in separating and quantifying specific compounds within a solution and this technique is applied to detach molecules grounded on their variances in construction/composition (Kupiec, 2004).

Proteolytic activity originating from both plasmin and bacterial proteases has negative effects on milk such as age gelation. Even low levels of these enzymes have the ability to result in protein degradation during milk storage. Sensitive techniques for protease detection are important for the dairy industry, however, there is no technique adopted for this purpose. Plasmin and bacterial proteases produce different peptides and RP-HPLC can be used to separate the peptides formed by these enzymes. Plasmin produces bigger and hydrophobic peptides that elute later than the peptides liberated by bacterial proteases which are smaller and more hydrophilic. This phenomenon forms the foundation for this RP-HPLC method, therefore, this technique can be used to measure proteolytic activity by examining the peptides, the quantity and the extent of proteolysis in milk and also to distinguish among plasmin and bacterial proteases (Datta & Deeth, 2003; Le *et al.,* 2006; Hattingh, 2017). This technique determines the absorbance at a specific wavelength of detached peptides that is based on mass. This makes RP-HPLC a sensitive technique since it can identify various sizes of

peptides. The RP-HPLC technique is, therefore, very precise, sensitive and trustworthy (Le *et al.,* 2006).

This RP-HPLC technique is used to determine the protease activity within milk samples with the added enzyme by measuring the increase in peptides when compared to control samples without the addition of enzyme. Different acids may be used to precipitate samples such as TCA or Hydrochloric acid (HCl). The different acids result in peptide variations since the strength and mechanism of protein precipitation of the acids differ, as established in previous research work (Hattingh, 2017).

2.5.6 MILQC software

The neural network software is the essential backbone in MILQC software programming. A neural network works on the same basis as human information processing. A neural network is very supportive with the documentation of preserved data and behaviour configurations are usually a prerequisite. This network also develops a model using the training data which is convenient when complex data is present where an algorithm exists or data that is not known or when too many variables are present (Smith, 1996).

This computer-assisted software may be used when the peak variances within the RP-HPLC chromatograms are not easily visually observed. Chromatograms of 20 repeats of a specific experiment are drawn into the program. An average chromatogram is then constructed. The MILQC produced chromatograms are thus illustrative of peptide peaks liberated by varying proteases, which makes the process of distinction easier. This computer software can be applied in order to differentiate between plasmin and microbial proteases as well as between various proteases from the thermo-tolerant origin.

2.6 Conclusions

It is clear from the literature that milk is an unstable medium since there are numerous factors that affect its stability, even the components within the milk. The difference between milk flocculation and age gelation is that milk flocculation is driven by a pH reduction and leads to casein micelle coalescence whereas age gelation involves a rise in thickness after extended storing and both processes occur within raw, pasteurised and UHT milk.

The components within milk such as the proteins, fat and proteolytic enzymes contribute to the occurrence of age gelation. Heat treatments applied to milk affect the behaviour and stability of proteins and in turn, have serious effects on the behaviour of the proteolytic enzymes resulting in age gelation whether through chemical action or enzymatic action. The main proteolytic enzymes that are role players in age gelation are microbial proteases and plasmin (the indigenous milk enzyme).

Age gelation is regarded to be very problematic for dairy manufacturing resulting in economic losses since milk with the presence of age gelation is rejected by consumers. The dairy industry currently does not have any specification of whether raw milk is fit for further procedures and that the products will not gel within the required shelf life since the culprit proteolytic enzymes that cause age gelation is extremely low and meaningful measuring techniques are lacking. Rapid and sensitive detection techniques are therefore essential. Some techniques can detect milk flocculation such as the Alizarol test whereas other techniques can detect proteolytic activity within milk such as the protease activity assay, milk agar plate technique, and RP-HPLC. All the detection techniques consist of their own advantages, disadvantages, and limitations and in most cases, more than one detection technique is needed.

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CHAPTER 3

Determination of peptide profiles for a wide range of microbial proteases using computer-assisted MILQC software

Objectives

The objective for Chapter 3 was to determine reverse-phase high-performance liquid chromatography (RP-HPLC) peptide profiles for proteases obtained from a wide range of thermo-tolerant bacteria (previously known as psychrotrophic bacteria). In addition to peptide profiles already obtained from *Bacillus* and *Pseudomonas* strains during previous research work (Master's study), peptide profiles derived from a wider range of microbial proteases will improve the current database of peptide profiles liberated by microbial proteases and plasmin.

3.1 Introduction

Proteolytic proteases have an extensive negative effect on milk quality since their existence can result in the phenomena of age gelation within ultra-high temperature (UHT) milk due to their capability to endure severe heat treatments for a short time interval. The main proteolytic proteases that play a role during age gelation are plasmin, the indigenous milk protease, and proteases of microbial origin. The primary microbial proteases that were researched for their characteristic peptide profiles in previous research work (Hattingh, 2017) were proteases from *Pseudomonas fluorescens* and *Bacillus licheniformis* (Datta & Deeth, 2001). A database of the respective peptide profiles liberated by the various bacterial proteases was developed using RP-HPLC and the computer-assisted MILQC software (Hattingh, 2017).

Low quantities of proteolytic enzymes have the ability to result in protein destruction during milk storing therefore sensitive, rapid and affordable detection techniques such as RP-HPLC are important for the dairy industry. Plasmin and microbial proteases liberate characteristic peptide profiles and RP-HPLC can be used to separate the peptides liberated by these enzymes. Plasmin produces more hydrophobic peptides that elute at a later time than the peptides liberated by bacterial proteases which are more hydrophilic. This phenomenon forms the foundation for this RP-HPLC technique, therefore, this can be used to measure the source of proteolytic activity by examining the peptide profiles and the extent of proteolysis in milk (Datta & Deeth, 2003; Le *et al.,* 2006; Hattingh, 2017).

Neural network software (run on the Wolfram Mathematica platform) is the best option to process the data imported from RP-HPLC proteolytic peptide profiles (the backbone in the programming of MILQC software). The logic of the neural network is a multiprocessor computer system similar to human information processing with regard to memory/processing. A neural network is very effective with

the identification of conserved data and behaviour patterns are usually a prerequisite. This network can also form part of a model using previously accumulated data (training data) which is convenient when working with complex data sets where an algorithm already exists or data that is not known or where more than one variable is present (Smith, 1996). This computer-assisted software may be used when the differences in peaks within the chromatograms from RP-HPLC are not always visible with the naked eye and small differences may be detectable between different preparations. The MILQC generated chromatograms are representative of conserved peptide peaks liberated by various enzymes, which makes it possible to effectively distinguish between the various peptide profiles. This MILQC computer software can be applied in order to differentiate between plasmin and microbial proteases as well as between various proteases from thermo-tolerant origin (Hattingh, 2017).

Peptide profiles for plasmin and proteases from *Pseudomonas fluorescens* and *Bacillus licheniformis* were successfully distinguished in previous research work (Hattingh, 2017). Therefore, the need arose to investigate a broader spectrum of microbial protease peptide profiles. The wider range of microbial proteases was selected based on the information available in the literature regarding the thermo-tolerant bacteria (previously known as psychrotrophic bacteria) that has an influence on milk with regard to the secretion of heat-stable proteases (Samaržija *et al*., 2012; Xin *et al.,* 2017). The various thermo-tolerant bacteria selected include; *Pseudomonas fluorescens, Pseudomonas fragi, Bacillus licheniformis*, *Bacillus cereus,* and *Bacillus subtilis*.

Keywords: Microbial proteases, Plasmin, Peptide profiles, RP-HPLC, MILQC

3.2 Materials

Milk

The milk used for the self-cultivation of enzymes was Pick and Pay Choice low fat UHT milk and was obtained from Pick and Pay in Langenhoven Park, Bloemfontein, Free State, South Africa.

Reagents

Bacteriological Agar powder (1%) was supplied by Quantum Biotechnologies, Randburg, South Africa.

The Nutrient broth was supplied by Merck, South Africa.

Hydrochloric acid AR (0.1 Normal [N] [HCl]) was used for the precipitation of samples prior to RP-HPLC analysis and was supplied by Merck, South Africa. According to literature, Trichloroacetic acid (TCA) precipitates the high pH proteins in milk (large molecules) whereas HCl precipitates only proteins soluble at pH 4.6 (iso-electric point [pI]) (Datta & Deeth, 2001).

Acetonitrile (CH_3CN), gradient grade for liquid chromatography, was supplied by Merck, South Africa.

Self-cultivated enzymes

Various bacterial species are known to have heat resistant proteases were cultivated and the proteolytic enzymes were harvested. Proteases were harvested from *Pseudomonas fluorescens, Pseudomonas fragi, Bacillus licheniformis*, *Bacillus cereus,* and *Bacillus subtilis.* These bacteria were supplied by the University of the Free State (UFS) bacterial collection maintained in the Department of Food Science, Faculty of Natural and Agricultural Sciences, UFS, South Africa.

3.3 Methods

3.3.1 Maintenance of bacterial cultures and preparation of pre-inoculum and nutrient agar slants

The various bacteria mentioned above were maintained on bacteriological agar (1%) slants and pre-inoculations prepared in nutrient broth (1.6%). The nutrient broth was prepared by dissolving 2g in 125mL of distilled water (DH_2O) in Erlenmeyer flasks as pre-inoculum. For the nutrient agar slants, 5g agar along with 8g nutrient broth was dissolved in 500mL of DH_2O and thoroughly mixed and 20mL aliquots were transferred in McCartney bottles. The Erlenmeyer flasks and McCartney bottles were sterilised by autoclaving for 20 minutes at 121°C in a Hirayama (Hiclave™, HV-85) autoclave (supplied by Labotec SA). The pre-inoculum flasks, along with the nutrient broth agar slants, were kept in a refrigerator at 4°C after sterilisation.

3.3.2 Self-cultivation of proteolytic enzymes

Five separate Erlenmeyer flasks (one for each organism) were filled with 250mL of low fat UHT milk, followed by sterilisation at 121°C for 20 minutes in a Hirayama (Hiclave™, HV-85) autoclave (supplied by Labotec SA).

The listed bacteria from the respective nutrient agar slants were inoculated into the pre-inoculums and incubated at 25°C with agitation (60 revolutions per minute [rpm]) for 12 consecutive hours or until the optical density for the individual organisms reached an absorption value of 2. A Cecil (CE 2021, 2000 series) spectrophotometer at 640 nanometers (nm) (supplied by Lasec SA) was used.

The pre-inoculums (1mL) were used to inoculate the Erlenmeyer flasks with sterile 250mL UHT milk. The flasks were slowly agitated (60 rpm) and incubated at 25°C for three days until bacteria growth reached the stationary phase. The flasks containing the bacteria in their stationary phase were incubated (lacking agitation) at 4°C for three days in order to cause milk to coagulate (Gaucher *et al.,* 2011).

3.3.3 Proteolytic peptide analysis by RP-HPLC

All the milk samples were iso-electrically precipitated with 250µL 0.1N HCl to 1mL of the milk to be analysed for peptide profile determination. The samples were subsequently centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) at 10 000 rpm for 20 minutes. The clear supernatant (200µL) was placed into HPLC vials for RP-HPLC analysis for proteolytic peptide profile determination.

The elution solvent used was acetonitrile (CH_3CN) and the acetonitrile concentration gradient ranged between 0 and 65% over a time interval of 90 minutes. The elution speed for the mobile phase was set at 1mL/min. Samples (20µL) were injected by an automatic injector (capable of injecting 100µL). A Phenomenex Jupiter C18 column with an internal diameter of 4.6mm was used. The temperature of the column was kept at 40°C. The ultraviolet (UV) detection at a wavelength of 214nm (Spectra System, UV 1000) was used and the data were integrated using data-reprocessing software provided by the HPLC supplier (Agilent Technologies, 1260 Infinity) (International Standard, 2005).

3.3.3.1 Computer-assisted identification of the proteolytic peptide profiles using MILQC software

RP-HPLC chromatograms of ±20 repeats of a specific experiment were drawn into the MILQC software using the Wolfram Mathematica 10 programme. From these, which may contain a difference in some peak sizes or presence/absence of some peaks, a computer-assisted chromatogram was constructed that represents the proteolytic profile for the various bacteria evaluated (Hattingh, 2017).

3.4 Results and discussions

The data in Table 4 depict the final optical density and the incubation time of the various bacteria (pre-inoculum) to reach the desired absorption value of 2 for each bacterium and a dilution factor of 5x was used.

Table 4. Timeframes for the various bacteria (pre-inoculum) to reach the optical density of 2 (A640 nm).

Bacterium	Absorption (A640nm)	Incubation time (hours)
Bacillus licheniformis	2.15	24
Pseudomonas fluorescens	2.15	17
Pseudomonas fragi	2	17
Bacillus cereus	2.3	12
Bacillus subtilis	2.05	20

MILQC Chromatograms

The computer-assisted MILQC peptide profiles were obtained for the individual bacterial proteases evaluated. The peptide profiles depicted in Figure 7 indicate the MILQC generated chromatograms for the self-cultivated *Pseudomonas* proteases. From this Figure, it was clear that differences existed between the two peptide profiles of the protease action from the two *Pseudomonas* species as indicated by the arrows and circles. *Pseudomonas fluorescens* protease had unique peaks between an elution time of 5-10, 10-15, 23 and 45-55 minutes as indicated by the green arrows and green circle. The unique peaks for *Pseudomonas fragi* protease action were between an elution time of 30-35, 40, 40-45 and 65-80 minutes as indicated by the red arrows and red circle. This difference is very important since it can be used to distinguish between the action of the two *Pseudomonas* proteases.

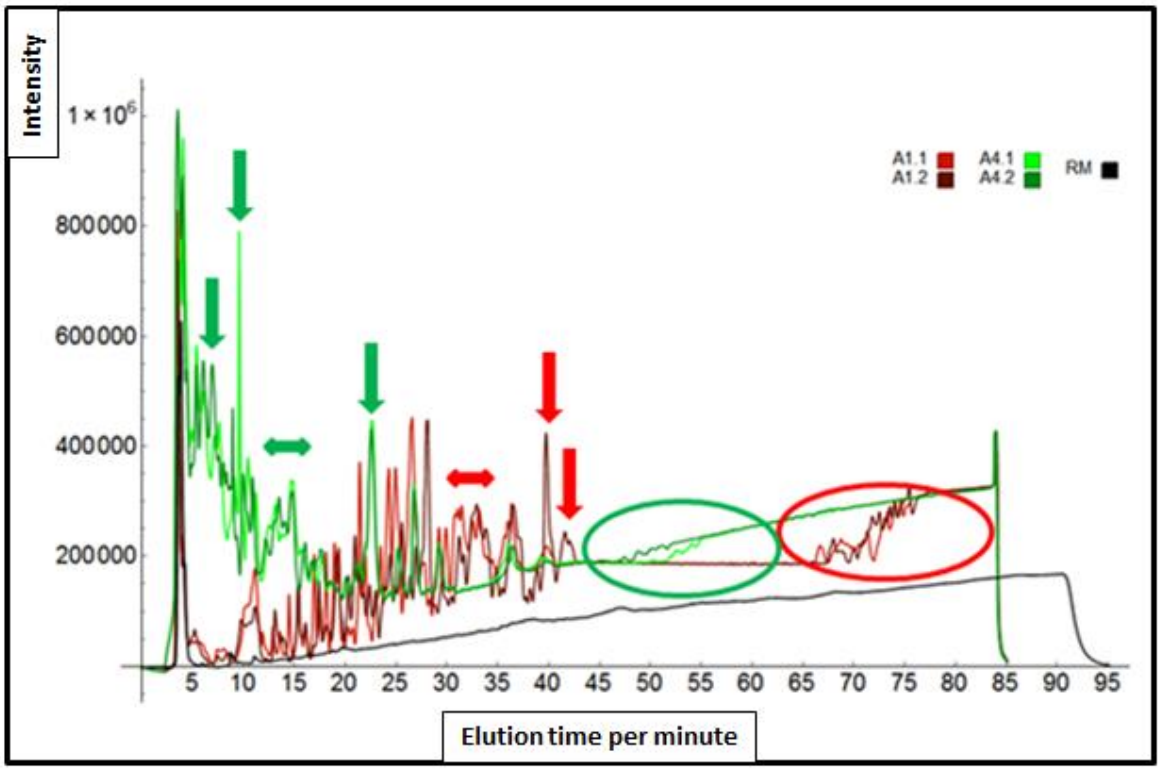

Figure 7. MILQC generated RP-HPLC chromatograms of the self-cultivated *Pseudomonas* proteases. The numbers in the legend represent the following; A 1.1 and A 1.2: *Pseudomonas fragi* protease, A 4.1, and A 4.2: *Pseudomonas fluorescens* protease. RM is raw milk which served as the control. The green arrows and green circle indicate unique peaks for *Pseudomonas fluorescens* protease whereas the red arrows and red circle indicate the area where *Pseudomonas fragi* protease shows unique peaks.

The MILQC generated chromatograms for the *Bacillus* proteases are indicated in Figure 8. It was evident that dissimilarities existed between the three *Bacillus* proteases as indicated by the circles. The *Bacillus licheniformis* protease had unique peaks between an elution time of 28 and 45-65 minutes as indicated by the maroon arrow and maroon circle. The unique peaks for *Bacillus cereus* protease were between an elution time of 43 and 60-75 minutes as indicated by the navy blue arrow and navy blue circle and lastly, the unique peaks for *Bacillus subtilis* protease were between an elution time of 30-40 and 60-70 minutes as indicated by the purple arrows. This difference is very important since it can be used to distinguish between the three *Bacillus* proteases.

After the MILQC processing, it became clear that all the proteases can be distinguished from one another since each protease had unique peaks in certain areas as already mentioned and also indicated in Figures 7 and 8. The major difference between the peptide profiles for *Pseudomonas* and *Bacillus* proteases was that characteristic peaks for *Pseudomonas* proteases were mainly at elution times 5-45 minutes (Figure 7) whereas for *Bacillus* proteases the unique peaks were at elution times between 45-75 minutes (Figure 8).

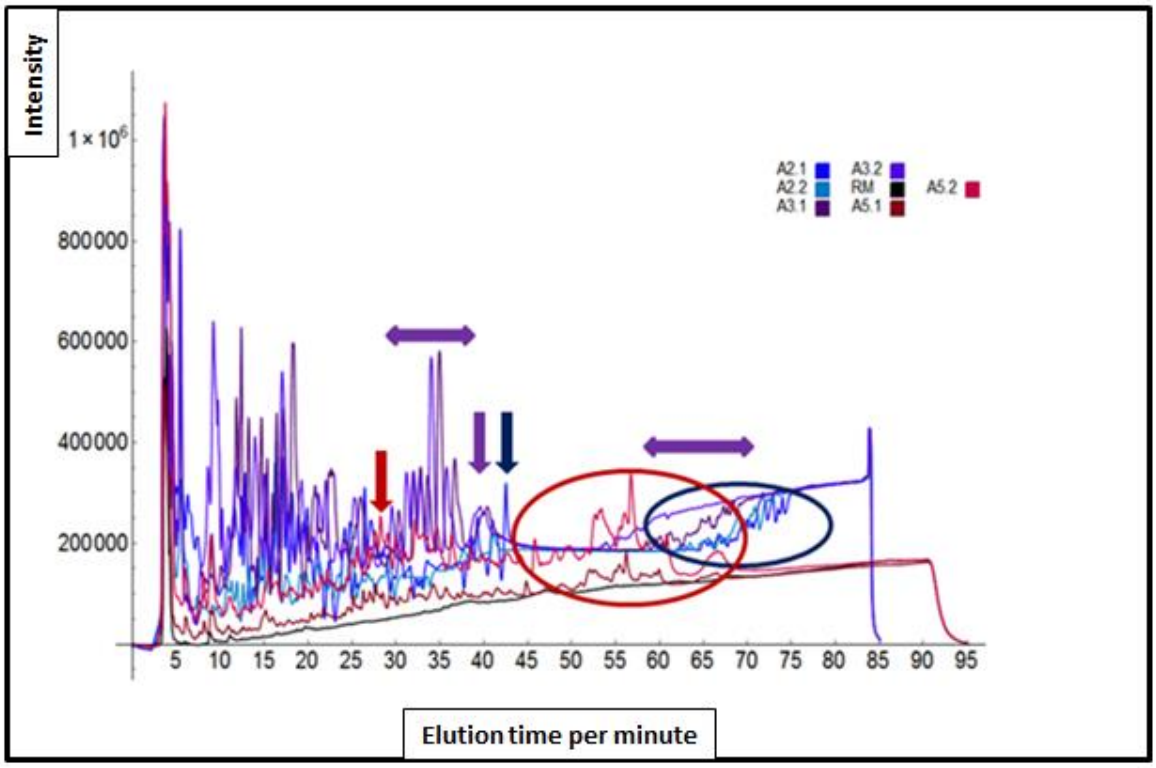

Figure 8. MILQC generated RP-HPLC chromatograms of the self-cultivated *Bacillus* proteases. The numbers in the legend represent the following; A 2.1 and A 2.2: *Bacillus cereus* protease, A 3.1, and A 3.2: *Bacillus subtilis* protease, A 5.1, and A 5.2: *Bacillus licheniformis* protease. RM is raw milk which served as the control. The maroon arrow and maroon circle indicate the area where *Bacillus licheniformis* protease shows unique peaks whereas the navy blue arrow and navy blue circle indicate unique peaks for *Bacillus cereus* protease. The purple arrows are indicative of the unique areas for *Bacillus subtilis* protease.

Figure 9 is previous research work taken from Hattingh, (2017) to be used for reference purposes only and the coloured squares on the diagram highlight the distinct peaks of peptides liberated by the two microbial proteases and indigenous plasmin. The green squares are indicative of plasmin-derived peptide profiles, orange squares from *Bacillus* protease whereas the red square from *Pseudomonas* protease.

The prominent peptide peaks liberated by plasmin and *Bacillus* proteases eluted between 50-60 minutes. Characteristically, all the peptide peaks from plasmin action at this elution time can be observed as a bell curve whereas, the peptide peaks liberated by *Bacillus* proteases at this elution time are spiky and this major difference can be used to easily distinguish between the characteristic profile peaks delivered from derived from plasmin and *Bacillus* proteases which were used in future research work. From Figure 9, it is evident that, after the MILQC processing, there were distinct conserved areas that displayed unique peptide profiles for each proteolytic enzyme.

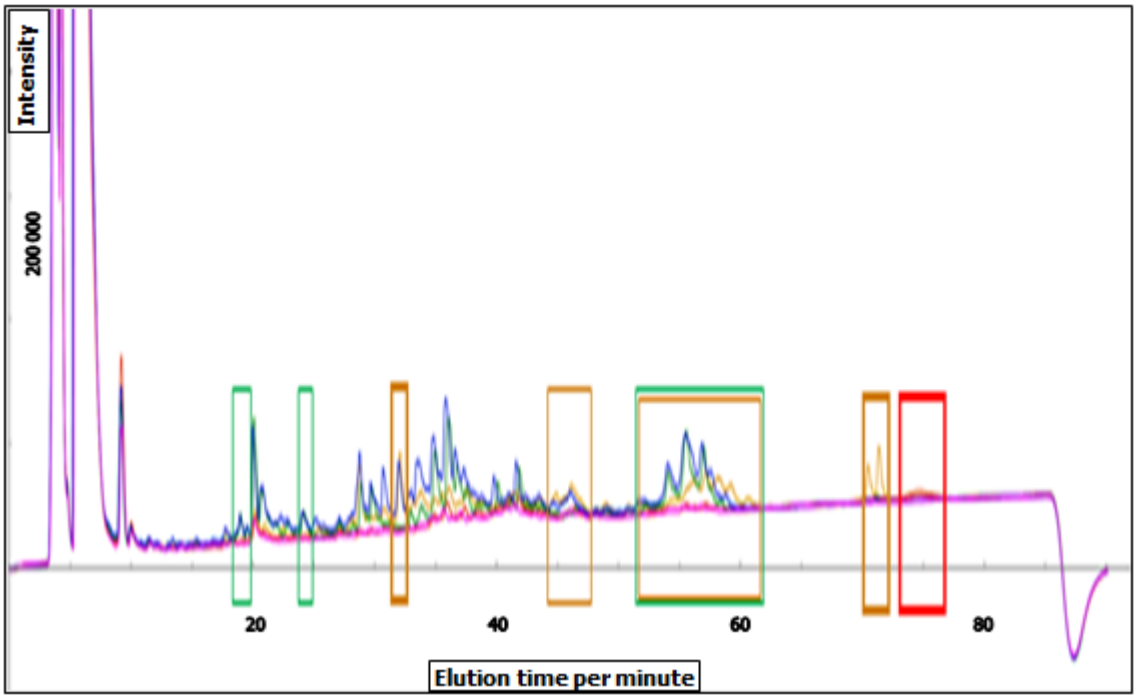

Figure 9. MILQC software-generated chromatograms of peptides liberated by Plasmin, *Bacillus* protease and *Pseudomonas* protease. The green squares represent distinct conserved areas for plasmin, orange for *Bacillus* protease and red for *Pseudomonas* protease (Hattingh, 2017).

3.5 Concluding remarks

It can be concluded from Chapter 3 that the MILQC processing simplified the process of prominent peak identification for each proteolytic enzyme since prominent peaks for the various self-cultivated *Pseudomonas* proteases as well as *Bacillus* proteases was successfully recognised. It can, therefore, be concluded that the self-cultivated proteases consisted of their own unique peptide profiles thus the *Pseudomonas* and *Bacillus* proteases can be accurately distinguished from one another.

Figure 9 is derived from previous research work (Hattingh, 2017) and was used for comparison purposes for the other two Figures in this Chapter since it consists of the fingerprint peaks for each group of proteolytic enzymes thus it was also used as a reference point for all the RP-HPLC peptide profiles to follow in order to easily identify the specific enzyme responsible for degradation.

3.6 References

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CHAPTER 4

Optimisation and validation of detection techniques for proteolytic activity

Objectives

The objectives for Chapter 4 were to optimise and validate detection techniques for proteolytic activity:

- **The milk agar plate technique.**
- **Investigation of various protease assays in order to establish a trustworthy and repeatable assay for plasmin and microbial proteases.**
- **The reverse-phase high-performance liquid chromatography (RP-HPLC) technique was also optimised in order to quantify the plasmin and microbial protease activity by relative area integration versus enzyme activity.**

4.1 Introduction

Detection techniques for proteolytic activity are highly desirable and essential and were established during previous research work (Hattingh, 2017). However, the need arose to develop new and more sensitive analytical techniques to detect casein digestion in milk.

The Alizarol test (a standard test for milk quality already in use in the industry for 75 years) is currently the preferred choice to evaluate the quality of milk with regard to milk flocculation. The Alizarol test is popular due to the ease at which it can be performed as well as the immediate availability of results much needed in dairy manufacturing. The objective of this test is to regulate the development of unacceptable acidity or alkalinity levels in milk which falls outside the generally acceptable norms (detects milk with pH 6.4 and lower) (Robertson, 2010).

The milk agar plate technique for protease detection is a modification of the milk agar plate technique generally used for the classification of microbes (Hanan, 2012; Vijayaraghavan & Vincent, 2013; Himedia Laboratories, 2015). This technique is mainly described as a qualitative determination method for protease activity (though the diameter of the halo gives a good indication of enzyme load). Casein forms an opaque suspension which causes milk to have a white appearance. When this casein is hydrolysed by proteases, clear halos develop (Hattingh, 2017). In this study, an attempt was to optimise the technique in terms of the quantity of agar and milk [casein] in the agar plates and also to monitor halo formation over time.

In this work, it is critical to accurately measure proteolytic activity. The spectrophotometric Merck protease assay is capable of measuring the total proteolytic activity within milk samples and this assay has the ability to identify the proteolytic activity levels for a wide variation of proteolytic enzymes such as plasmin and the proteases produced by both *Bacillus* and *Pseudomonas* (Hattingh, 2017).

Plasmin assays are used for the detection of plasmin activity within milk samples. An artificial chromophore substrate is normally used where the release of the chromophore is measured with a spectrophotometer (Bastian & Brown, 1996; Saint-Denis *et al.,* 2001).

Keywords: Detection, Alizarol, Milk agar plates, Plasmin, Proteases, Assay

4.2 Materials

Milk

The milk used for the optimisation of the milk agar plate technique and the validation of the RP-HPLC technique was Pick and Pay Choice low fat ultra-high temperature (UHT) milk and was obtained from Pick and Pay in Langenhoven Park, Bloemfontein, Free State, South Africa.

Reagents

Bacteriological Agar powder (1%) was supplied by Quantum Biotechnologies, Randburg, South Africa.

Sodium azide was supplied by Merck, South Africa.

In the case of the plasmin assay, the four possible plasmin chromophore based substrates were supplied by Sigma-Aldrich, South Africa and included the following; Gly-Arg-*p*-nitroanilide dihydrochloride urokinase (G8148); N_a-*p*-Tosyl-L-arginine methyl ester hydrochloride (T4626); N-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (T6140) and D-Val-Leu-Lys 4-nitroanilide dihydrochloride (V0882).

The spectrophotometric Merck protease assay kit (Calbiochem no. 539125) was supplied by Merck Millipore, South Africa.

Trichloroacetic acid (5% TCA) was supplied by Merck, South Africa.

The following reagents for the 0.1 Molar (M) trisaminomethane (Tris) Hydrochloric acid AR (HCl) buffer (pH 7.4) were supplied by Merck, South Africa.

The following reagent used for the proteolytic peptide profiles (RP-HPLC) was supplied by Merck, South Africa. The acid solution (0.1 Normal [N] HCl) was used to precipitate samples prior to centrifugation.

Acetonitrile (CH_3CN), gradient grade for liquid chromatography, was supplied by Merck, South Africa.

The 68% Alizarol solution was supplied by Selectech, South Africa.

Proteolytic enzymes

Plasmin (EC 3.4.21.7) was supplied by Sigma-Aldrich, South Africa (10602370001). The plasmin (5 U/mL) originated from bovine plasma.

Microbial protease Type VIII (11 U/mg) (*Bacillus licheniformis;* EC 3.4.21.62) was in powder form and was supplied by Sigma-Aldrich, South Africa (P5380).

Self-cultivated enzymes

The self-cultivation of enzymes was described in detail in Chapter 3, Section 3.3.2.

4.2.1 The preparation of various batch solutions

Milk with sodium azide

Sodium azide, 0.5g, was added to the low fat UHT milk prior to sterilisation for the preparation of the milk agar plates as a preservative.

Safety note: Sodium azide was used as per instructions from the supplier (Merck, South Africa).

Tris HCl buffer

0.1M Tris HCl buffer (pH 7.4) stock solution. The Tris base was prepared by mixing 12.1g Tris with 1L distilled water (DH_2O). The buffer was prepared by mixing 100mL Tris base with 84mL 0.1N HCl.

Plasmin substrates

The stock solutions for the four plasmin substrates were prepared by mixing 5mg of the substrate with 1mL Tris HCl buffer (pH 7.4).

4.2.2 Stock solutions for the various commercial proteolytic enzymes

4.2.2.1 Plasmin

Assay purposes

A stock solution of the commercial plasmin was mixed in DH_2O to ensure ease of handling and ensure a uniform level of activity over the time of research and was stored at -20°C. A 10x plasmin dilution was prepared by adding 900µL DH_2O to 100µL plasmin (0.5 U/mL).

RP-HPLC standard curve

Milk samples (900µL) was treated with plasmin at the following increasing activity levels; 1µL, 4µL, 8µL, 10µL, 12µL, 15µL, and 20µL respectively.

4.2.2.2 *Bacillus licheniformis* protease

RP-HPLC standard curve

Bacillus protease was subdivided in 500µL aliquots of which the stock solution contained 10mg of *Bacillus* protease powder along with 10mL DH_2O and was stored at -20°C. During experimental use, the *Bacillus* protease stock solution was diluted 10x. Milk samples (900µL) was treated with *Bacillus* protease at the following increasing activity levels; 1µL, 4µL, 8µL, 10µL, 12µL, 15µL, and 20µL respectively.

4.3 Methods

4.3.1 Optimisation of the milk agar plate technique for protease detection

4.3.1.1 Concentrations of agar and UHT milk

Various concentrations of agar and low fat UHT milk (casein) were prepared for optimisation in order to establish the best concentration to be used which will present the clearest halos. The two agar concentrations evaluated were 0.5% and 1%. Different volumes [casein] of low fat UHT milk evaluated were 100mL, 150mL, 200mL and 250mL respectively. The final volume for each concentration agar and low fat UHT milk mixture was 500mL.

4.3.1.2 Preparation of the milk agar plates

A total volume of 500mL of milk agar (bacteriological) solution (2x 250mL Erlenmeyer flasks) was prepared using 250mL low fat UHT milk and 250mL DH_2O with 1% agar. The various volumes of low fat UHT milk in the agar were adjusted according to a final volume of 500mL. The first flask contained the low fat UHT milk along with 0.5g sodium azide and was heated to 55°C in a water bath. The second flask, containing the agar, was heated in a boiling water bath for 15 minutes in order to melt the agar, after which it was also placed at 55°C for 30 minutes to cool. The content of the two flasks was mixed in a 1L Schott bottle and sterilised by autoclaving at 121°C for 20 minutes in a Hirayama (Hiclave™, HV-85) autoclave (supplied by Labotec SA). After sterilisation, the milk-agar suspension was again placed in a water bath at 55°C for 20 minutes. The sterile milk-agar suspension (10mL for thin agar layer and 20mL for a thick agar layer) was aseptically poured into Petri dishes and stored at 4°C until needed.

4.3.1.3 The effectiveness of proteolytic activity using the milk agar plates

Before optimisation

Commercial proteolytic enzymes (plasmin and *Bacillus* protease) as well as the various self-cultivated enzymes (*Pseudomonas* and *Bacillus* proteases), were pipetted (5µL) respectively on top of the milk agar within the Petri dishes. The plates were then incubated upside down at 32°C for 24 hours and observed for possible halo formation.

After optimisation

To measure the time for halo formation, 5µL and 10µL of commercial plasmin and *Bacillus* protease, as well as the self-cultivated *Pseudomonas fluorescens* protease, were pipetted respectively on top of the milk agar within the Petri dishes using the optimised milk agar plate (1% agar and 100mL milk [20mL thick agar layer]) and incubated upside down at 32°C for 24 hours. At time intervals of 0 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 5 hours, 6 hours, 12 hours and 24 hours, the formation of halos were photographically monitored.

4.3.2 The screening for a superior plasmin assay

4.3.2.1 Plasmin substrates

The four plasmin chromophore based substrates that were evaluated included; Gly-Arg-*p*-nitroanilide dihydrochloride urokinase (hereafter referred to as substrate 1); N_{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (hereafter referred to as substrate 2); N-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (hereafter referred to as substrate 3) and D-Val-Leu-Lys 4-nitroanilide dihydrochloride (hereafter referred to as substrate 4).

4.3.2.2 Procedure for the plasmin assay

The diluted plasmin samples for the plasmin assay were prepared according to the Sigma-Aldrich assay protocol (Enzymatic assay of Plasmin protocol). Table 5 below indicates the specific content of each sample.

Table 5. The layout of the samples analysed with the plasmin assay.

Sample	Tris HCl buffer (µL)	Plasmin substrate (µL)	DH_2O (µL)	Plasmin dilution (µL)
Reagent Blank (x3)	500	125	50	0
Plasmin samples (x3)	500	125	0	50

The 500µL Tris HCl buffer (pH 7.4) with the various substrates (125µL) included were pre-heated to 37°C for 5 minutes, thereafter the diluted plasmin samples (50µL) were added (refer to Table 1). According to the protocol the various substrates in the presence of the various plasmin loads were incubated for 10 minutes.

Immediately after incubation, the samples were placed in ice water for 10 minutes in order to halt any enzymatic reactions. The absorbance was measured at 405 nanometers (nm) in a Cecil (CE 2021, 2000 series) spectrophotometer (supplied by Lasec SA) against the reagent blank samples (no plasmin present) in order to determine the total plasmin activity. The calculation formula used for enzymatic activity determination was supplied from the Sigma-Aldrich protocol (Enzymatic assay of Plasmin protocol).

CALCULATION FORMULA

$$\text{U/mL Enzyme} = \frac{(\Delta A\ 405nm\ Test - \Delta A\ 405nm\ Blank)(0.675)(10)}{(10.5)(0.05)}$$

Where,

0.675 = Total reaction volume (mL) of assay

10 = Dilution factor

10.5 = Micromolar extinction constant for plasmin substrates 1 to 4

0.05 = Volume (mL) of enzyme used

4.3.3 Spectrophotometric Merck protease assay

The spectrophotometric Merck protease assay kit contained the following; Fluorescein thiocarbamoyl casein (FTC) that consisted of 10mL of 0.6% FTC in 50 millimolar (mM) Tris-HCL (pH 7.3), the incubation buffer that was 10mL of 200mM Tris-HCL (pH 7.8), 20mM $CaCl_2$, 0.1% NaN_3, the assay buffer which was 120mL of 500mM Tris-HCL (pH 8.8), 0.1% NaN_3 and the protease positive control that consisted of 1mL of 1mg/mL trypsin in phosphate-buffered saline (PBS) with 10mg/mL bovine serum albumin (BSA).

The principle for this protease assay is based on the hydrolysis of the FTC in the milk sample when incubated at 37°C for 24 hours. The protease activity in the sample cleaves FTC into smaller, TCA soluble, FTC-peptides to release the yellow chromophore (Twining, 1984; Wiesner & Troll, 1982).

4.3.3.1 Assay procedure

The samples were assayed according to the protocol (Calbiochem user protocol, 2007) and incubated in a water bath at 37°C for 24 hours (refer to Table 6 for the layout of the samples).

Table 6. The layout of the spectrophotometric Merck protease assay for plasmin samples.

Sample	FTC* (µL)	Incubation buffer* (µL)	DH_2O (µL)	Diluted plasmin (µL)
Reagent Blank (x3)	25	25	50	0
Plasmin samples (x3)	25	25	0	50

*Components of the spectrophotometric Merck protease assay

The samples were incubated in a water bath at 37°C for 24 hours, after which the enzymatic reaction was terminated by the addition of 250µL 5% TCA in order to precipitate any remaining FTC. The reaction samples were then centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) at 10 000 rpm (standard rotor head) for 20 minutes in order to obtain a clear supernatant. Two hundred µL of the clear supernatant from each sample was transferred into separate Eppendorf tubes. The assay buffer (300µL) was added and carefully mixed. The absorbance was read at 492nm in a Cecil (CE 2021, 2000 series) spectrophotometer (supplied by Lasec SA) against the reagent blank sample in order to determine the total protease activity. No calculations were needed since the protocol states that the value obtained with the spectrophotometer is the activity value for the sample (activity in U/mL).

4.3.4 The evaluation of the RP-HPLC technique

Low fat UHT milk was treated with various enzyme loads (1µL, 4µL, 8µL, 10µL, 12µL, 15µL, and 20µL) of both plasmin (5 U/mL) and *Bacillus* protease (11 U/mg) and the resulting peptide profiles analysed with RP-HPLC.

The various enzyme loads were added to 900µL low fat UHT milk in each Eppendorf tube and subsequently placed into the water bath for incubation at 37°C for 6 hours (Crudden *et al.,* 2005). Refer to Table 7 for the layout of the samples. After the completion of incubation, the samples were transferred to a water bath at 90°C for 15 minutes in order to halt all enzymatic actions by heat (Denis *et al.,* 2001).

Table 7. The layout of the samples for the evaluation of the RP-HPLC technique.

Samples	Low fat UHT milk (µL)	Plasmin protease (µL)	*Bacillus* protease 10x dilution (µL)	DH_2O (µL)
Control	900	0	0	100
Plasmin 1µL	900	1	0	99
Plasmin 4µL	900	4	0	96
Plasmin 8µL	900	8	0	92
Plasmin 10µL	900	10	0	90
Plasmin 12µL	900	12	0	88
Plasmin 15µL	900	15	0	85
Plasmin 20µL	900	20	0	80
***Bacillus* protease 1µL**	900	0	1	99
***Bacillus* protease 4µL**	900	0	4	96
***Bacillus* protease 8µL**	900	0	8	92
***Bacillus* protease 10µL**	900	0	10	90
***Bacillus* protease 12µL**	900	0	12	88
***Bacillus* protease 15µL**	900	0	15	85
***Bacillus* protease 20µL**	900	0	20	80

n=3 (Triplicate)

4.3.4.1 The Alizarol test

All the above milk samples with different enzyme loads were subjected to the Alizarol test to determine if some samples will test positive with the Alizarol test and also to possibly visualise the onset of the degradation process.

Different alcohol levels are used in various Alizarol tests. In this study, 68% of Alizarol solution was used. Equal volumes (200µL) of Alizarol solution were transferred to the various milk samples (200µL). The milk and Alizarol solution was thoroughly mixed by inversion (important not to shake). The criteria for sample evaluation were specifically for flake formation and the corresponding colour formation, refer to Table 3 and Figure 6 in Chapter 2, Section 2.5.1 for the interpretation of the Alizarol test and colour chart (Robertson, 2010).

4.3.4.2 RP-HPLC analysis of peptide profiles

The various milk samples with different enzyme loads analysed with RP-HPLC as prescribed in detail previously in Chapter 3, Section 3.3.3.

4.4 Results and discussions

4.4.1 The milk agar plate technique

The various milk agar plates which contained different concentrations of low fat UHT milk are depicted in Figure 10.

Agar concentrations

Plate A in Figure 10 consisted of 0.5% agar and 250mL of UHT milk. The problem with 0.5% agar was that the resolution of halos was poorly developed. The 1% agar proved to be the best option (Plate B).

The thickness of the agar layer

Plates B and C in the Figure was the plate with 100mL UHT milk along with 1% agar. Two agar layers were poured, a thin layer (10mL) and a thick layer (20mL). The thick agar layer (Plate C) proved to be the best option since the proteolytic halos with a higher resolution were formed.

UHT volume [Casein concentration]

Both 150mL (Plate D) and 200mL (Plate E) UHT milk plates exhibited poor quality halos with low resolution.

Plate F (Plate used in previous research work [Hattingh, 2017]) showed clear halos, however, halos in Plate C had the highest resolution which was clear and distinguishable.

Finally, it is clear that the combinations (1% agar, 20mL/plate layer thickness, and 100mL UHT milk [casein]) used for Plate C was the optimised plate thus this was the best option to be used for further studies.

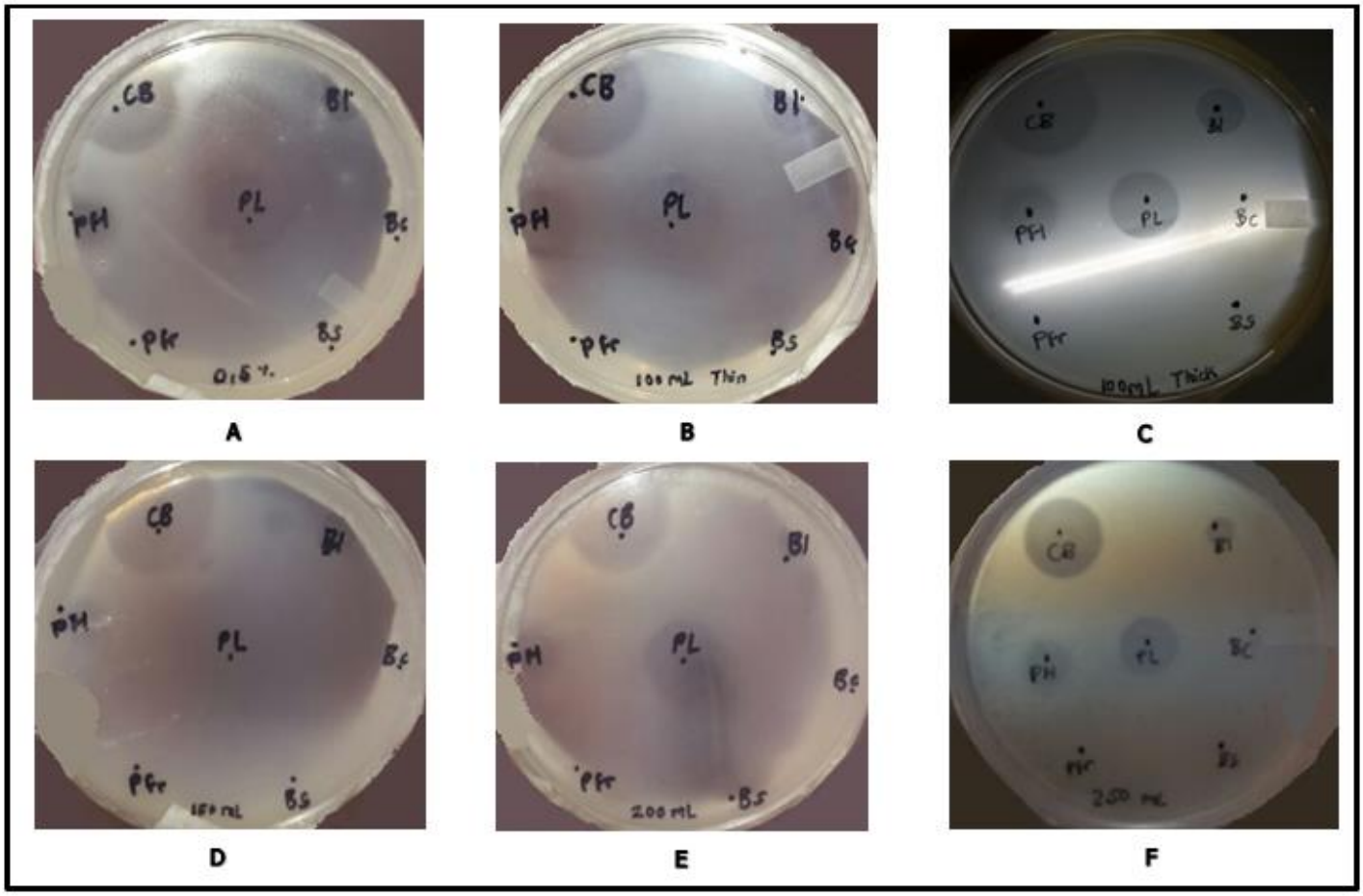

Figure 10. Milk agar plates with different concentrations of milk and agar. Plate A: 0.5% agar and 250mL UHT milk; B: 1% agar (10mL layer) and 100mL UHT milk; C: 1% agar (20mL layer) and 100mL UHT milk; D: 1% agar and 150mL UHT milk; E is 1% agar and 200mL UHT milk and F is 1% agar and 250mL UHT milk.

The halo diameters (mm) of plasmin protease, commercial *Bacillus* protease and self-cultivated *Pseudomonas fluorescens* protease that was incubated on a milk agar plate at 32°C for 24 consecutive hours at the various time intervals are depicted in Table 8. Halos became unmistakably visible and larger as a function of time, after 1 hour of incubation time the halo diameters were approximately 5mm wide. After 24 hours all three proteolytic enzymes were similar in size (15mm).

The milk agar plates in Figure 11 was monitored and photos were taken at intervals of 0 minutes (Plate A), 30 minutes (Plate B), 60 minutes (Plate C), 90 minutes (Plate D), 120 minutes (Plate E), 5 hours (Plate F), 6 hours (Plate G), 12 hours (Plate H) and finally at 24 hours (Plate I). Halos developed visibly after 1 hour of incubation time and as incubation time progressed, the halos increased in size.

Table 8. Halo diameters of proteolytic enzymes monitored on a milk agar plate for 24 hours.

Time	Halo diameter (mm)					
	Plasmin protease		***Bacillus* protease**		***Pseudomonas* protease**	
	5µL	**10µL**	**5µL**	**10µL**	**5µL**	**10µL**
0 min	0	0	0	0	0	0
30 min	0	0	0	0	0	0
60 min	5	5	5	5	5	5
90 min	5	7	7	10	5	5
120 min	5	7	7	10	7	10
5 h	7	10	10	12	7	10
6 h	10	10	10	15	7	15
12 h	13	15	15	15	10	15
24 h	15	15	15	15	15	15

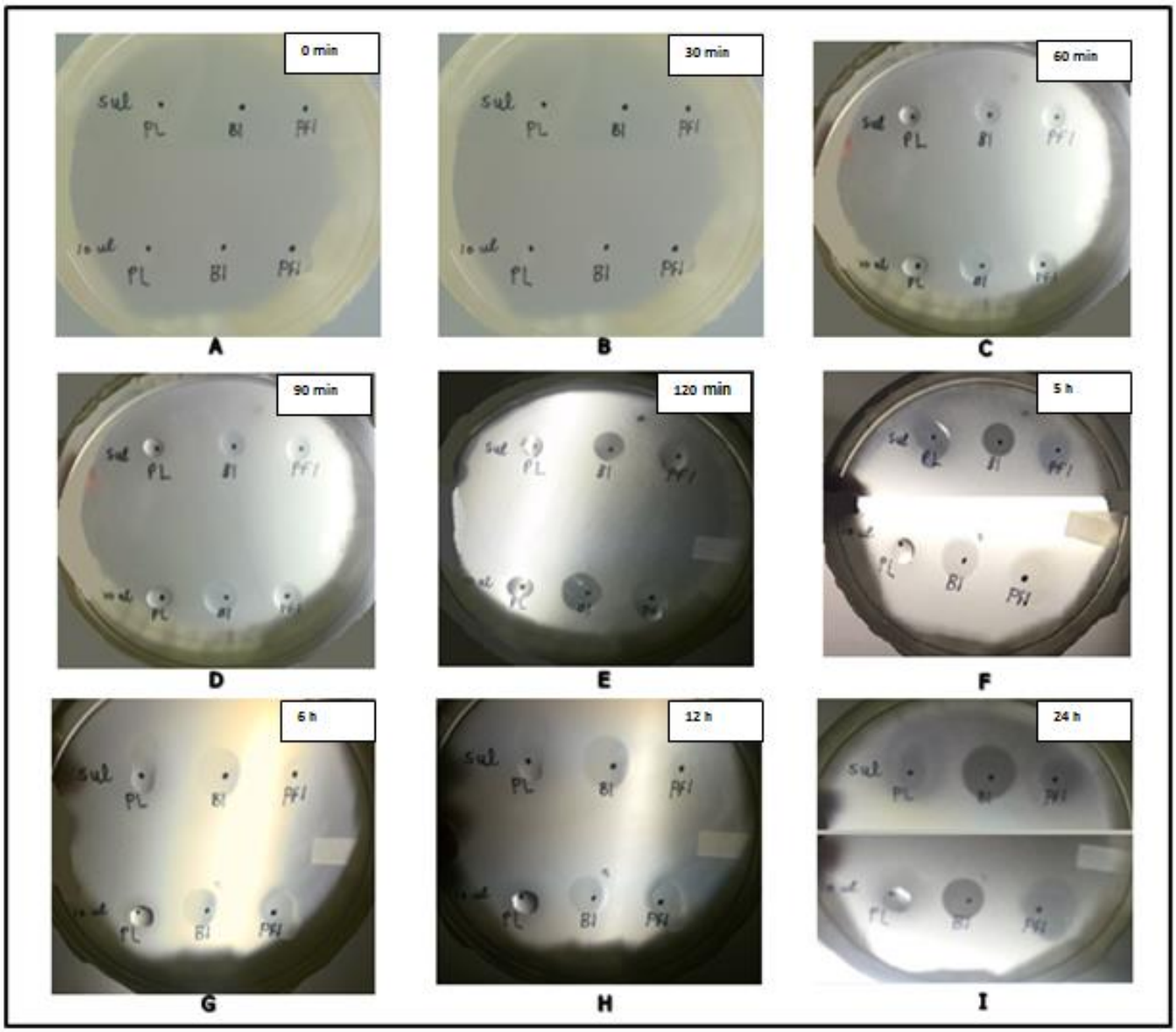

Figure 11. Milk agar plates that have been monitored for 24 hours. The dots on the plates represent the following: plasmin (PL), commercial *Bacillus licheniformis* protease (Bl) and self-cultivated *Pseudomonas fluorescens* protease (Pfl).

4.4.2 Plasmin assay and the Spectrophotometric Merck protease assay

The results from the various plasmin assay substrates are depicted in Table 9. From the results, it is evident that substrate 1 and 2 did not work. The substrates that delivered the most accurate results were substrates 3 and 4. Thus it can be concluded that substrate 3 gave the best results when comparing the final activity to the 0.5 U/mL of commercial plasmin added. From the data, it is evident that substrate 3 was best to be used within a spectrophotometric assay. Unfortunately, the cost of the substrate 3 was very high and the cost for one assay will run in the vicinity of R600/sample, making it totally uneconomical to be used on a daily basis. Fortunately, the Merck protease assay (economical) also delivered similar (Table 10) plasmin activity values and therefore was used during the rest of the study.

Table 9. The various enzyme activities as measured with the plasmin assay using the four substrates after the addition of 0.5 U/mL plasmin.

Substrates	Final enzyme activity (U/mL)	Standard deviation
Substrate 1		
Plasmin	0.365	0.009
Reagent Blank	0.339	0.006
Substrate 2		
Plasmin	0.457	0.002
Reagent Blank	0.456	0.002
Substrate 3		
Plasmin	0.593	0.008
Reagent Blank	0.348	0.000
Substrate 4		
Plasmin	0.609	0.003
Reagent Blank	0.355	0.001

n=3, Substrate 1=Gly-Arg-*p*-nitroanilide dihydrochloride urokinase; Substrate 2=N_{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride; Substrate 3=N-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt and Substrate 4 was D-Val-Leu-Lys 4-nitroanilide dihydrochloride

Table 10. Enzyme activities obtained with the spectrophotometric Merck protease assay.

Sample	Enzyme activity (U/mL)	Standard deviation
Plasmin	0.450	0.013
Reagent Blank	0.340	0.009

n=3

4.4.3 The evaluation of the RP-HPLC technique

The purpose of this study was to detect the level of protease (plasmin and *Bacillus* protease) needed to cause flocculation with the Alizarol test and the results are depicted in Figure 12.

The samples (both plasmin and *Bacillus* protease) that tested positive (flake formation) with the Alizarol test were from tube 5 (10µL) and upwards to tube 8 (20µL) as indicated by the red numbers in photo series A and B. Furthermore, synereses could be witnessed within the *Bacillus* protease samples that contained 12µL up until 20µL of protease. No major colour changes could be witnessed among the various samples when compared to the control samples since all of the samples displayed a lilac colour (Robertson, 2010). This is an indication that the pH remained similar for all of the samples (if a colour change occurs it would be indicative of pH differences).

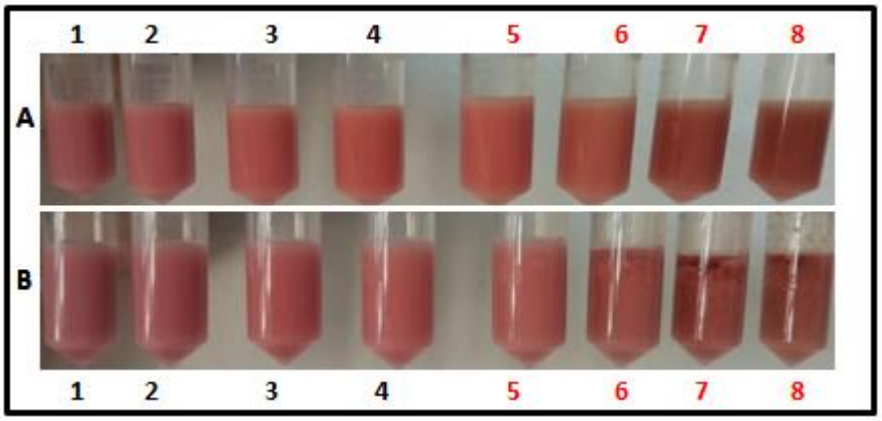

Figure 12. Alizarol results of samples treated with both plasmin (photo series A) and *Bacillus* protease (photo series B). The numbers on all the tubes indicate the following (for photo series A and photo series B); tube 1 was the control samples, tube 2 contained 1µL protease, tube 3 contained 4µL protease, tube 4 contained 8µL protease, tube 5 contained 10µL protease, tube 6 contained 12µL protease, tube 7 contained 15µL protease and tube 8 contained 20µL protease. The samples with red numbers are indicative of the samples tested positive for milk flocculation.

The RP-HPLC peptide profiles liberated from the various enzyme activity loads are depicted in Figure 13 (*Bacillus* protease) and Figure 14 (plasmin). The purpose of this study was to inspect whether the sensitivity of the RP-HPLC technique is good enough to discriminate when the milk was treated with lower and higher enzyme activity loads. Samples that were treated with 20µL of the protease were not included in the RP-HPLC chromatograms since the peaks ran out off-scale (too high).

From Figures 13 and 14 it can be concluded that the peptide profile increased as a function of enzyme load. For *Bacillus* protease treated samples, there was a characteristic peak area between an elution time of 50-60 minutes. For plasmin treated samples, the characteristic plasmin peak area is between an elution time of 45-55 minutes.

Finally, it can be concluded that the RP-HPLC technique is sensitive enough to detect enzyme activity loads from as low as 1µL when compared to the Alizarol test which only detected enzyme activity loads from 10µL and higher. Therefore, in the case of milk, the RP-HPLC technique is 10x more sensitive than the Alizarol test.

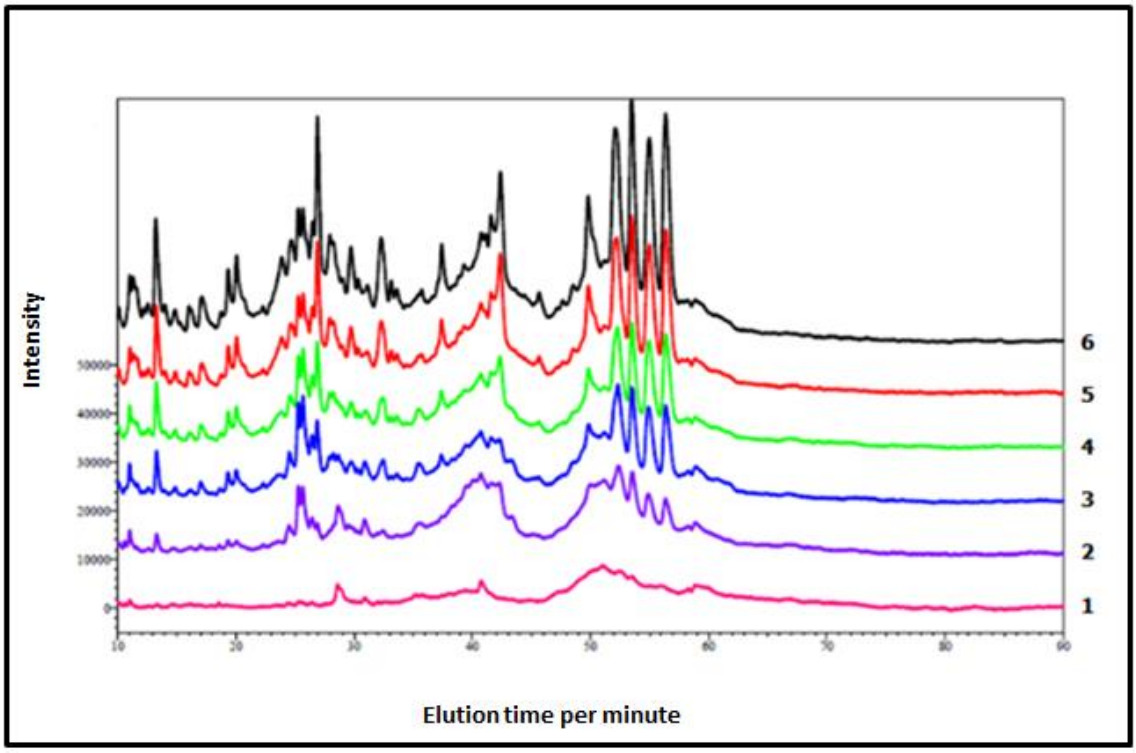

Figure 13. RP-HPLC chromatogram for the various *Bacillus* protease treated milk samples using a gradient of enzyme loads. The peptide profile numbers represent the following; 1: sample treated with 1µL *Bacillus* protease, 2: sample treated with 4µL *Bacillus* protease, 3: sample treated with 8µL *Bacillus* protease, 4: sample treated with 10µL *Bacillus* protease, 5: sample treated with 12µL *Bacillus* protease. Peptide profile number 6 was treated with 15µL *Bacillus* protease.

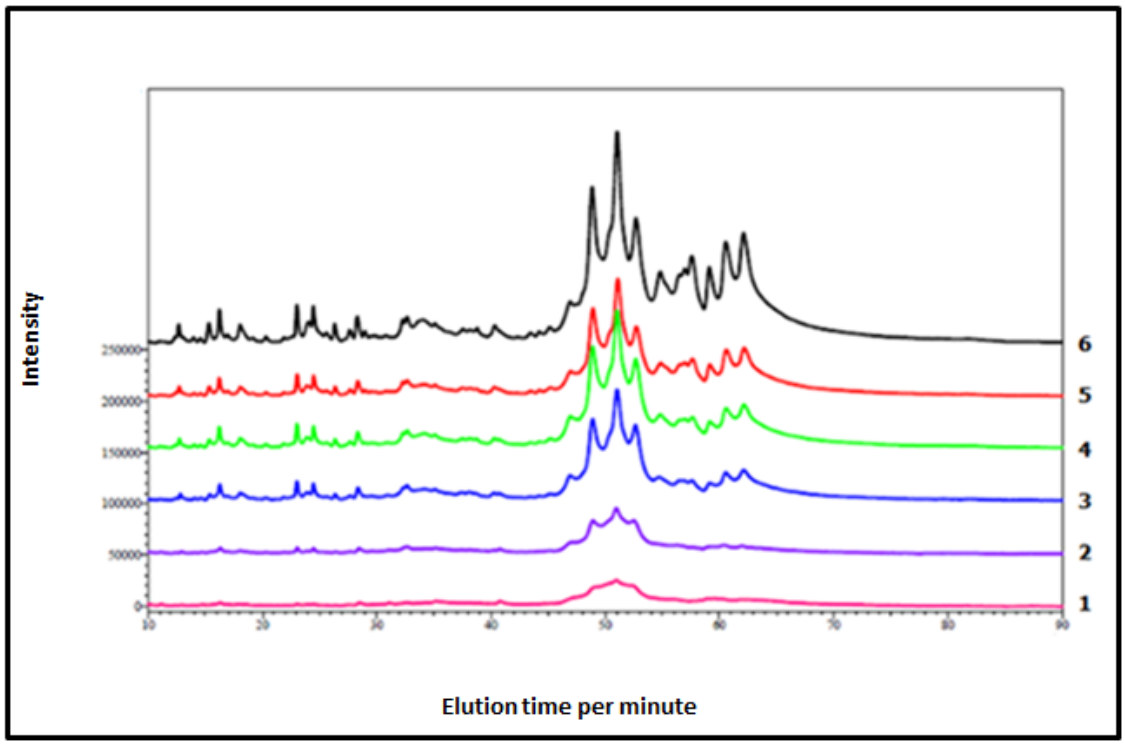

Figure 14. RP-HPLC chromatogram for the various plasmin treated milk samples using a gradient of enzyme loads. The peptide profile numbers represent the following; 1: sample treated with 1µL plasmin, 2: sample treated with 4µL plasmin, 3: sample treated with 8µL plasmin, 4: sample treated with 10µL plasmin, 5: sample treated with 12µL plasmin. Peptide profile number 6 was treated with 15µL plasmin.

The standard curve in Figure 15 can be used to determine the proteolytic activity load of unknown samples for the quantification of plasmin and microbial enzyme activity.

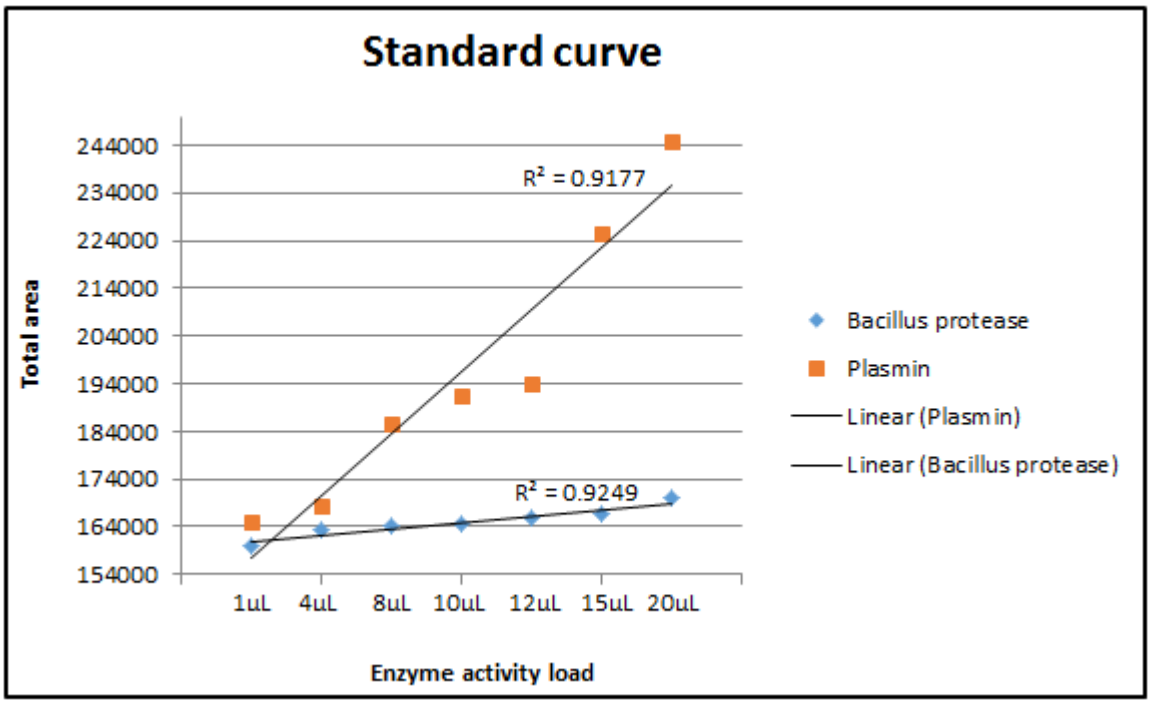

Figure 15. The standard curve (RP-HPLC) obtained from *Bacillus* protease and plasmin treated milk samples with various enzyme activity loads.

4.5 Concluding remarks

All of the objectives laid down for Chapter 4 were successfully optimised and validated.

The optimisation of the milk agar plate technique delivered desirable results since the optimised milk agar plate made it easy to visually observe the clear halos and the time at which halos became visible was established at one hour after the onset of incubation at 32°C.

The plasmin assay and the spectrophotometric Merck assay was successful in the determination of plasmin activity. The spectrophotometric Merck protease assay is considerably more cost-effective than the plasmin assay. Therefore, the spectrophotometric Merck protease assay is the preferable option to be used.

The RP-HPLC was compared with the Alizarol test to determine the proteolytic stability of milk. The RP-HPLC technique was 10x more sensitive. The data from the standard curve is also very useful since it makes it possible to determine the proteolytic damage on caseins for any milk samples (raw and UHT milk).

4.6 References

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CHAPTER 5

Investigation towards plasminogen activation

Objectives

The objectives for Chapter 5 were to investigate plasminogen activation:

- **Determine the possible activation of plasminogen by bioactive peptides from microbial proteases.**
- **The impact of milk pre-heat treatment at 60°C on plasmin activity.**
- **The activation of plasminogen to plasmin by abnormal milk (mastitis and colostrum milk).**

5.1 Introduction

Plasminogen is the inactive part of plasmin and activators are of high importance throughout the activation process (Gazi *et al*., 2014). Plasminogen activators are classified as serine proteases consisting of the responsibility to convert inactive plasminogen to active plasmin (Crudden *et al*., 2005). Plasminogen activators are resistant towards heat since processes such as pasteurisation do not affect them, however, they can slightly be inactivated during the ultra-high temperature (UHT) procedure due to very high temperatures (Datta & Deeth, 2001; Datta & Deeth, 2003; Ismail & Nielsen, 2010). According to literature, the two main activators are tissue type (t-PA) and urokinase-type (u-PA) (Aslam & Hurley, 1997; Enright *et al*., 1999; Ismail & Nielsen, 2010).

Plasminogen activation can be substantially affected by microbial proteases cultivated by thermo-tolerant bacteria throughout the storing of milk (Schroeder *et al*., 2008). The cultivation of *Pseudomonas* species (spp.) has the capability to destruct the casein micelles and cause variations in pH levels thus greatly affecting the stability of the micelles. Therefore, this result in the discharge of intact plasminogen from micelles into the whey portion of milk (Fajardo-Lira, 1999; Ismail & Nielsen, 2010). In this study, the aim was to inspect whether peptides from *Bacillus* proteases consist of the ability to activate plasminogen.

The inactivation of plasmin plays a vital role in general milk stability as well as shelf life stability (Van Asselt *et al.,* 2008). Heat processes such as pasteurisation and UHT treatment characteristically cannot lead to the inactivation of plasminogen and plasmin due to their resistance against heat. Higher plasmin levels may even be present after pasteurisation due to the activator-inhibitors being inactivated by heat and also the unfolding of plasminogen which leads to increased accessibility of the plasminogen activation sites (Gazi *et al.,* 2014). In this study, milk was exposed to a pre-heat treatment at 60°C for 1 hour in order to inspect the effect on plasminogen activation.

Plasmin plays a major role in milk during mastitis infection since this enzyme is present at elevated levels due to greater levels of somatic cells (Chen *et al.*, 2003; Forsbäck, 2010). This can be attributed due to higher levels of plasminogen activators present within the somatic cells which result in enhanced proteolysis, therefore, mastitis milk is more prone towards age gelation than normal milk (Bastian & Brown, 1996; Datta & Deeth, 2001; Fernandez *et al.*, 2008; Ismail & Nielsen, 2010; Chavan *et al.*, 2011). Mastitis milk was obtained for this study to investigate the possibility of plasminogen activation by the constituents within this milk.

Colostrum can be defined as the preliminary milk that is formed in the mammalian gland within the first 5 to 7 days directly after calving (Penchev Georgiev, 2008). The presence (level) of plasmin is ten times more superior in colostrum than in normal milk and a reduction in plasmin activity throughout the conversion from colostrum to normal milk is common. Ethanol stability is the lowest concentration of additional ethanol that results in coagulation and colostrum is regarded to consist of poor ethanol stability thus in combination with the high plasmin content, colostrum is therefore very prone to milk flocculation (McGrath *et al.,* 2016). The purpose of using colostrum (48 hours) milk was to study whether the constituents can activate plasminogen.

Keywords: Plasminogen, Plasmin, Proteases, Peptides, Mastitis, Colostrum

5.2 Materials

Enzymatically treated milk

The milk used was Pick and Pay Choice low fat UHT milk and was obtained from Pick and Pay in Langenhoven Park, Bloemfontein, Free State, South Africa. This milk was used for the preparation of the milk agar plates and *Bacillus* protease peptide hydrolysates (freeze-dried *Bacillus* peptides).

The milk used for the pre-heat treated samples were Pick and Pay Choice low fat pasteurised milk and were obtained from Pick and Pay in Langenhoven Park, Bloemfontein, Free State, South Africa.

Abnormal milk

The raw, mastitis and colostrum milk (Holstein) were obtained by three different milk producers. The raw milk was obtained by a milk producer from Dealesville, Free State, South Africa. The mastitis milk (somatic cell counts [SCC] >500 000/mL) was obtained from a milk producer near Dewetsdorp, Free State, South Africa. The colostrum milk (48 hours postpartum [obtained from the first calving period]) was obtained by a milk producer within the district of Bloemfontein, Free State, South Africa.

Indigenous plasminogen activation in raw milk

All of the samples were added to raw milk for the activation of plasminogen thereafter the hydrolysis peptide profiles of the casein were followed by reverse-phase high-performance liquid chromatography (RP-HPLC) analysis. The raw milk was obtained from Dairy Corporation, Bloemfontein, Free State, South Africa.

Reagents

Glycerol, supplied by Sigma-Aldrich, South Africa, was used for the storage of the plasminogen buffer during freezing.

Bacteriological Agar powder (1%) was supplied by Quantum Biotechnologies, Randburg, South Africa.

Sodium azide was supplied by Merck, South Africa and was added to the milk as a preservative where needed.

Phosphate buffer saline tablets were supplied by Sigma-Aldrich, South Africa.

Potassium iodate (KIO_3) was supplied by Merck, South Africa.

The spectrophotometric Merck protease assay kit (Calbiochem no. 539125) was supplied by Merck Millipore, South Africa.

Trichloroacetic acid (TCA) (5%) was supplied by Merck, South Africa.

Hydrochloric acid AR (0.1 Normal [N] HCl) was used for the precipitation of samples prior to RP-HPLC analysis and was supplied by Merck, South Africa.

Acetonitrile (CH_3CN), gradient grade for liquid chromatography, was supplied by Merck, South Africa.

The 68% Alizarol solution was supplied by Selectech, South Africa.

Proteolytic enzymes

Microbial protease Type VIII (11 U/mg) (*Bacillus licheniformis;* EC 3.4.21.62) was in powder form and was supplied by Sigma-Aldrich, South Africa (P5380).

Plasmin (EC 3.4.21.7) was supplied by Sigma-Aldrich, South Africa (10602370001). The plasmin (5 U/mL) and originated from bovine plasma.

Bovine plasminogen (0.32 U/mg) (EC 232.641.9) was supplied by Sigma-Aldrich, South Africa (P9156).

5.2.1 The preparation of various batch solutions

Milk with sodium azide:

Milk agar plates

Sodium azide (0.5g/500mL) was added to the low fat UHT milk for the milk agar plates.

Evaluation of plasminogen activation

Sodium azide (0.1g/100mL) was added to raw milk and subsequently used.

Safety note: Sodium azide was used as per instructions from the supplier (Merck, South Africa).

Phosphate buffer

Phosphate buffer stock solution was prepared by dissolving one phosphate buffer saline tablet in 200mL distilled water (DH_2O) in order to consist of a 0.1 Molar (M) phosphate buffer solution (pH 7.4).

KIO_3

The KIO_3 was prepared as a control for the purpose of plasminogen activation (Enright *et al.,* 1999). A 0.2M KIO_3 stock solution was prepared by dissolving 8.56g in 200mL DH_2O.

Proteolytic enzymes:

Bacillus licheniformis protease

Commercial *Bacillus* protease was suspended in DH_2O to ensure ease of handling (pipetting) and ensures a uniform level of activity over the time of research and was stored at -20°C. *Bacillus* protease was subdivided in aliquots of 500µL.

For experimental use, the *Bacillus* protease stock solution was diluted 10x (20µL *Bacillus* protease stock solution and 180µL DH_2O) at a final respective activity of 0.0055 U/mL (*Bacillus* protease dilution).

Plasmin

A stock solution of the commercial plasmin was prepared in DH_2O to ensure ease of handling and ensure a uniform level of activity over the time of research and was stored at -20°C. A volume of 10µL of commercial plasmin, which contained 0.05 U/mL, was used for experimental purposes.

Plasminogen buffer

Bovine plasminogen was prepared as a stock solution by dissolving 20mg of the powder in 2mL DH_2O together with 600µL glycerol. For experimental purposes, 8mL of the phosphate buffer solution was added to the above plasminogen and glycerol solution (2mL).

During the abnormal milk (raw, mastitis and colostrum milk) studies, the concentration was increased 4 fold.

5.3 Methods

5.3.1 The impact of peptides liberated by *Bacillus* proteases on plasminogen

5.3.1.1 The preparation of freeze-dried *Bacillus* protease peptide hydrolysates

The *Bacillus* protease peptides were prepared by the addition of 20mL of low fat UHT milk with 100µL of the *Bacillus* protease dilution into a Falcon tube and incubated in a water bath at 37°C for 6 hours. Subsequently, all enzymatic actions were halted by heat in a water bath at 90°C for 15 minutes (Denis *et al.,* 2001). The casein fractions within the sample were iso-electrically precipitated using 0.1N HCl (5mL) followed by a centrifugation step at 10 000 revolutions per minute (rpm) for 20 minutes to obtain a clear supernatant. The supernatant with the peptides was freeze-dried at -60°C for 24 hours in order to obtain *Bacillus* protease freeze-dried powder which was used for further experimental use.

5.3.1.2 Preparation of samples for possible plasminogen activation

The *Bacillus* peptides freeze-dried solution (3mL DH_2O added to the entire batch of freeze-dried powder, concentrated 6.67x) was added to the plasminogen buffer to a final reaction volume of 2mL and incubated for 3 hours at 37°C in order for plasminogen activation to occur (refer to Table 11 for the layout of the samples). A positive control sample that contained KIO_3 along with the plasminogen buffer was also included.

Table 11. The layout of samples that contained the freeze-dried peptides liberated through *Bacillus* proteolytic action along with plasminogen buffer and KIO_3 solution.

Sample	*Bacillus* peptides freeze-dried solution (µL)	Plasminogen buffer (µL)	0.2M KIO_3 solution (µL)
FDB 1	10	1990	0
FDB 2	50	1950	0
FDB 3	100	1900	0
KIO	0	1995	5

FDB=Freeze-Dried *Bacillus*+plasminogen buffer, KIO=plasminogen buffer+KIO_3

5.3.1.3 Evaluation of possible plasminogen activation by *Bacillus* protease liberated peptides

5.3.1.3.1 Milk agar plates

Subsequently, the samples from Table 11 were pipetted (5, 10 and 50µL) respectively on top of the milk agar within the Petri dishes using the optimised milk agar plate (Chapter 4) and incubated upside down at 32°C for 24 hours. Halo formation was monitored and when a halo developed it was an indication of plasminogen activation. The detailed procedure for the milk agar plate preparation is discussed previously in Chapter 4, Section 4.3.1.2.

5.3.1.3.2 Spectrophotometric Merck protease assay

The spectrophotometric Merck protease assay in plasminogen activated samples (halos) was also performed. The samples were prepared according to the assay protocol (Calbiochem user protocol, 2007). The detailed spectrophotometric Merck protease assay procedure is discussed previously in Chapter 4, Section 4.3.3.

5.3.1.3.3 Preparation and incubation of samples for RP-HPLC analysis

The plasminogen activated samples in Table 11 were added to raw milk with sodium azide (0.1g/100mL) to a final reaction volume of 1mL (refer to Table 12 for the layout of samples). The samples were hydrolysed in a water bath for 6 hours at 37°C. Thereafter the enzymatic actions were halted by heat in a water bath for at 90°C for 15 minutes (Denis *et al.,* 2001). The casein fractions within the samples were iso-electrically precipitated with 0.1N HCl and centrifuged. The clear supernatant of the samples was analysed with RP-HPLC (Chapter 3, Section 3.3.3).

Table 12. The layout of the plasminogen activated samples analysed by RP-HPLC.

Sample	Raw milk with sodium azide (µL)	Samples (µL)				DH_2O (µL)	*Bacillus* peptides freeze-dried solution (FDBP) (µL)
		FDB 1	FDB 2	FDB 3	KIO		
1	900	0	0	0	50	50	0
2	900	0	0	50	0	50	0
3	900	0	50	0	0	50	0
4	900	50	0	0	0	50	0
5	900	0	0	0	0	90	10
6	900	0	0	0	0	100	0

FDB=Freeze-Dried *Bacillus*+plasminogen buffer (Table 11), KIO=plasminogen buffer+KIO_3

5.3.2 The impact of milk pre-heat treatment on plasmin activity/activation

5.3.2.1 Pre-heat treatment

Low fat pasteurised milk was subjected to an additional pre-heat treatment at 60°C for 1 hour to assess the effect of pre-heat treatment on plasmin activity. Control samples were not subjected to any additional pre-heat treatment.

5.3.2.2 Exposure of pre-heat treated samples to plasmin, plasminogen, and KIO_3

The low fat pasteurised milk that was exposed to additional pre-heat treatment and the control samples were treated with plasmin, plasminogen buffer, and KIO_3 (refer to Table 13 for the layout of the sample treatment).

Table 13. The layout of the samples and control samples treated with plasmin, plasminogen, and KIO_3.

Sample	Low fat pasteurised milk (µL)	DH_2O (µL)	Plasmin (µL)	KIO_3 (µL)	Plasminogen buffer (µL)	Pre-heat treatment
C 1	900	100	0	0	0	No
C 2	900	100	0	0	0	No
C 3	900	100	0	0	0	No
CH 1	900	100	0	0	0	Yes
CH 2	900	100	0	0	0	Yes
CH 3	900	100	0	0	0	Yes
CH 4	900	100	0	0	0	No
CH 5	900	100	0	0	0	No
CHP 1	900	90	10	0	0	Yes
CHP 2	900	90	10	0	0	Yes
CHP 3	900	90	10	0	0	Yes
CHP 4	900	90	10	0	0	No
CHP 5	900	90	10	0	0	No
KIO 1	900	95	0	5	0	Yes
KIO 2	900	95	0	5	0	Yes
KIO 3	900	95	0	5	0	Yes
KIO 4	900	95	0	5	0	No
KIO 5	900	95	0	5	0	No
KIOP 1	900	0	0	5	150	Yes
KIOP 2	900	0	0	5	150	Yes
KIOP 3	900	0	0	5	150	Yes
KIOP 4	900	0	0	5	150	No
KIOP 5	900	0	0	5	150	No

C 1-3=Milk without pre-heat treatment, CH 1-5=Milk with pre-heat treatment, CHP 1-3=Milk with plasmin with pre-heat treatment, CHP 4-5=Milk with plasmin without pre-heat treatment, KIO 1-3=Milk with KIO_3 with pre-heat treatment, KIO 4-5=Milk with KIO_3 without pre-heat treatment, KIOP 1-3=Milk with plasminogen buffer and KIO_3 with pre-heat treatment, KIOP 4-5=Milk with plasminogen buffer and KIO_3 without pre-heat treatment

5.3.2.3 Evaluation of the level of plasminogen activation

The above-treated milk samples were subjected to hydrolysis in a water bath at 37°C for 6 hours. After this incubation period, all the samples were heat-treated in a water bath at 90°C for 15 minutes to halt any possible plasmin actions (Denis *et al.,* 2001). These samples were subjected to RP-HPLC.

5.3.2.4 Plasmin activity detection

5.3.2.4.1 Milk agar plates

All the samples above (Table 13) were pipetted (5µL) on top of the milk agar within the Petri dishes and incubated upside down at 32°C for 24 hours. The detailed procedure for the milk agar plate preparation was discussed previously in Chapter 4, Section 4.3.1.2.

5.3.2.4.2 Spectrophotometric Merck protease assay

All the samples in Table 13 were subjected to the spectrophotometric Merck protease assay. Each sample was prepared according to the assay protocol (Calbiochem user protocol, 2007). The detailed spectrophotometric Merck protease assay procedure was discussed previously in Chapter 4, Section 4.3.3.

5.3.3 Investigation of plasminogen activation to plasmin when exposed to abnormal milk

A variety of milk (raw, mastitis and colostrum) were incubated in the presence of plasminogen buffer. The purpose of this study was to assess whether constituents within the abnormal milk can act as plasminogen activators.

Prior to commencement, the three types of milk (raw, mastitis and colostrum) were tested with the Alizarol test to establish the onset quality. The procedure for the Alizarol test is discussed thoroughly previously in Chapter 4, Section 4.3.4.1.

Thereafter, 0.1N HCl (250µL) was added to the three samples (1mL) followed by vortex mixing in order to iso-electrically precipitate all the caseins. The samples were then centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) in an Eppendorf centrifuge tube for 20 minutes at 10 000 rpm. The clear supernatant was obtained.

This clear supernatant was used in all further experimental procedures. The various samples were prepared by the addition of plasminogen buffer to the clear supernatant to a final reaction volume of 2mL. The control samples contained KIO_3 (refer to Table 14 for the layout of the samples). The samples were then incubated for 6 hours at 37°C in order for possible plasminogen activation to occur. The samples were heat-treated in a water bath at 90°C for 15 minutes to halt any possible plasmin activity (Denis *et al.,* 2001).

Table 14. The layout of samples that contained raw, mastitis and colostrum milk along with plasminogen buffer and KIO_3 solution.

Sample	Raw milk supernatant (µL)	Mastitis milk supernatant (µL)	Colostrum milk supernatant (µL)	Plasminogen buffer (µL)	0.2M KIO_3 solution (µL)	DH_2O (µL)
R	2000	0	0	0	0	0
M	0	2000	0	0	0	0
C	0	0	2000	0	0	0
RP	100	0	0	1900	0	0
MP	0	100	0	1900	0	0
CP	0	0	100	1900	0	0
PB	0	0	0	1900	0	100
PK	0	0	0	1900	10	90

n=3 (Triplicate), Supernatants from; R=Raw milk, M=Mastitis milk, C=Colostrum, RP=Raw milk+plasminogen buffer, MP=Mastitis milk+plasminogen buffer, CP=Colostrum+plasminogen buffer, PB=plasminogen buffer, PK=plasminogen buffer+KIO_3

5.3.3.1 Milk agar plates

All the samples depicted in Table 14 were pipetted (10µL) on top of the milk agar within the Petri dishes and incubated upside down at 32°C for 24 hours. The detailed procedure for the milk agar plate preparation was discussed previously in Chapter 4, Section 4.3.1.2.

5.3.3.2 Spectrophotometric Merck protease assay

All of the samples depicted in Table 14 were prepared according to the assay protocol (Calbiochem user protocol, 2007). The detailed spectrophotometric Merck protease assay procedure was discussed previously in Chapter 4, Section 4.3.3.

5.3.3.3 RP-HPLC

The samples depicted in Table 14 were analysed with RP-HPLC. The detailed procedure for preparation and analysis by RP-HPLC was prescribed in Chapter 3, Section 3.3.3.

5.4 Results and discussions

5.4.1 The impact of peptides liberated by *Bacillus* proteases on plasminogen

The results of the milk agar plates with the various freeze-dried *Bacillus* peptide, plasminogen buffer, and KIO_3 samples are shown in Figure 16. Plate A shows the results from the sample which only contained freeze-dried *Bacillus* peptide solution and it was suspected that this sample would not form any halo since no proteolytic activity is present thus it is critical that no halo was formed for this sample.

Plate B shows the results for all the samples with various freeze-dried *Bacillus* peptide and plasminogen buffer. The halos show that plasmin activity was present for all the samples. Therefore, it is clear that plasminogen was activated to plasmin. The sharp halo edge is characteristic of plasmin action (Hattingh, 2017). These results were a major breakthrough and clearly, showed that peptides liberated by *Bacillus* protease were able to activate plasminogen to plasmin. This brought an entirely fresh perspective towards age gelation in UHT milk.

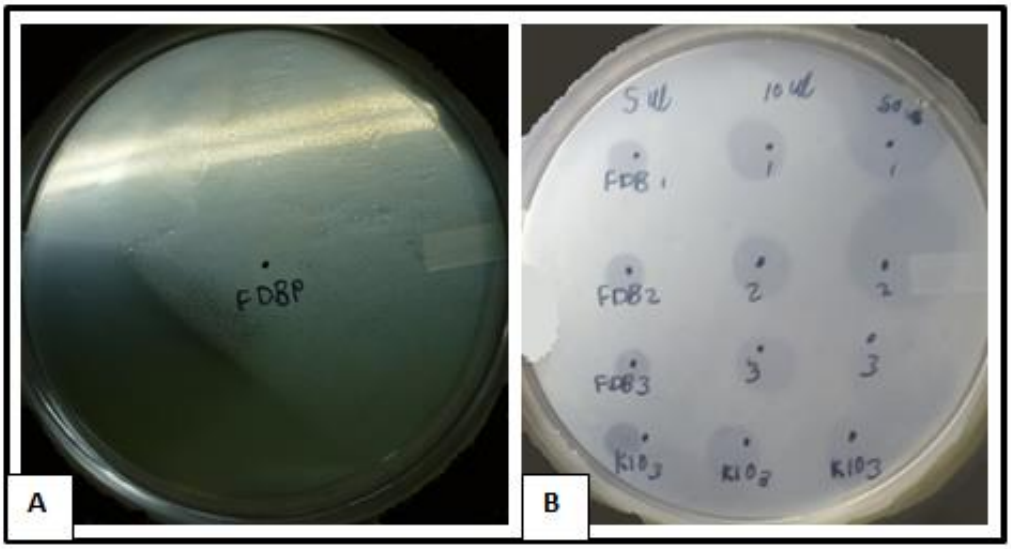

Figure 16. Milk agar plates with the various freeze-dried *Bacillus* peptide, plasminogen, and KIO_3 samples. Plate A: freeze-dried *Bacillus* peptide solution (no halo) and Plate B: all the samples with various freeze-dried *Bacillus* peptide and plasminogen/plasmin solutions. The dots on the plates represent the following: FDB 1 is 10µL freeze-dried *Bacillus* peptide solution and 1990µL plasminogen buffer; FDB 2 is 50µL freeze-dried *Bacillus* peptide solution and 1950µL plasminogen buffer; FDB 3 is 100µL freeze-dried *Bacillus* peptide solution and 1900µL plasminogen buffer and KIO_3 is 5µL KIO_3 stock solution and 1995µL plasminogen buffer.

It is evident from Figure 17 that plasminogen activation took place since the graph clearly indicates that the proteolytic activity levels were higher for samples FDB 1, FDB 2, FDB 3 and KIO than the proteolytic activity level for the control sample which was FDBP. Refer to Table 15 for proteolytic activity levels obtained with the spectrophotometric Merck protease assay.

Table 15. Spectrophotometric Merck protease assay activity values for the various freeze-dried *Bacillus* peptide, plasminogen, and KIO_3 samples.

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.094	0.003
Protease Positive Control	0.102	0.001
FDBP	0.151	0.002
FDB 1	0.410	0.013
FDB 2	0.390	0.005
FDB 3	0.363	0.004
KIO	0.374	0.003

n=3, FDB=Freeze-Dried *Bacillus*+plasminogen buffer, KIO=plasminogen buffer+KIO_3

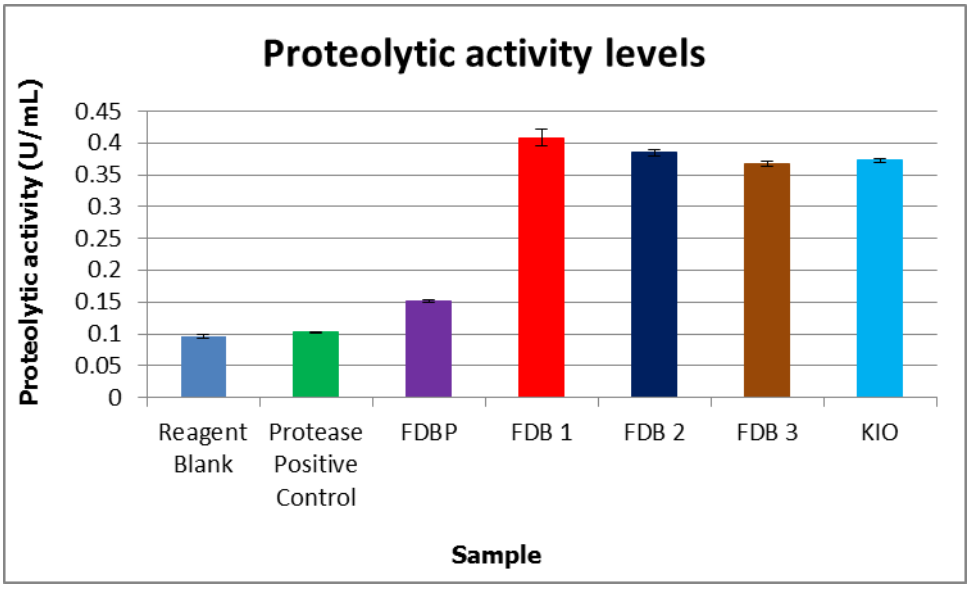

Figure 17. The proteolytic activity levels for the various freeze-dried *Bacillus* peptide, plasminogen, and KIO_3 samples analysed with the spectrophotometric Merck protease assay. The reagent blank and protease positive control was part of the protease assay kit. FDBP: Sample with freeze-dried *Bacillus* peptide solution, FDB 1: Sample with 10µL freeze-dried *Bacillus* peptide solution and 1990µL plasminogen buffer, FDB 2: Sample with 50µL freeze-dried *Bacillus* peptide solution and 1950µL plasminogen buffer, FDB 3: Sample with 100µL freeze-dried *Bacillus* peptide solution and 1900µL plasminogen buffer. KIO is a sample with 5µL KIO_3 solution and 1995µL plasminogen buffer.

The RP-HPLC chromatogram with the various freeze-dried *Bacillus* peptide and plasminogen buffer samples are depicted in Figure 18. The purpose of this was to prove whether *Bacillus* proteases can activate plasminogen to plasmin. Peptide profile number 5 contained only freeze-dried *Bacillus* peptide solution which was expected to look similar to the control (number 6) since no proteolytic activity was present within this sample (as confirmed by the milk agar plate as well as with the protease assay).

It was very interesting that the peptide profiles numbers 2, 3 and 4 showed the plasmin peak at an elution time of 60 minutes (as indicated by the black arrows) since it was evident that plasminogen activation took place and *Bacillus* protease peptides were responsible. This plasminogen activation was also confirmed with the milk agar plates and the protease assay.

Peptide profile number 1 was the sample that did not contain freeze-dried *Bacillus* peptides, however, this sample contained KIO_3 (known to act as a plasminogen activator) solution along with plasminogen buffer (Kennedy & Kelly, 1997). This peptide profile also indicated the plasmin peak at an elution time of 60 minutes.

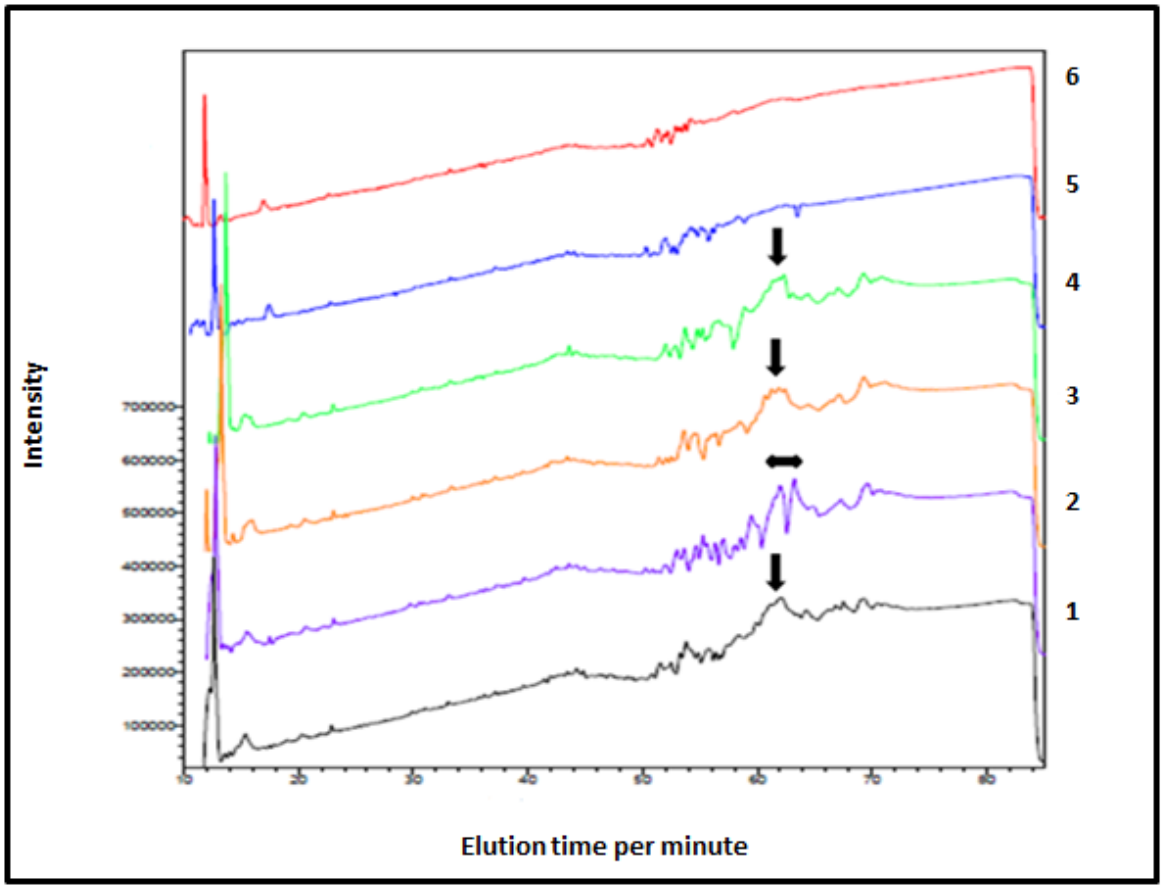

Figure 18. RP-HPLC chromatogram for the various freeze-dried *Bacillus* peptide and plasminogen samples analysed with RP-HPLC. The peptide profile numbers represent the following; 1 was a sample with 5µL KIO_3 solution and 1995µL plasminogen buffer, 2: Sample with 100µL freeze-dried *Bacillus* peptide solution and 1900µL plasminogen buffer, 3: Sample with 50µL freeze-dried *Bacillus* peptide solution and 1950µL plasminogen buffer, 4: Sample with 10µL freeze-dried *Bacillus* peptide solution and 1990µL plasminogen buffer, 5: Sample with only freeze-dried *Bacillus* peptide solution and peptide profile number 6 was UHT milk served as the control. The black arrows indicate the characteristic plasmin peak which was the most important observation.

5.4.2 The impact of milk pre-heat treatment on plasmin activity/activation

The milk agar plate with the various pre-heat treated samples is shown in Figure 19. This plate showed that halos were present for every sample, however, the halos presented a white precipitate in the middle which can be attributed to whey-casein complexes which formed throughout the pre-heat treatment and thus cannot be iso-electrically precipitated. The reason for this is unclear, however, it was only visible when pre-heat treatments were applied to the samples. It must be stated that the clear plasmin halo was still formed despite the presence of the precipitate.

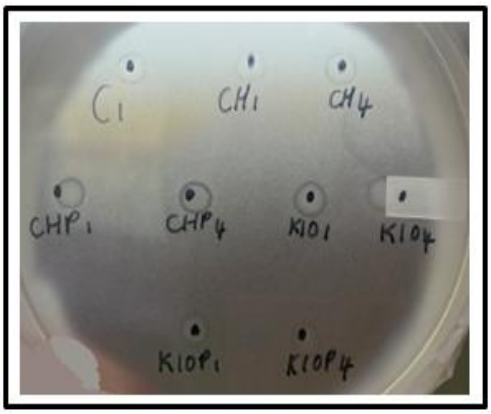

Figure 19. Milk agar plate with the various pre-heat treated samples. C 1: pasteurised milk without pre-heat treatment, CH 1: pasteurised milk with pre-heat treatment, CH 4: pasteurised milk without pre-heat treatment, CHP 1: pre-heated pasteurised milk with added plasmin, CHP 4: pasteurised milk with added plasmin without pre-heat treatment, KIO 1: pre-heated pasteurised milk with added KIO_3, KIO 4: pasteurised milk with added KIO_3 without pre-heat treatment, KIOP 1: pre-heated pasteurised milk with added KIO_3 and plasminogen and KIOP 4 was pasteurised milk with added KIO_3 and plasminogen without pre-heat treatment.

The results for the various pre-heat treated samples analysed with the spectrophotometric Merck protease assay are depicted in Table 16 and Figure 20. It is clear that the pre-heat treatment at 60°C for 1 hour had an impact on the proteolytic activity. The pre-heat treatment step enhanced the final plasmin activity since the samples exhibited higher proteolytic activity than the samples without the pre-heat treatment step.

Table 16. Proteolytic activity values for the various pre-heat treated samples analysed with the spectrophotometric Merck protease assay.

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.106	0.002
Protease Positive Control	0.095	0.002
C1	0.387	0.001
CH1	0.371	0.004
CH4	0.378	0.001
CHP1	0.459	0.003
CHP4	0.393	0.018
KIO1	0.414	0.006
KIO4	0.122	0.001
KIOP1	0.433	0.006
KIOP4	0.142	0.002

n=3, C 1=Milk without pre-heat treatment, CH 1+4=Milk with pre-heat treatment, CHP 1=Milk with plasmin with pre-heat treatment, CHP 4=Milk with plasmin without pre-heat treatment, KIO 1=Milk with KIO_3 with pre-heat treatment, KIO 4=Milk with KIO_3 without pre-heat treatment, KIOP 1=Milk with plasminogen buffer and KIO_3 with pre-heat treatment, KIOP 4=Milk with plasminogen buffer and KIO_3 without pre-heat treatment

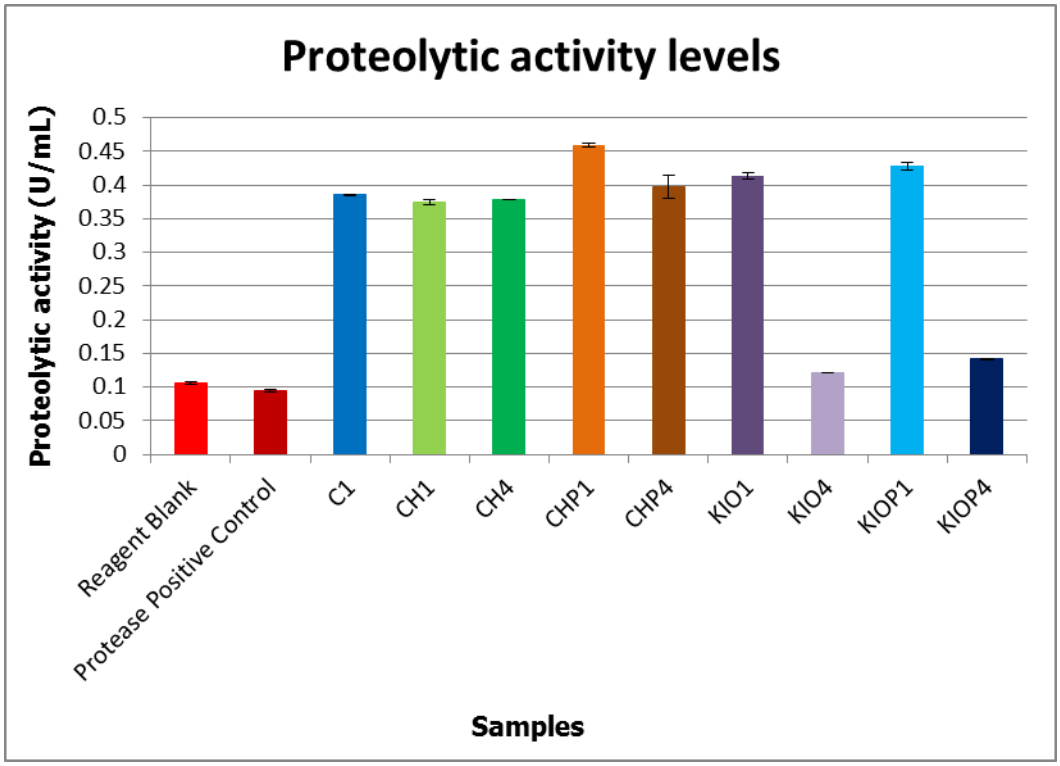

Figure 20. The proteolytic activity levels for the various pre-heat treated samples analysed with the spectrophotometric Merck protease assay. The reagent blank and protease positive control was part of the protease assay kit. C 1: pasteurised milk without pre-heat treatment, CH 1: pasteurised milk with pre-heat treatment, CH 4: pasteurised milk without pre-heat treatment, CHP 1: pre-heated pasteurised milk with added plasmin, CHP 4: pasteurised milk with added plasmin without pre-heat treatment, KIO 1: pre-heated pasteurised milk with added KIO_3, KIO 4: pasteurised milk with added KIO_3 without pre-heat treatment, KIOP 1: pre-heated pasteurised milk with added KIO_3 and plasminogen and KIOP 4 was pasteurised milk with added KIO_3 and plasminogen without pre-heat treatment.

The results for the various pre-heat treated samples analysed with RP-HPLC are shown in Figure 21. It is clear that the pre-heat treatment at 60°C for 1 hour had an impact on the proteolytic activity. The pre-heat treatment enhanced proteolytic activity since the samples that received pre-heat treatment have higher peaks than the samples without the application of pre-heat treatment. This is indicated by peptide profile number 8 which was pre-heated pasteurised milk with added plasmin and peptide profile number 7 which was pasteurised milk with added plasmin without pre-heat treatment of which the latter had smaller peaks (between an elution time of 60 and 80 minutes for both peptide profiles). This observation was the same for both the samples with KIO_3 (peptide profiles numbers 5 and 6) as well as the samples with KIO_3 and plasminogen buffer (peptide profiles numbers 3 and 4) of which the characteristic peaks are at an elution time of 75 minutes.

All the results obtained from this study were in correlation with literature that states that pre-heat treatment of milk between 50-60°C enhances plasminogen activation (Newstead *et al.*, 2006; Prado *et al.*, 2007; Ryan *et al.,* 2012). An additional pre-heat treatment step is thus not recommended since it resulted in milk that is more susceptible to proteolytic attack by plasmin.

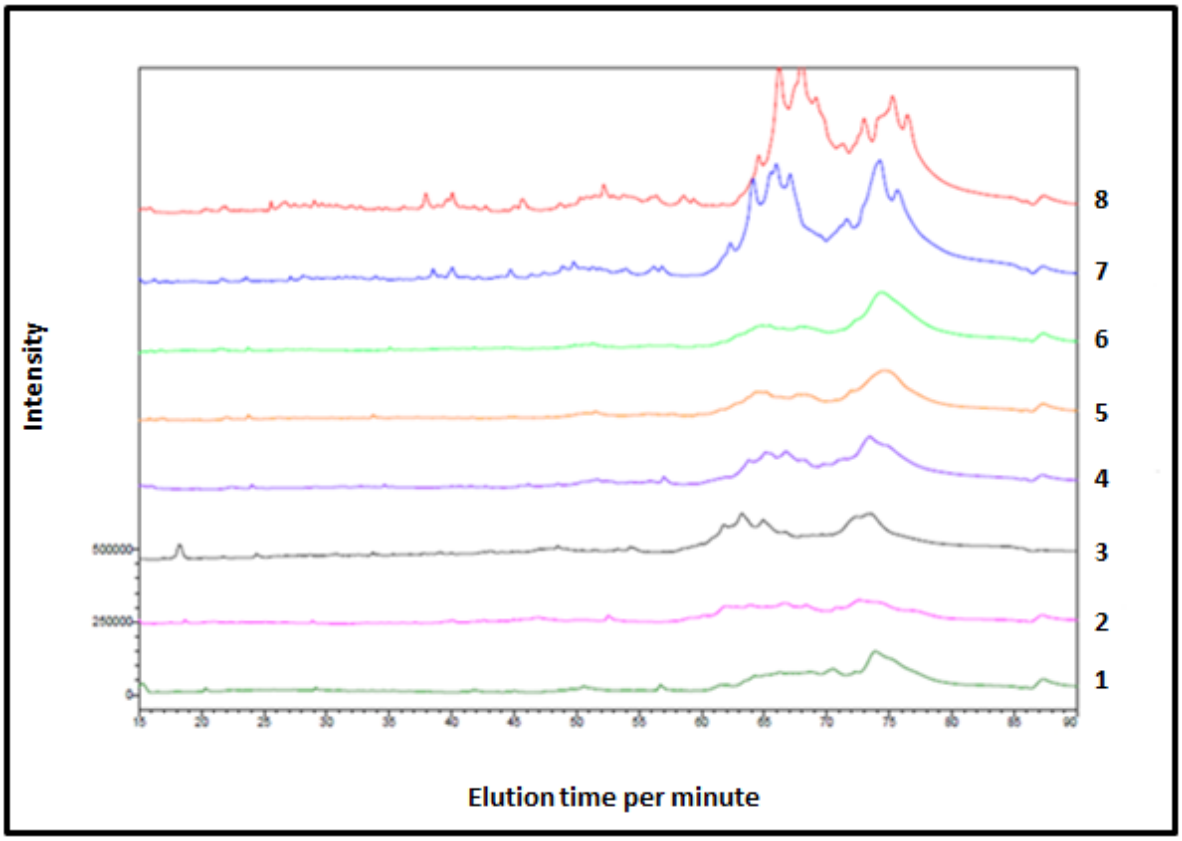

Figure 21. RP-HPLC chromatogram for the various pre-heat treated samples. The peptide profile numbers represent the following; 1: pasteurised milk without pre-heat treatment, 2: pasteurised milk with pre-heat treatment, 3 was pasteurised milk with added KIO_3 and plasminogen without pre-heat treatment, 4: pre-heated pasteurised milk with added KIO_3 and plasminogen, 5: pasteurised milk with added KIO_3 without pre-heat treatment, 6: pre-heated pasteurised milk with added KIO_3, 7: pasteurised milk with added plasmin without pre-heat treatment and peptide profile number 8 was pre-heated pasteurised milk with added plasmin.

5.4.3 Investigation of plasminogen activation to plasmin when exposed to abnormal milk

The Alizarol test results depicted in Figure 22 shows that the raw milk passed the Alizarol test since it displayed a lilac colour and no flakes were present. The mastitis and colostrum milk did not pass the Alizarol test since it exhibited an off colour, flakes were visible as well as synereses could be visually observed (Robertson, 2010).

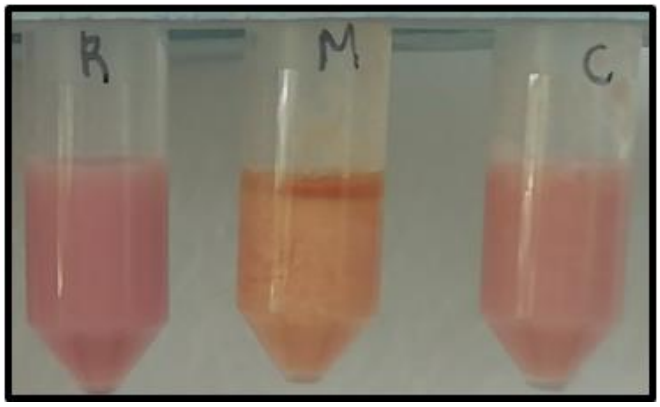

Figure 22. Outcomes of the Alizarol test samples for the raw (R), mastitis (M) and colostrum (C) milk.

The results for the raw, mastitis and colostrum milk samples evaluated with the milk agar plates for plasminogen activation are shown in Figure 23. Plate A shows the results for the control samples; plasminogen buffer alone (PB1 and PB2) and plasminogen buffer with a KIO_3 solution (PK1 and PK2). The data from this plate was important since it shows that plasminogen buffer alone does not have any activity present whereas the samples that contained plasminogen buffer along with KIO_3 solution had the presence of halos and it is known that KIO_3 acts as a plasminogen activator (Kennedy & Kelly, 1997).

Plate B shows the results from the milk samples that contained only the supernatants from raw (R1 and R2), mastitis (M1 and M2) and colostrum (C1 and C2) milk. No halos were present thus it can be concluded that there was no plasmin existent within these samples.

Plate C shows the results for all the supernatants incubated along with plasminogen buffer. The halos show that activity was present for all the samples thus it is clear that plasminogen was activated to plasmin.

These results were a major breakthrough and clearly, showed that constituents within mastitis and colostrum milk are capable to activate plasminogen to plasmin.

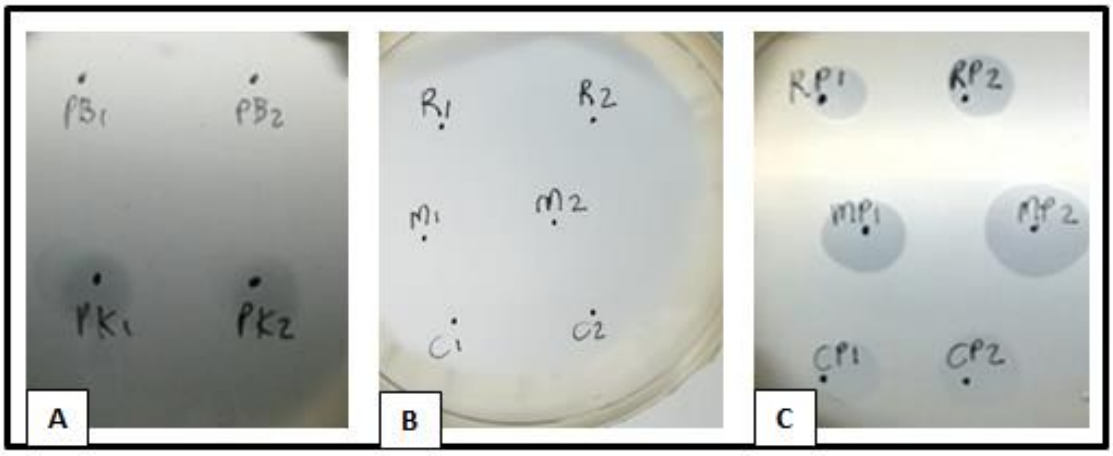

Figure 23. Milk agar plates with the various raw, mastitis and colostrum samples. Plate A: plasminogen buffer alone (PB1 and PB2) and plasminogen buffer with a KIO_3 solution (PK1 and PK2) which served as the control samples. Plate B: all the supernatants alone; raw milk supernatant (R1 and R2), mastitis supernatant (M1 and M2) and colostrum supernatant (C1 and C2). Plate C: raw supernatant with plasminogen buffer (RP1 and RP2), mastitis supernatant with plasminogen buffer (MP1 and MP2) and colostrum supernatant with plasminogen buffer (CP1 and CP2). Samples with number 1 were not incubated (controls) whereas samples with number 2 were incubated at 37°C for 6 hours.

The plasmin activity induced by raw, mastitis and colostrum milk samples is depicted in Table 17 and Figure 24. It was not surprising that the level of enzyme activity for all the samples incubated at 37°C for 6 hours (samples ending with number 2) was higher than the samples which were not subjected to incubation (samples ending with number 1).

Table 17. Results from the spectrophotometric Merck protease assay for the raw, mastitis and colostrum milk samples that were incubated in the presence of plasminogen buffer (samples with the number 1 were the controls whereas the samples with the number 2 were incubated for possible plasminogen activation).

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.098	0.003
Protease Positive Control	0.095	0.002
R1	0.086	0.000
R2	0.117	0.001
M1	0.087	0.002
M2	0.094	0.003
C1	0.116	0.004
C2	0.137	0.003
RP1	0.097	0.002
RP2	0.122	0.001
MP1	0.085	0.001
MP2	0.108	0.001
CP1	0.094	0.001
CP2	0.115	0.001
PB1	0.095	0.001
PB2	0.111	0.002
PK1	0.095	0.002
PK2	0.113	0.001

n=3, Supernatants from; R 1+2=Raw milk, M 1+2=Mastitis milk, C 1+2=Colostrum, RP 1+2=Raw milk+plasminogen buffer, MP 1+2=Mastitis milk+plasminogen buffer, CP 1+2=Colostrum+plasminogen buffer, PB 1+2=plasminogen buffer, PK 1+2=plasminogen buffer+KIO_3

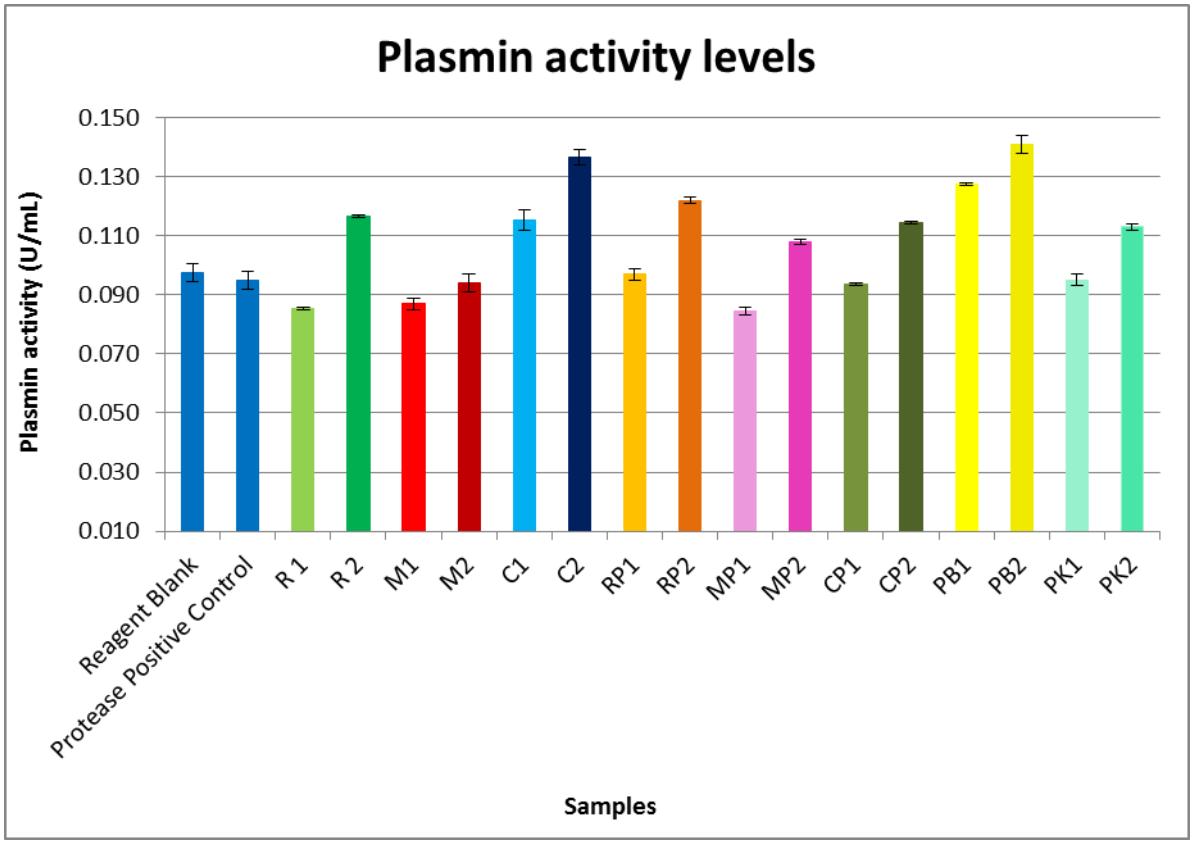

Figure 24. The plasmin activity levels of the raw, mastitis and colostrum milk samples analysed with the spectrophotometric Merck protease assay that were incubated in the presence of plasminogen buffer (samples ending with number 1 were the controls whereas the samples ending with number 2 were incubated for possible plasminogen activation). Supernatants from; R 1+2: raw milk, M 1+2: mastitis milk, C 1+2: colostrum, RP 1+2: raw milk+plasminogen buffer, MP 1+2: mastitis milk+plasminogen buffer, CP 1+2: colostrum+plasminogen buffer, PB 1+2: plasminogen buffer, PK 1+2: plasminogen buffer+KIO_3.

The RP-HPLC chromatogram with the various raw, mastitis and colostrum samples that contained plasminogen buffer is shown in Figure 25. It is clear that raw, mastitis and colostrum milk induced plasminogen activation since the characteristic plasmin peak can be observed at an elution time around 60 minutes.

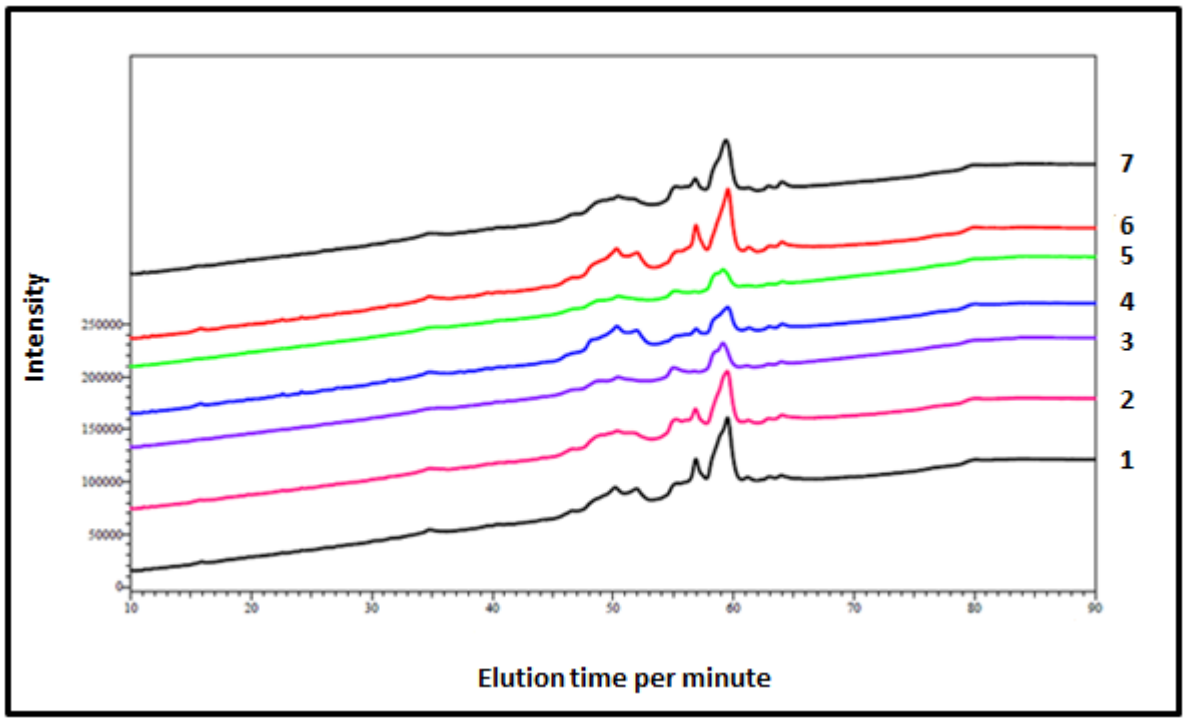

Figure 25. RP-HPLC chromatogram for the various raw, mastitis and colostrum milk samples. The peptide profile numbers represent the following; 1 was the positive control which was plasminogen buffer along with the KIO_3 solution, 2: raw milk supernatant with plasminogen buffer, 3: raw milk supernatant, 4: mastitis supernatant with plasminogen buffer, 5: mastitis supernatant, 6: colostrum supernatant with plasminogen buffer and peptide profile number 7 was colostrum supernatant.

5.5 Concluding remarks

Plasminogen activation is regarded as a complex process since in Chapter 5 it was proved that there are many other potential constituents that can act as plasminogen activators. This warrants further investigation.

The freeze-dried peptides liberated by *Bacillus* protease, surprisingly activated plasminogen to plasmin as shown by the milk agar plate technique, spectrophotometric Merck protease assay, and RP-HPLC.

Plasmin is also highly affected by heat treatments since pre-heat treatment enhanced plasminogen activation. It can thus be concluded that the additional pre-heat treatment step results in milk that is more susceptible to proteolytic attack and is therefore not recommended.

Constituents within the raw, mastitis and colostrum milk were also able to activate plasminogen to plasmin as shown with the milk agar plate technique, spectrophotometric Merck protease assay, and RP-HPLC.

Finally, it can be concluded that the above-mentioned brought a totally new angle towards plasminogen activation (age gelation) in milk since it was proven that peptides liberated by *Bacillus* protease and constituents within mastitis and colostrum milk can act as plasminogen activators.

5.6 References

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CHAPTER 6

The investigation of the levels of proteases present within milk available at a South African outlet level

Objectives

The objective of Chapter 6 was to evaluate the presence and levels of proteases in milk that is available at the outlet level in South Africa. From previous studies, it is clear that proteases have a negative impact on milk quality and stability. Various techniques were developed to evaluate the presence of proteases in South Africa milk and to quantify and classify it as being plasmin or from a bacterial source.

6.1 Introduction

From previous research work covered in this study, it is evident that proteases have a negative effect on milk quality and stability. Moreover, techniques (standard or newly developed) were used to detect the existence of proteases in milk which includes; the standard Alizarol test generally used in the industry, the milk agar plate technique, the commercial spectrophotometric Merck protease assay and reverse-phase high-performance liquid chromatography (RP-HPLC) peptide profiles.

Various brands and types of milk were purchased at the outlet level in order to investigate the protease load within milk that is available for purchase by South African consumers. It was decided to obtain these various milk samples during the problematic months (beginning of March and end of April 2019) when age gelation is a common phenomenon. The main goal was to obtain as many different brands as possible in South Africa and the main types of heat-processed milk which included; pasteurised, ultra-pasteurised and ultra-high temperature (UHT) (raw milk was also included). It was also important to include both full cream and low fat for all the brands and types when available.

The above milk samples were subjected to the Alizarol test, milk agar plate test, spectrophotometric Merck protease assay, and RP-HPLC in order to establish the original native protease load within all the milk samples and also to quantify and identify it as being plasmin or from a microbial source. The various milk samples were also incubated in the presence of plasminogen buffer to investigate whether the constituents within the milk samples consisted of the ability to activate plasminogen to plasmin.

Keywords: Proteases, Techniques, Commercial, Assay, Milk agar plates, RP-HPLC

6.2 Materials

Milk

The milk used for the preparation of the milk agar plates was Pick and Pay Choice low fat UHT milk and was obtained from Pick and Pay in Langenhoven Park, Bloemfontein, Free State, South Africa.

The various brands and types of milk that were collected at the outlet level for the evaluation of protease levels were obtained from Checkers in Langenhoven Park, Bloemfontein, Free State, South Africa. The various fat contents evaluated included pasteurised (low fat and full cream), ultra-pasteurised (low fat and full cream) and UHT (low fat and full cream).

The raw milk for the final incubations of samples analysed with RP-HPLC for plasminogen activation was obtained from Dairy Corporation, Bloemfontein, Free State, South Africa.

Addendum A was included at the end of this Chapter with the various codes that were used for the respective milk samples.

Reagents

The 68% Alizarol solution was supplied by Selectech, South Africa.

Glycerol, supplied by Sigma-Aldrich, South Africa, was used for the storage of the plasminogen buffer during freezing.

Phosphate buffer saline tablets were supplied by Sigma-Aldrich, South Africa.

Potassium iodate (KIO_3) was supplied by Merck, South Africa.

Bacteriological Agar powder (1%) was supplied by Quantum Biotechnologies, Randburg, South Africa.

Sodium azide was supplied by Merck, South Africa and was used for the milk as a preservative where needed.

The spectrophotometric Merck protease assay kit (Calbiochem no. 539125) was supplied by Merck Millipore, South Africa.

Trichloroacetic acid (TCA) (5%) was supplied by Merck, South Africa.

Hydrochloric acid AR (0.1 Normal [N] HCl) was used for the precipitation of samples prior to RP-HPLC analysis and was supplied by Merck, South Africa.

Acetonitrile (CH_3CN), gradient grade for liquid chromatography, was supplied by Merck, South Africa.

Proteolytic enzymes

Bovine plasminogen (0.32 U/mg) (EC 232.641.9) was supplied by Sigma-Aldrich, South Africa (P9156).

6.2.1 The preparation of various batch solutions

Milk with sodium azide

Sodium azide (0.5g) was added to 100mL low fat UHT milk for the milk agar plates and 0.1g/100mL to raw milk for the evaluation of plasminogen activation samples (RP-HPLC analysis).

Safety note: Sodium azide was used as per instructions from the supplier (Merck, South Africa).

Phosphate buffer

Phosphate buffer stock solution was prepared by dissolving one phosphate buffer saline tablet into 200mL distilled water (DH_2O) in order to consist of a 0.1 Molar (M) phosphate buffer solution (pH 7.4).

KIO_3

The KIO_3 stock solution was prepared for the purpose of plasminogen activation (Enright *et al.,* 1999). A 0.2M KIO_3 solution was prepared by dissolving 8.56g in 200mL DH_2O.

Plasminogen buffer

The bovine plasminogen was prepared by dissolving 80mg of the powder in 8mL DH_2O along with 2400µL glycerol. For experimental purposes, 32mL of the phosphate buffer solution was added to the above plasminogen and glycerol solution (8mL).

6.3 Methods

6.3.1 The Alizarol test

All the various milk that was purchased at the outlet level was tested with the Alizarol test to investigate the initial quality upon purchase. The detailed procedure for the Alizarol test is indicated in Chapter 4, Section 4.3.4.1.

6.3.2 Milk agar plate technique for the detection of proteolytic activity

The various milk samples evaluated (10µL), were pipetted respectively on top of the milk agar within the Petri dishes using the optimised milk agar plate (Chapter 4) and incubated upside down at 32°C for 24 hours. If halos (zones of hydrolysis) are formed, it will be an indicator of the presence of proteases within the milk. The detailed procedure for the milk agar plate preparation is presented in Chapter 4, Section 4.3.1.2.

6.3.3 Spectrophotometric Merck protease assay

The spectrophotometric Merck protease assay was performed according to the assay protocol (Calbiochem user protocol, 2007) on all the samples purchased at the outlet level to determine whether proteolytic activity is present. The detailed spectrophotometric Merck protease assay procedure is presented in Chapter 4, Section 4.3.3.

6.3.4 RP-HPLC analysis of peptide profiles

The various milk samples were prepared and analysed with RP-HPLC as prescribed earlier in Chapter 3, Section 3.3.3.

6.3.5 Preparation of samples for plasminogen activation

The various purchased milk samples and raw milk were incubated with plasminogen buffer. Following the incubation, the various samples were subjected to the milk agar plate test, spectrophotometric Merck protease assay and RP-HPLC to determine whether constituents within the milk can act as an activator for plasminogen. The detailed procedure is mentioned in Chapter 5, Section 5.3.3.

6.4 Results and discussions

6.4.1 The Alizarol test

The various purchased milk samples were subjected to the Alizarol test within one hour after it was obtained to test for the presence of milk flocculation (milk quality).

The results are depicted in Figure 26, Figure 27 and Figure 28. The results for the two different batches of raw milk are depicted in Figure 26. Figure 27 shows the results of the milk samples that were purchased at the beginning of March 2019 and the results of the milk samples purchased at the end of April 2019 are shown in Figure 28.

The raw milk and the various branded milk samples purchased during March 2019 regularly tested negative with the 68% Alizarol test where the majority of the samples displayed a lilac colour and had no visible flakes present which is the ideal. However, there was a sample that displayed a lilac colour but had flakes present which was the full cream ultra-pasteurised sample (Figure 27, number 8). The sample that tested positive with the Alizarol test was a pasteurised milk sample (Figure 27, number 7) which displayed a yellow colour, synereses and the presence of flakes which is a perfect example of Alizarol positive/flocculated milk. There were no Alizarol positive samples among the various milk purchased during April 2019 as seen in Figure 28 with all the milk samples displayed a lilac colour and no flakes were present (Robertson, 2010).

From these results, it is evident that most of the various branded milk purchased at outlet level tested negative with the Alizarol test which was ideal since this is the milk that is available to consumers who rely on good quality milk.

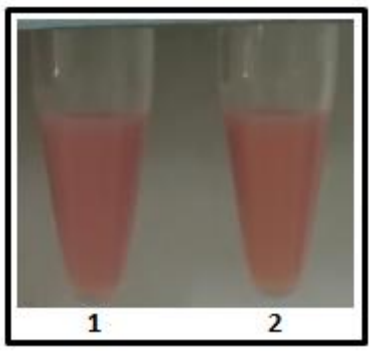

Figure 26. Outcomes of the Alizarol test for the two batches of raw milk obtained from milk producers at the beginning of March 2019 (number 2) and at the end of April 2019 (number 1).

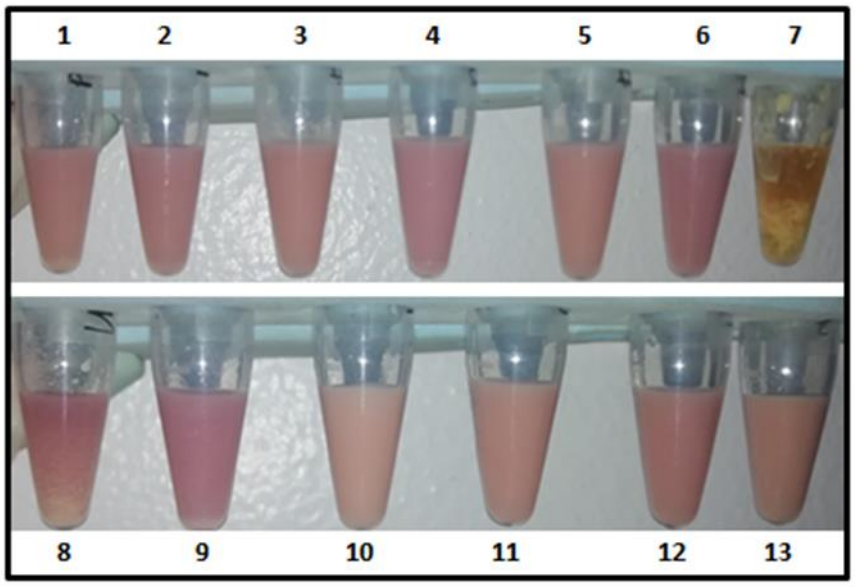

Figure 27. Outcomes of the Alizarol test for the various branded milk samples purchased at the outlet level at the beginning of March 2019. The numbers indicate the type of milk that was purchased. Number 1 represent P1V, 2: P1L, 3: P2V, 4: P2L, 5: P3V, 6: P3L, 7: P4, 8: UP1V, 9: UP1L, 10: UH1V, 11: UH1L, 12: UH2 and 13: UH3.

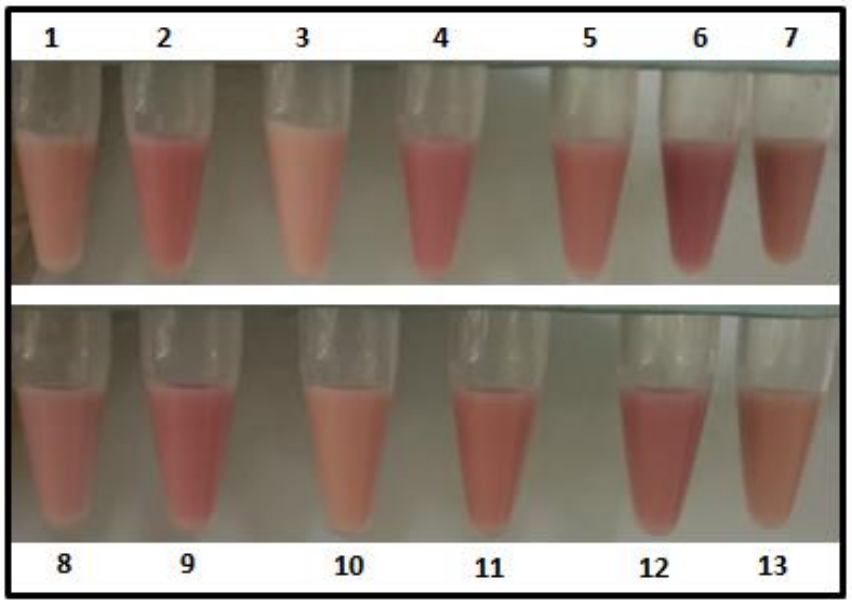

Figure 28. Outcomes of the Alizarol test for the various branded milk samples purchased at the outlet level at the end of April 2019. The numbers indicate the type of milk that was purchased. Number 1 represent P1V, 2: P1L, 3: P2V, 4: P2L, 5: P3V, 6: P3L, 7: P4, 8: UP1V, 9: UP1L, 10: UH1V, 11: UH1L, 12: UH2 and 13: UH3.

6.4.2 Milk agar plates

The various purchased milk samples and raw milk samples were also exposed to the milk agar plate test to see whether proteolytic activity was present. No halos were observed for the milk samples obtained during March and April 2019 as seen in Figures 29, 30 and 31, which makes these results desirable since this is the milk that consumers obtain at the outlet level. The samples that tested positive with the 68% Alizarol test have most likely undergone flocculation due to chemical reasons rather than proteolytic flocculation since no halos were visible.

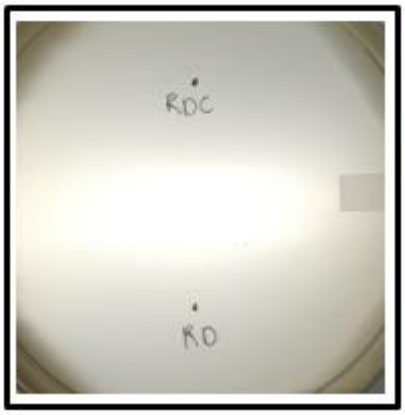

Figure 29. Results obtained with the milk agar plate test for the two batches of raw milk samples obtained from milk producers at the beginning of March 2019 (RD) and at the end of April 2019 (RDC).

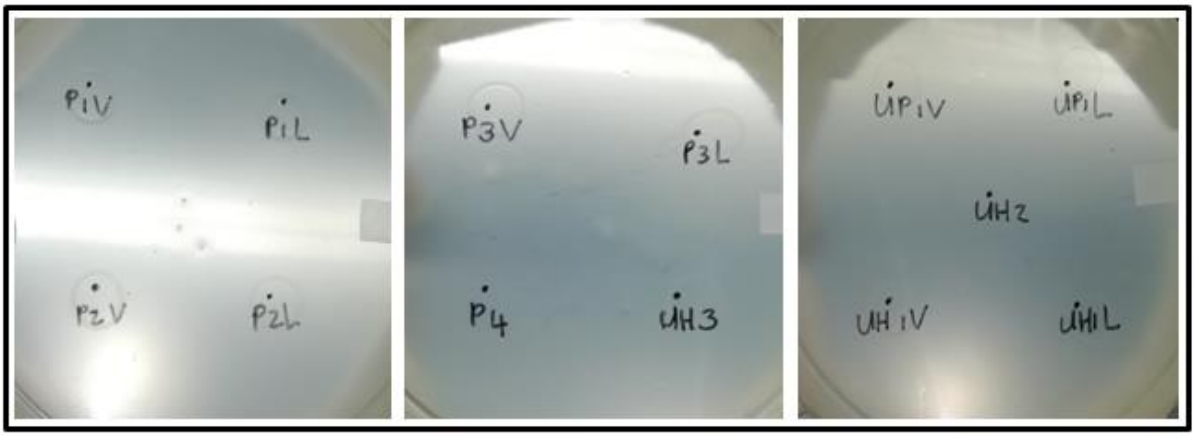

Figure 30. Results obtained with the milk agar plate test for the various branded milk samples purchased at the outlet level at the beginning of March 2019.

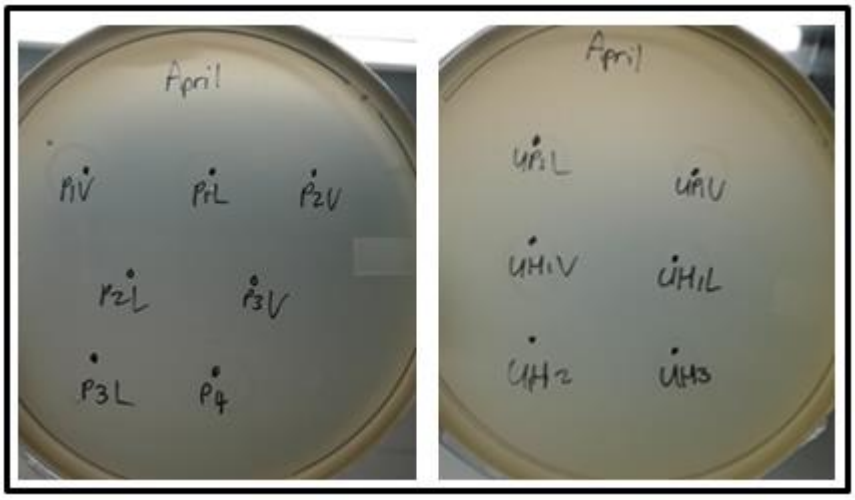

Figure 31. Results obtained with the milk agar plate test for the various branded milk samples purchased at the outlet level at the end of April 2019.

Plasminogen activation

The milk agar plate test was also used for all the various purchased milk samples that were incubated in the presence of plasminogen buffer in order to investigate whether plasmin activity was present. All of the milk agar plates evaluated (Figures 32 and 33) had the presence of halos thus it is clear that plasmin activity is present. This is in correlation with the results previously reported in Chapter 5 where it was also evident that peptides from *Bacillus* proteases and constituents within mastitis and colostrum milk had the capability to activate plasminogen to plasmin.

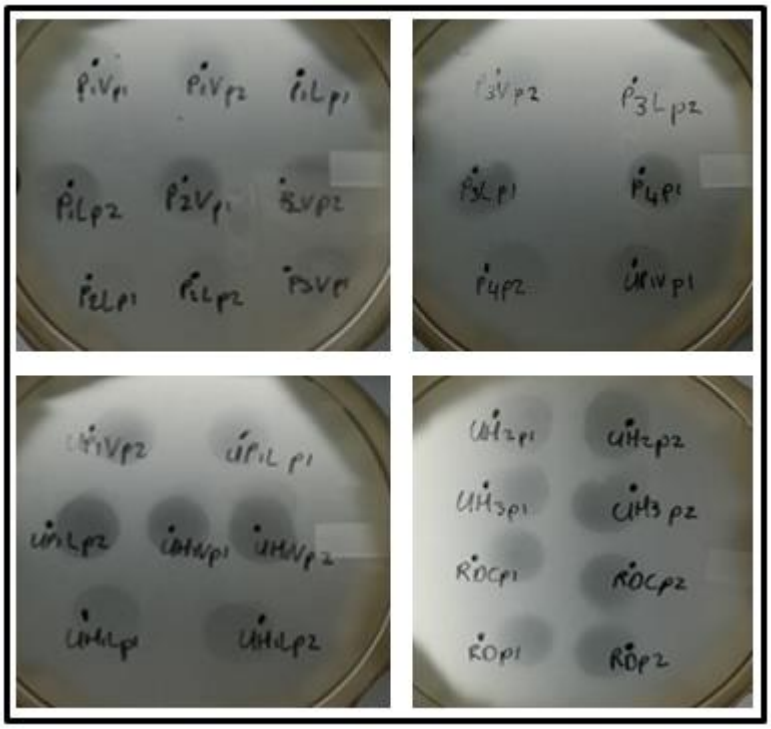

Figure 32. Results obtained with the milk agar plate test for the various branded milk samples purchased at outlet level at the beginning of March 2019 that was incubated in the presence of plasminogen buffer.

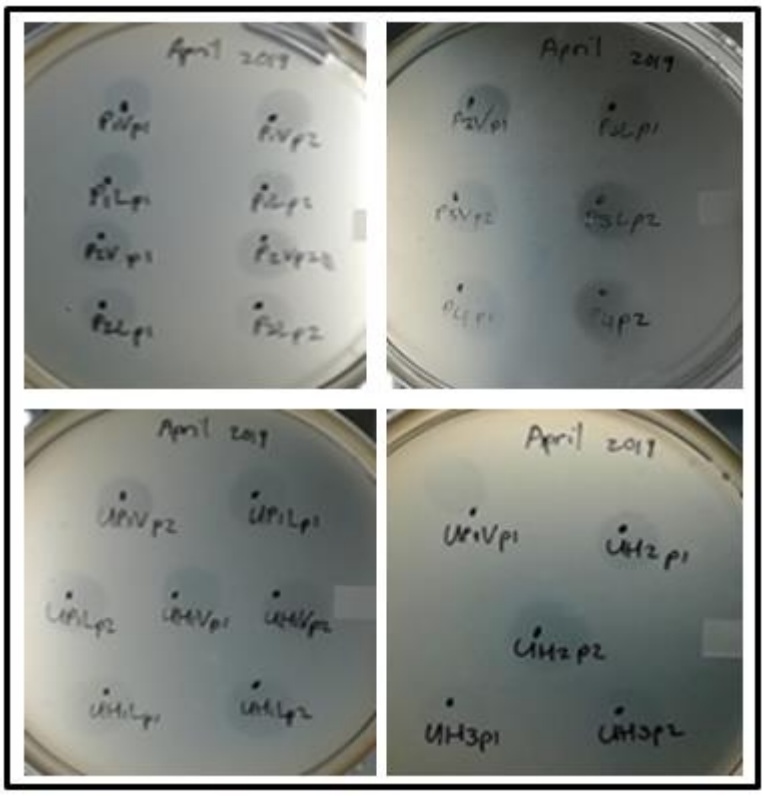

Figure 33. Results obtained with the milk agar plate test for the various branded milk samples purchased at the outlet level at the end of April 2019 that were incubated in the presence of plasminogen buffer.

6.4.3 Protease assay

The various purchased milk samples were subjected to the spectrophotometric Merck protease assay within one hour after it was obtained to investigate whether the proteolytic activity was present within these samples. The same procedure was followed for the samples purchased at the beginning of March 2019 as well as at the end of April 2019 (60 days apart). Results for the March 2019 samples are depicted in Table 18 and Figure 34 whereas results for the April 2019 samples are depicted in Table 19 and Figure 35.

Proteolytic activity levels were low for all the March 2019 samples in comparison with the reagent blank sample. There were a few exceptions with higher activity (two pasteurised milk samples and one UHT milk sample (P2L, P4, and UH1L), refer to Figure 34.

The proteolytic activity levels for all the April 2019 samples exhibited higher proteolytic activity among the various samples when compared to the reagent blank sample (Figure 35).

All the samples evaluated (March and April 2019) had low levels of proteolytic activity thus this milk is at no risk for later age gelation experienced by consumers (Champagne *et al.*, 1994; Samaržija *et al.*, 2012).

Table 18. Proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the beginning of March 2019.

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.097	0.002
Protease positive control	0.102	0.001
RD	0.096	0.001
P1V	0.095	0.003
P1L	0.100	0.001
P2V	0.096	0.001
P2L	0.121	0.002
P3V	0.106	0.003
P3L	0.102	0.002
P4	0.141	0.004
UP1V	0.107	0.002
UP1L	0.105	0.003
UH1V	0.100	0.001
UH1L	0.118	0.002
UH2	0.100	0.002
UH3	0.106	0.001

n=3

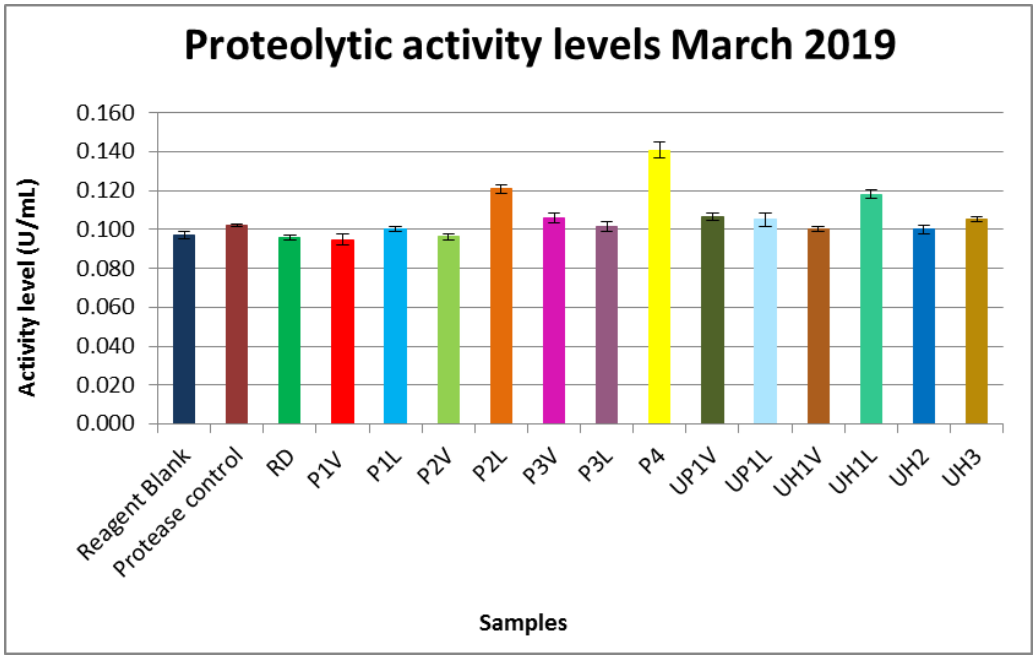

Figure 34. The proteolytic activity levels of the various branded milk samples purchased at the beginning of March 2019.

Table 19. Proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the end of April 2019.

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.097	0.002
Protease positive control	0.102	0.001
RDC	0.099	0.001
AP1V	0.129	0.001
AP1L	0.139	0.002
AP2V	0.108	0.001
AP2L	0.117	0.001
AP3V	0.102	0.001
AP3L	0.101	0.001
AP4	0.113	0.001
AUP1V	0.129	0.001
AUP1L	0.106	0.001
AUH1V	0.102	0.001
AUH1L	0.132	0.001
AUH2	0.114	0.001
AUH3	0.121	0.001

n=3

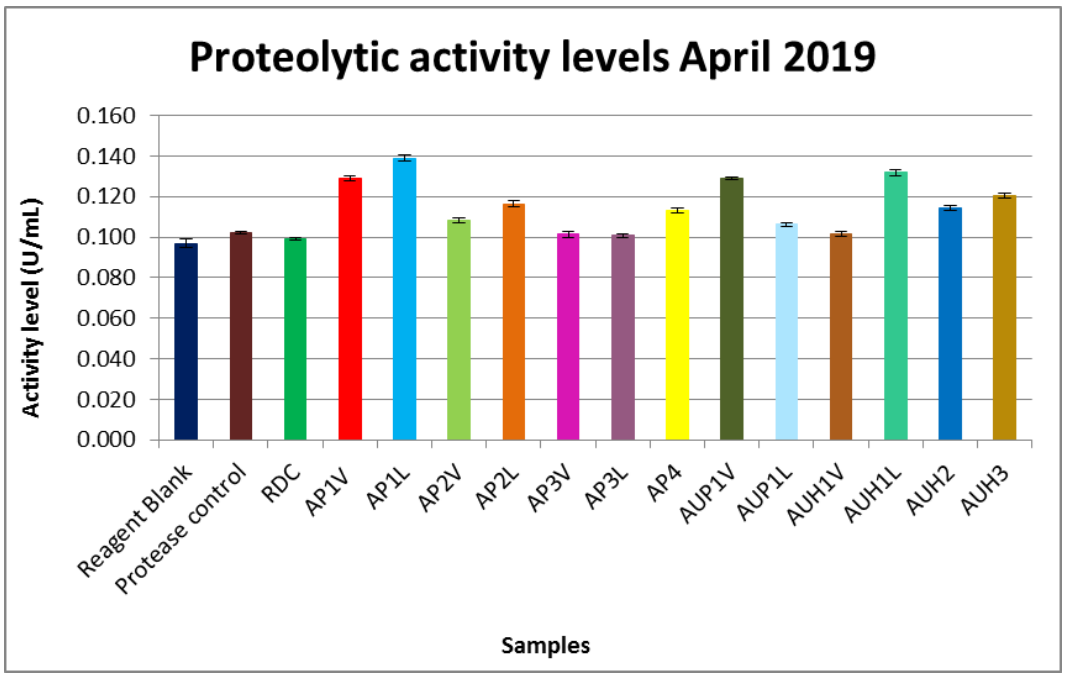

Figure 35. The proteolytic activity levels of the various branded milk samples purchased at the end of April 2019.

Plasminogen activation

The various purchased milk samples were incubated in the presence of plasminogen buffer to inspect whether the proteolytic action of the proteases existent (peptides) potentially had the ability to lead to plasminogen activation. Since this plasminogen activation was previously reported in Chapter 5, it was essential to expose all the purchased milk samples to this evaluation. The samples in Figures 36 and 37 exhibits higher proteolytic activity levels as the previous results shown in Figures 34 and 35 hence it is evident that plasminogen activation took place.

All of the purchased milk samples were incubated with plasminogen buffer. Samples ending with number x1 were included as control whereas samples ending with number x2 were incubated at 37°C for 6 hours. The samples ending with number x2 exhibited higher proteolytic activity than the samples ending with number x1 thus plasminogen was activated to plasmin.

Table 20. Proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the beginning of March 2019 that was incubated in the presence of plasminogen buffer (samples ending with number P1 were the controls whereas the samples ending with number P2 were incubated for possible plasminogen activation).

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.096	0.002
Protease control	0.102	0.001
PB 1	0.104	0.001
PB 2	0.103	0.001
RD P1	0.117	0.002
RD P2	0.165	0.001
P1V P1	0.237	0.001
P1V P2	0.320	0.001
P1L P1	0.217	0.001
P1L P2	0.262	0.002
P2V P1	0.229	0.001
P2V P2	0.317	0.001
P2L P1	0.215	0.001
P2L P2	0.313	0.001
P3V P1	0.225	0.002
P3V P2	0.295	0.002
P3L P1	0.214	0.001
P3L P2	0.331	0.001
P4 P1	0.262	0.001
P4 P2	0.383	0.001
UP1V P1	0.233	0.001
UP1V P2	0.283	0.001
UP1L P1	0.221	0.001
UP1L P2	0.337	0.002
UH1V P1	0.273	0.001
UH1V P2	0.318	0.001
UH1L P1	0.280	0.001
UH1L P2	0.332	0.002
UH2 P1	0.213	0.001
UH2 P2	0.315	0.001
UH3 P1	0.204	0.001
UH3 P2	0.253	0.001

n=3

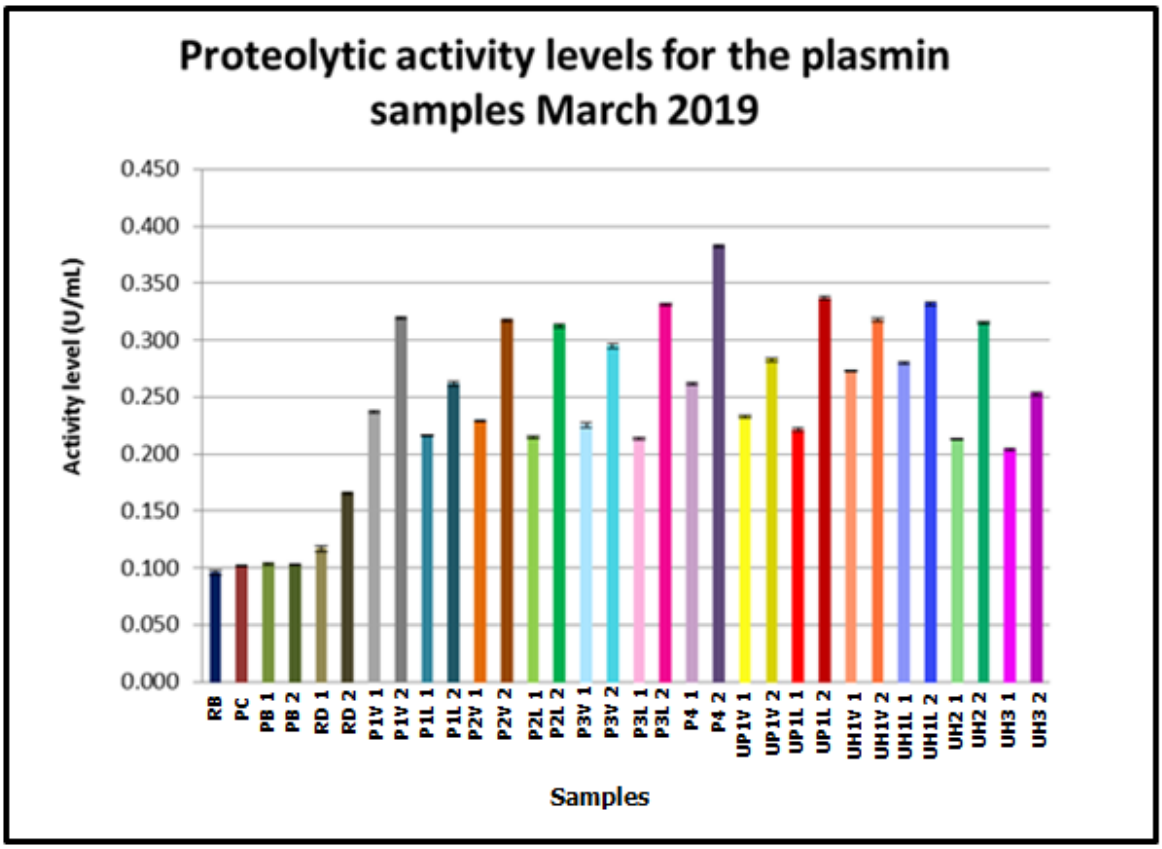

Figure 36. The proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the beginning of March 2019 that was incubated in the presence of plasminogen buffer (samples ending with number x1 were the controls whereas the samples ending with number x2 were incubated for possible plasminogen activation).

Table 21. Proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the end of April 2019 that was incubated in the presence of plasminogen buffer (samples ending with number P1 were the controls whereas the samples ending with number P2 were incubated for possible plasminogen activation).

Sample	Average (U/mL)	Standard deviation
Reagent Blank	0.096	0.002
Protease control	0.102	0.001
PB 1	0.104	0.001
PB 2	0.103	0.001
RDC P1	0.103	0.001
RDC P2	0.169	0.001
AP1V P1	0.239	0.001
AP1V P2	0.330	0.001
AP1L P1	0.227	0.001
AP1L P2	0.280	0.001
AP2V P1	0.239	0.001
AP2V P2	0.397	0.001
AP2L P1	0.235	0.001
AP2L P2	0.329	0.002
AP3V P1	0.245	0.002
AP3V P2	0.285	0.001
AP3L P1	0.314	0.001
AP3L P2	0.436	0.004
AP4 P1	0.276	0.001
AP4 P2	0.362	0.001
AUP1V P1	0.253	0.001
AUP1V P2	0.256	0.001
AUP1L P1	0.219	0.001
AUP1L P2	0.327	0.002
AUH1V P1	0.293	0.001
AUH1V P2	0.307	0.001
AUH1L P1	0.304	0.001
AUH1L P2	0.319	0.001
AUH2 P1	0.233	0.001
AUH2 P2	0.319	0.001
AUH3 P1	0.208	0.001
AUH3 P2	0.233	0.001

n=3

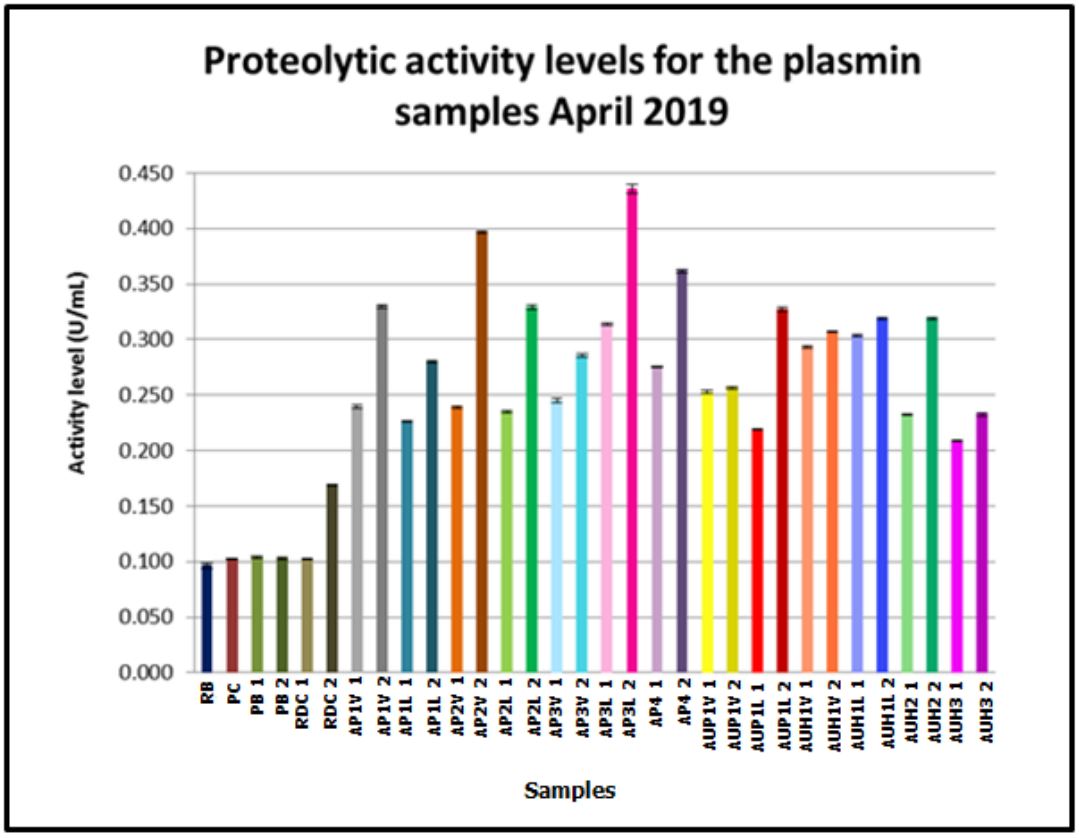

Figure 37. The proteolytic activity levels obtained with the spectrophotometric Merck protease assay for the various branded milk samples purchased at outlet level at the end of April 2019 that was incubated in the presence of plasminogen buffer (samples ending with number x1 were the controls whereas the samples ending with number x2 were incubated for possible plasminogen activation).

6.4.4 RP-HPLC

The RP-HPLC chromatogram for the various purchased milk samples analysed in order to investigate the peptide profiles since proteolytic activity was present as established with the spectrophotometric Merck protease assay are depicted in Figure 38. There were 28 samples in total for March and April 2019 and all of the samples exhibited the same peptide profiles when analysed with RP-HPLC, therefore only one representative peptide profile is shown.

The peptides liberated by these samples are characteristically that from microbial proteases since the elution time of the peaks is between 30 and 40 minutes (the blue horizontal arrow) when compared to Figures 7 and 8 in Chapter 3.

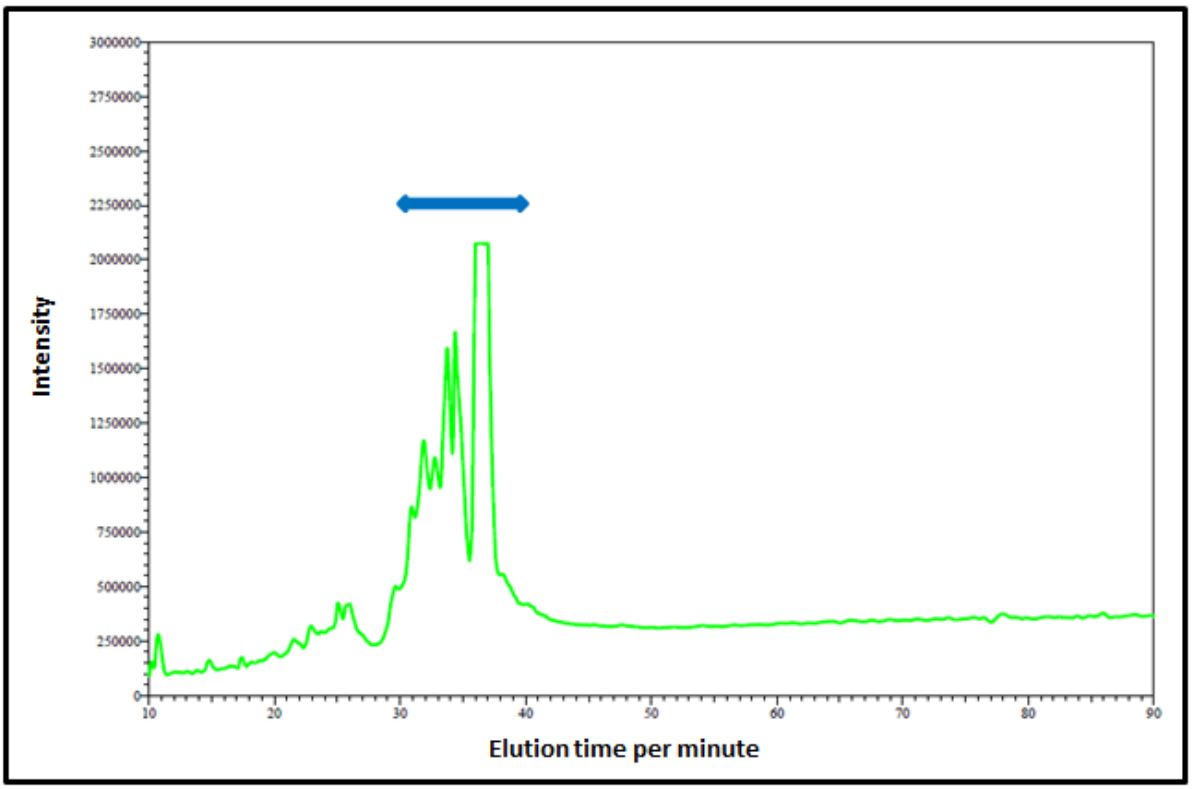

Figure 38. RP-HLPC chromatogram for the purchased milk samples analysed. The blue horizontal arrow is indicative of a characteristic peptide profile liberated by microbial proteases.

The various purchased milk samples were incubated in the presence of plasminogen buffer to inspect whether the proteolytic action of the proteases present (peptides) potentially had the ability to lead to plasminogen activation. The milk samples were analysed with RP-HPLC and the peptide profile liberated in Figure 39 is a characteristic peptide profile liberated by plasmin hydrolysis of milk casein. As in previous cases, the peptide profiles liberated were replicas and thus only one representative peptide profile is shown.

The characteristic peptide profile liberated by plasmin is shown by the purple horizontal arrow between elution times 45-55 minutes. The characteristic peptide profile liberated by plasmin was expected as it coincides with the data reported with the milk agar plates and the spectrophotometric Merck protease assay that also confirms that plasminogen activation took place.

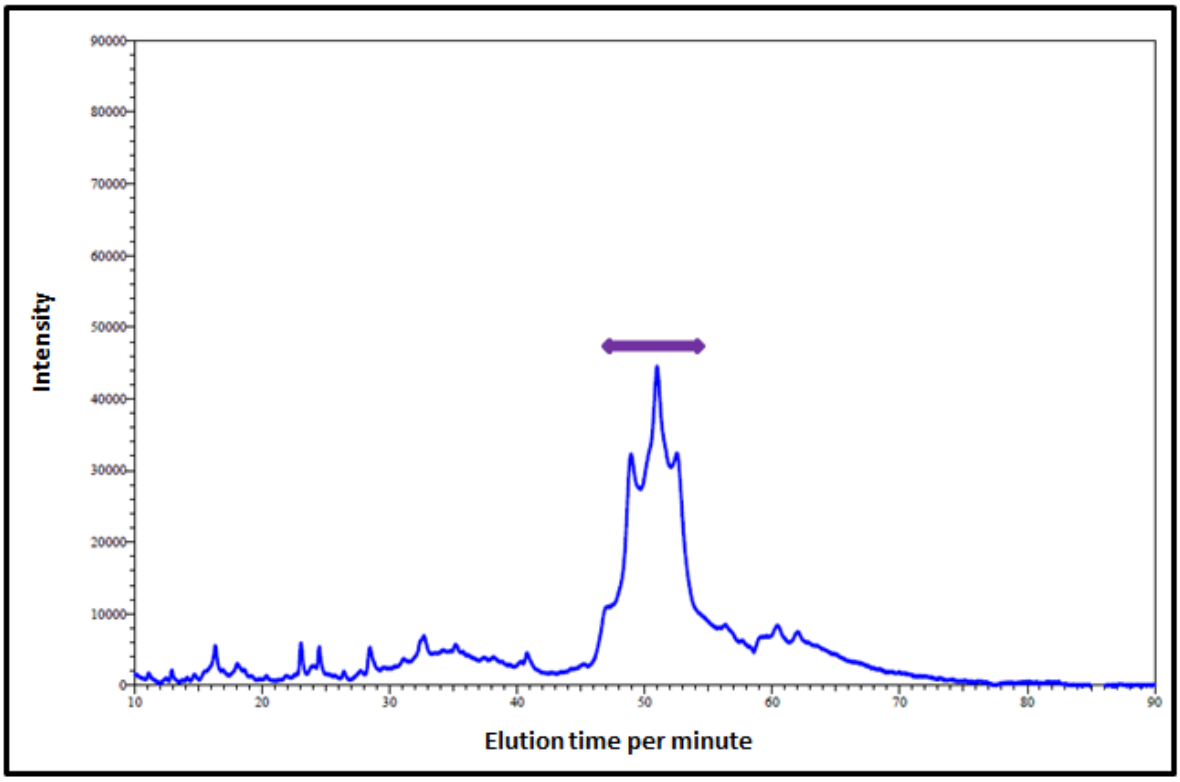

Figure 39. RP-HPLC chromatogram for the purchased samples incubated in the presence of plasminogen buffer. The purple horizontal arrow is indicative of a characteristic peptide profile liberated by plasmin.

6.5 Concluding remarks

Commercial milk in South Africa collected during the peak age gelation period (March and April) was of good quality after evaluation with the various techniques mentioned in this Chapter (Alizarol test, milk agar plate test, the Merck protease assay, and RP-HPLC).

This reflects good news for the consumer since it can be concluded that the hygiene levels in the tank were up to standard which is indicative of good cleaning in place (CIP).

Although the milk samples tested, were of good quality, it exhibited microbial proteolytic activity on the casein derived from the RP-HPLC peptide profiles, therefore, it was essential to evaluate for potential plasminogen activation that possibly can cause gelation later.

All of the milk samples had the ability to lead to plasminogen activation to plasmin with the exception of the internal controls (raw milk). This is problematic for milk stored for longer periods such as ultra-pasteurised and UHT due to the fact that plasminogen is still intact and can be activated to plasmin resulting in potential age gelation during prolonged storage which may have a negative effect on the consumer upon purchase of this milk.

6.6 References

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<u>Addendum A</u>: Description of the various milk types that were purchased at the outlet level with the codes used in the discussion.

Type of milk	Code used
Pasteurised full cream	P1V
Pasteurised low fat	P1L
Pasteurised full cream	P2V
Pasteurised low fat	P2L
Pasteurised full cream	P3V
Pasteurised low fat	P3L
Pasteurised full cream	P4
Ultra-pasteurised full cream	UP1V
Ultra-pasteurised low fat	UP1L
UHT full cream	UH1V
UHT low fat	UH1L
UHT low fat	UH2
UHT full cream	UH3
Raw milk	RDC
Raw milk	RD

A was added in front of the codes for milk purchased at the end of April 2019, P1 was added at the end of the codes for control samples during plasminogen activation, P2 was added at the end of the codes for the samples that were incubated for plasminogen activation

CHAPTER 7

General conclusions

Due to the fact that age gelation is a major problem for the dairy industry, broad, intensive research is important. It was essential to investigate all the possible factors that are involved in the occurrence of age gelation. The factors that were investigated in this study included; proteolytic enzymes, plasminogen activation, and relevant detection techniques.

Reverse-phase high-performance liquid chromatography (RP-HPLC) proteolytic peptide profiles are a very helpful tool in the detection of the presence of proteolytic enzymes within a milk sample. This technique is sensitive, repeatable and very accurate. Fingerprint peak identification for each group of proteolytic enzymes (plasmin and microbial) was successfully applied.

The Alizarol test is a successful benchmark for the evaluation of milk quality, however, when compared to RP-HPLC, it was concluded that the latter is 10x more sensitive.

The milk agar plate test is a simple, accurate and very cost-effective technique for the detection of proteolytic activity. Optimisation in terms of % agar and casein was very successful since clear characteristic halos (plasmin and microbial) developed within one hour of incubation time.

It was important to establish a trustworthy protease (plasmin) assay. Various substrate options were investigated and it was concluded that the spectrophotometric Merck protease assay was the best suitable option to be used due to simplicity and low-cost implications.

Plasminogen activation plays a vital role in the occurrence of age gelation and according to literature, there are only a few activators reported (tissue-type [t-PA], urokinase-type [u-PA] and potassium iodate [KIO_3]). This study included the investigation towards possible plasminogen activation by freeze-dried peptides derived from *Bacillus* protease and constituents within abnormal milk (mastitis and colostrum). Both cases above proved that plasminogen activation took place thus it can be concluded that the activation procedure is very complex. All the above-mentioned detection techniques were sufficient to detect plasmin activity.

Commercial milk in South Africa was evaluated for the presence of proteolytic activity and it was concluded that hygiene levels were up to standard which is finally very important to consumers. However, this study also established that possible plasminogen activation might take place during prolonged storage (ultra-pasteurised and ultra-high temperature [UHT]) which may result in the purchase of high-risk milk towards age gelation before the set expiry date.

Finally, to conclude, plasminogen activation to plasmin is an on-going process that cannot be stopped, but rather be retarded for age gelation to occur only after the expiration date within ultra-pasteurised and UHT milk. Thus the consumer will not be affected upon purchase.

CHAPTER 8

Summary

Age gelation is regarded as a process that arises in milk after prolonged storage and involves physical and chemical changes which can be characterised as; decreased fluidity, increased viscosity, and sedimentation in the form of a gel at the bottom of the milk container.

Milk is regarded as an unstable medium since the components within milk along with heat treatments affect milk stability. The main reason for the occurrence of age gelation can be attributed to the presence of heat resistant proteolytic enzymes and their behaviour on the milk caseins. The main proteolytic enzymes responsible are the indigenous milk protease (plasmin) and proteases from microbial origin.

Plasmin plays an enormous role in age gelation and the raw plasminogen can be activated by a wide range of constituents, other than the activators previously reported in the literature, as evidently proved in this study. The plasminogen activation process can be regarded as very complex and is an important factor that plays a major role in the occurrence of age gelation.

Age gelated milk is not suitable for human/animal consumption and causes enormous economic losses to the dairy industry. Pro-actively, the proteolytic detection techniques studied are thus very important to detect high-risk proteolytic milk which is therefore prone to age gelation.

Age gelation cannot be inhibited, however, the timeline can be retarded which falls beyond the expiration date and thus will not have an impact on the shelf life of ultra-high temperature (UHT) milk.