The effects of pH-stat long-term lactic acid bacterial activity prior to curd formation on the development of cheese structure in a fat free model cheese

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Abstract

Cheese ripening is an important step in most cheese production practices during which a tasteless fresh cheese is converted to a tasty and flavourful product with specific textural attributes. However, the complexity of its composition (pH, solubilisation of calcium from the colloidal casein proteins, salt concentration, acid production rate, indigenous and added enzymes, residual activity of the enzymes etc.) coupled with the length of time associated with manufacturing which in some cases can be in excess of two years has made it a complicated area of study. The influences of the composition and process contributors are confounded and it is impossible to connect the impact of one particular parameter on cheese making steps and quality attributes. pH has proven to be an important influential factor to influence the extent of effects of other parameters with significant influence on other ruling parameters in milk, curd and cheese. Proteolysis during ripening is the most important physicochemical pathway to define the quality of cheese. One of the major factors governing cheese ripening reactions is the starter bacteria.

This study has aimed to characterise the effects of starter bacteria activity on curd formation and resultant cheese textural attributes of the long fermented cheesemilk. By developing a pH-stat system, long fermentations carried out to assess the proteolytic activity of selected starter lactic acid bacteria (LAB) on a milk based medium before rennet addition. It was attempted to assess the degree of hydrolysis of cheesemilk through extended bacterial fermentation, conducted under pH-stat conditions, prior to curd formation. The effects of the bacterial activity on casein proteins during pH-stat long term (PSLT) fermentations were evaluated by assessing proteolysis index from pH4.6 soluble nitrogen as a fraction of total nitrogen (pH4.6SN/TN). The proteolysis of proteins during PSLT was further assessed by doing reverse-phase high performance liquid chromatography (RP-HPLC) on 70%Ethanol soluble (70%EtOHS) and insoluble (70%EtOHI) fractions of pH4.6 soluble fraction of the samples. The effects of PLST fermentation on formation of small-size peptides were assessed by quadrupole time-of-flight mass spectrometry (Q-ToF MS) on the 70%EtOHS fraction. The effect of PLST fermentation on 'depth of proteolysis' during

cheese ripening were assessed by analysing the quantity of free amino acid (FAA) formed in resultant cheese after 12 months storage at 4°C. The impact of PLST fermentation on gel formation attributes were assessed by doing dynamic law amplitude oscillatory rheometry (DLAOR). The consequent effects on resultant cheese texture were evaluated using texture profile analysis (TPA). The impact of PSLT on microstructure were assessed by confocal laser scanning microscopy (CLSM).

The results provided evidence for the adequacy of developed fermentation to conduct PSLT with reproducible results. High correlation between the parameters of the PSLT fermentation system were obtained. The proteolysis index measured from the PSLT fermentations with different durations showed evidences on the significance of LAB proteolytic system on cheese milk prior to curd formation. The proteolysis index for the longest fermentation prior to curd formation was 5% which was comparable to day one cheese proteolysis index, in presence of rennet, in most cheeses varieties. Peptide profiling of the 70%EtOHS and 70%EtOHI sub-fractions of pH4.6S showed significant ($p<0.05$) effects arising from PSLT fermentations. Analysis of FAA of ripened cheese also showed a significant increase ($p<0.05$) in the samples with longer PSLT (20 times increase in total free amino acids compared to non-fermented treatment) fermentations. The differences in gelation behaviour of the sample and textural attributes of cheese and microstructure of final cheese were connected to the extent of proteolytic activity of LAB during PSLT fermentations. The hardness of cheese significantly ($p<0.05$) decreased (up to ~60%) by increasing fermentation duration over the studied timescale.

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List of abbreviations and symbols

°C: Celsius degree

AA:Aminoacid

pI: Isoelectric pH

ANOVA: Analysis of variance

a_w: Water activity

CCP: Colloidal calcium phosphate

CFU: Colony forming unit

CLSM: Confocal laser scanning microscopy

CMP: Caseinomacropeptide

Da: Dalton

DLAOR: Dynamic low amplitude oscillatory rheometry

EtOH: Ethanol

FAA: Free amino acid

g: gram

G': Elastic modulus

G": Viscous modulus

GMP: Glycomacropeptide

h: Hour

HPLC: High performance liquid chromatography

LAB: Lactic acid bacteria

Lc.: Lactococcus

LEP: Lactose elimination point

LTLT: Low temperature long time

MALDI-ToF: Matrix-assisted laser desorption/ionization time of flight

min: Minute

mL: Millilitre

MM: Molecular Mass

MPC: Milk protein concentrate

NSLAB: Non-starter lactic acid bacteria

PAGE: Polyacrylamide gel electrophoresis

PEP-PTS: Phosphoenol pyruvate phosphotransferase system

PSLT: pH-stat long-term

PTA: Phosphotungestic acid

P-ß-gal: Phosphor-ß-galactosidase

Q-ToF: Quadrupole time of flight

RCT: Rennet coagulation time

RP-HPLC: Reverse phase high performance liquid chromatography

RSM: Reconstituted skim milk

s: Second

TCA: Trichloroacetic

t_g: Gelation time

η: Complex viscousity*

TPA: Texture profile analysis

UF: Ultrafiltration

ß-gal: ß-galactosidase

Chapter One Introduction

1.1 Introduction

Cheese is one of the most important dairy products and plays a significant part in our diet either as an ingredient or actual product. Over thousands of years, cheese making has been improved by trial and error to become a well-established art and eventually what is currently known as 'cheese science', which is responsible for the production of around 700 various types of cheeses.

During cheese manufacturing, milk is converted to a coagulum (by enzyme, by acid, or a combination, with the help of heat when required), which is consequently converted to cheese curd. This curd is further processed (depending on the variety) into the final cheese.

Prior to renneting, the casein proteins are colloidally dispersed in milk serum. They have a micellar aggregate form that is stabilized by the presence of κ-caseins on the surface of micellar particles. The stabilization effect mostly arises from the charged glycosylated terminal moiety on the κ-caseins with hydrophilic nature, which protrudes into the serum around the micelle at the natural pH of milk, ~6.7. Casein proteins have an isoelectric point of ~4.6. Changes in pH approaching that point cause the charge to decrease and, consequently, reduces the inter-chain repulsion, destabilizing the micelles.

Caseins contain calcium–phosphate in their structure (colloidal), which is in equilibrium with the soluble calcium and phosphate contained in the serum (in-organic). The effect of this equilibrium on the stability of milk is significant. This equilibrium is impacted by pH, and any fluctuation in the pH will result in structural changes to the casein proteins. Lowering pH from the natural pH, for example, results in the solubilisation of colloidal calcium-phosphate, and this will impact the behaviour of casein proteins during any subsequent steps in processing.

Thus, the textural attributes of gel, curd and cheese are pH-dependent. The storage modulus, G', complex viscosity, η*, and gel firmness of coagulum will increase as the pH decreases towards the isoelectric point of casein proteins. Curd processing is also affected by the changes in pH. The level of syneresis is pH-dependent to such an extent that a very finely tuned pH manipulation is required to reach the desired curd texture (i.e. in terms of rigidity, syneresis, wheying-off etc.) and, in turn, cheese quality attributes.

In the majority of cheese types, ripening is an important step during which the characteristic texture of cheese is developed. Biochemically, three important procedures take place during ripening: proteolysis of casein and its hydrolysates, glycolysis of lactose, and lipolysis of milk fat. Proteolysis is by far the most important in texture development. Although a number of factors take part in the proteolysis during ripening, the role of starter bacteria is essential. As all these parameters, especially pH, are normally confounded, and any variation in one results in changes in behaviour of the others, the significance of the starter bacteria on the texture and flavour of cheese needs to be investigated separately. Therefore the aim of this study is set to evaluate the merely effects of lactic acid bacteria activity (in absence of other proteolytic agents) on cheese milk.

In order to achieve the above goal, the following objectives were considered:

- To determine an appropriate milk-based medium that would enable the production of a range of cheese-milks with varying fermentation times while maintaining the same final composition;
- To select an appropriate bacteria strain (or strains) to conduct the experiments based on an ability to overcome unwanted parameters, and to achieve acceptable proteolytic activity;
- To develop an appropriate fermentation method for the long term fermentation of the milk-base medium at a controlled, constant pH;

- To characterise the influence of bacterial proteolytic activity on the treated milk-based medium;
- To evaluate the rennet gelation characteristics of the treated medium;
- To evaluate the textural and structural attributes of the final cheese product.

1.2 Research questions

- Does long term bacterial fermentation of the cheese milk under defined conditions (pH-stat, controlled acid production rate, controlled temperature and controlled conductivity) have a significant effect on casein proteins and their ability to form a rennet-induced gel?
- How would the selected strain (or strains) of lactic acid bacteria (LAB) change the peptide profile of the casein proteins?
- How will the curd formation of a sample obtained from pH-stat long fermentation be affected?
- Is it possible to make a cheese curd from the long fermented samples under pH-stat conditions?
- Is there any difference between the textural attributes of normal cheese and the cheese produced under long fermentation conditions?

Chapter Two Literature Review

2.1 Cheese

Cheese is a fermented dairy product produced from milk. Official recommendations recognize milk and other dairy products as one of the five major food groups in the diet (Miller, Jarvis, & McBean, 2000). Due to its balanced nutritional composition, milk and other dairy products such as cheese are consumed to obtain a desirable growth, development and maintenance for the body. The typical formulation of milk is presented in Table 2-1.

Table 2-1 Approximate composition of milk (Walstra, Wouters, & Geurts, 2006).

Component	Average Content in Milk (% w/w)	Range [a] (% w/w)	Average Content in Dry Matter (% w/w)
Water	87.1	85.3-88.7	—
Solids-not-fat	8.9	7.9–10.0	—
Fat in dry matter	31	22-38	—
Lactose	4.6	3.8–5.3	36
Fat	4	2.5–5.5	31
Protein [b]	3.3	2.3–4.4	25
Casein	2.6	1.7–3.5	20
Mineral substances	0.7	0.57–0.83	5.4
Organic acids	0.17	0.12–0.21	1.3
Miscellaneous	0.15	—	1.2

Note: Typical for milks of lowland breeds.

a These values will rarely be exceeded, e.g., in 1 to 2% of samples of separate milking of healthy individual cows, excluding colostrum and milk drawn shortly before parturition.

b Non-protein nitrogen compounds not included

2.1.1 Milk

Milk is a colloidal solution in which proteins are dispersed in an aqueous medium alongside with fat, milk sugar (lactose) and minerals (Walstra et al., 2006). Bovine milk contains two distinct groups of proteins: caseins and whey proteins. Casein proteins consist of about 80% of bovine milk proteins and are normally in the form of highly hydrated casein micelles that allows the milk to appear as a liquid. Casein proteins and their unique characteristics enable them to form a gel

during cheesemaking process, therefore, it is important to have a thorough look at their characteristics in detail.

2.1.2 Casein proteins

Casein proteins are made in mammary glands and the assembly method and the governing parameters of their formation have been subject of various researches (Boisgard, Chanat, Lavialle, Pauloin, & Ollivier-Bousquet, 2001).

Caseins are a group of proteins specific to milk consisting of four sub-unit proteins with different chemical structures, namely α_{s1}-, α_{s2}, β- and κ-casein, representing 93% of the micelle's dry mass (Fox & Brodkorb, 2008; Walstra et al., 2006). Some of their general specifications are presented in Table 2.2.

Casein proteins are unique in some aspects. They are highly heat resistant- an important aspect which enables milk to be heat treated at fairly high temperatures due to the presence of high amounts of proline in the primary structure of the amino acid sequence present in of α_{s1}-, α_{s2}-, β-proteins. Proline is a cyclic structured amino acid that prevents the formation of secondary and tertiary structure in protein . κ-casein contains less amounts of proline, and therefore has some secondary structure.

Casein proteins differ in their conformation in primary structure, the extent of disulphide bonding, and the degree of the glycosylation. These differences define the behaviour of casein micelle in milk and also dairy products. The source of the differences arise from the unique amino acid (AA) composition of the proteins. α_{s1}- and β-caseins don't have any cysteine in their amino acid composition where both α_{s2}- and κ-caseins contain two cysteine residues.

κ-casein is the only glycosylated casein molecule. It is glycosylated at its C-terminal and these hydrophilic molecules play an important role in the formation of the native micellar form of caseins.

As shown in Figure 2-1, the last 64 peptide residues towards the C-terminal is called glycomacropeptide (GMP) and the number of carboxylic groups in its protein chain is considerably high (14 out of 64).

Table 2-2: Some general specifications of casein proteins in milk (O'Mahony and Fox 2013)

Protein	mmol/m³ milk	g/kg milk	g/100 g protein	Molar mass	Remarks
Casein	1120	26	78.3	—	IEP≈4.6
αS1-Casein	450	10.7	32	~23600	Phosphoprotein
αS2-Casein	110	2.8	8.4	~25200	Same, contains –S–S–
β-Casein	360	8.6	26	23983	Phosphoprotein
κ-Casein	160	3.1	9.3	~19550	"Glycoprotein"
γ-Casein	40	0.8	2.4	~20500	Part of β-casein

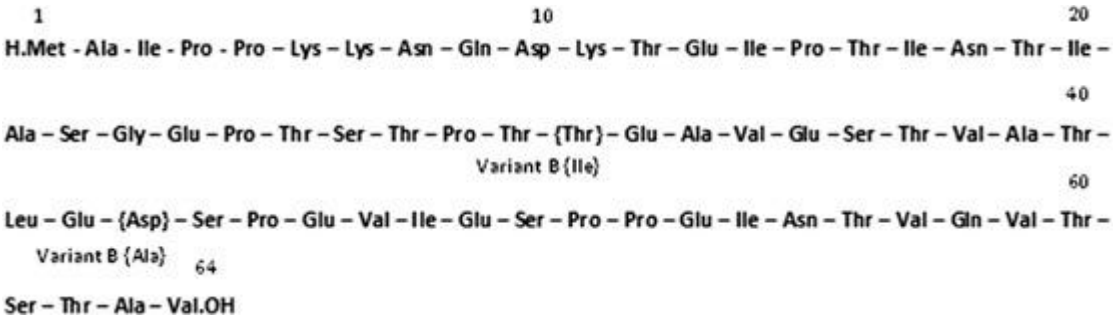


Figure 2-1 Amino acid sequence of glycomacropeptide (AA 106-169) in κ-casein chain of bovine milk protein (Eigel, Butler et al., 1984)

Casein proteins are phosphoproteins that precipitate at 20 °C by acidification down to pH~4.6; their isoelectric point. A very important aspect of casein proteins in cheese making is their phosphate content. The extent of phosphorylation between casein proteins are different (J. A. O'Mahony & P. F. Fox, 2013). These phosphate groups exert high charge on casein molecules, mostly esterified with serine residues (hydroxyl group -CH_2OH) and form serine phosphor-monoesters (Figure 2-2). In presence of a divalent ion, these steric groups act as two legs of a bridge between two different casein molecules. Hence, the higher the phosphorylation level of

casein molecule, the higher susceptibility of the molecule to calcium bonding. The impact of this phosphorylation on Ca^{2+} bonding is presented in Figure 2.3.

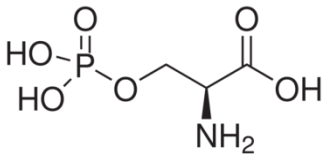


Figure 2-2 Phosphoserine

Figure 2-3. Ca^{2+} binding tendency of casein molecules. The ester-bond phosphate content is presented for each casein protein (mmol.g^{-1} casein), (Walstra, Wouters et al. 2006).

2.1.2.1 Casein micelle assembly- Proposed models

An overview of different models to explain the story has been reviewed by Fox (2003).

The term 'micelle' is commonly used for casein proteins in their stable form in milk, however they are not real micelles based on micelle definition with one hydrophobic and one hydrophilic tail (amphipathic molecules) and head (Dalgleish, 2011; Fox & Brodkorb, 2008).

The early researches on the structural model for these so-called micelles goes back to late 19th century (Fox & Brodkorb, 2008). Several models have been proposed to explain the overall structure of casein micelles which can be categorized into three main models (McMahon and McManus (1998) including coat-core model, internal structure model and subunit model. The

coat-core model (initially proposed in 1965 (Waugh & Noble Jr, 1965)) suggests that the interior part of the casein is made of different proteins compared to the exterior parts. The internal model (initially proposed in 1969 (Rose, 1969)) postulates the κ-casein as the chain terminator arising from a nonlinear polymerization. This model later on was developed by Horne (Horne, Parker, & Dalgleish, 1989) as the dual binding model. The subunit models of casein micelles assume that some distinct subunits link together to form a larger network. This model was initially proposed by Slattery and Evard (Slattery & Evard, 1973) and evolved continuously by other researchers (Schmidt, 1982; Walstra, 1990). Another model was proposed by McMahon and Oommen proposes an 'interlocking lattice' system of casein micelles, according to which, casein proteins extend from the core of a micelle as a chain and end with κ-caseins on the outer layer.

A newer model is also proclaimed by (Holt, 1992) named nanoclusters model. This model proposes that the formation of casein micelle is defined by the existence of so-called 'calcium phosphate nanoclusters'. On their journey from secretory vesicle to the cell membrane, the phosphorylated casein (and for κ-casein glycosylated) molecules are introduced into a medium with higher calcium phosphate concentration and here is the place where these nanoclusters are being formed (Dalgleish, 2011). This eventually results in formation of a structure where higher serine content casein molecules (see earlier this section) are located in central parts of the casein and κ-casein with lower serine residues forms the majority of the outer layer.

There is still a considerable research going on a better the understanding of casein micelles and their behaviour when they are exposed to different conditions and treatments.

There are similarities between these models specially the most frequently referenced ones i.e. subunit model and nanocluster model. Both of these models can efficiently explain the high content of calcium phosphate and the presence of higher κ-casein on the surface of micelle and are supported by the electron microscopy images of the micelle. Considering its comprehensive utilization and recent supporting developments, in this research we will refer to the nanocluster model where necessary. A schematic of this model is presented in Figure 2-4.

Figure 2-4. Schematic structure of the casein micelle (Dalgleish 2011) with α-caseins in red, β-casein in blue, κ-casein in green and calcium phosphate nanoclusters in grey (not to scale).

As shown in the schematic, κ-caseins are located on the surface of casein micelles. This can describe most of the casein micelle's behaviour in terms of its stability in milk at normal conditions and also in some technological aspects of dairy products. The pH dependency of calcium-phosphate equilibrium between the micellar form (colloidal) and mineral form (soluble in the serum) is one of the most influential aspects of casein proteins' study.

In this research a constant pH was targeted to control this parameter. This was selected based on the stability of micelles during pH reduction. Some pH ranges (pH between 6.7-5.8) reported to be moderate for the stability of caseins and no significant destabilizing effects were reported for the overall behaviour of micelles within that range. The rationale of pH selection will also be reviewed in detail.

2.1.2.2 Casein micelle stability

The basis of cheese making from milk is to transform the sole state of cheese milk to a gel. For this transformation, the casein micelles need to be destabilized in order to aggregate (to trigger gelation) and eventually coagulate (to form a curd). Cheese making is normally based on utilization and manipulation of one or more of these stabilizing/destabilizing parameters.

The stability of casein micelle in milk arises from both its casein molecules charges and assembly scheme together in the form of final micelle. These stabilizing forces include hydrophobic interactions, ionic and electrostatic interactions, hydrogen bonds and steric stabilization (McMahon & Oommen, 2013).

Major parameters affecting casein stability include:

2.1.2.2.1 *Calcium*

In normal milk conditions calcium (32mM) is present in three different forms including ionic, colloidal (also known as casein-bound or micellar calcium) and compound. The colloidal form (22 mM) itself can be found in two forms: directly bound to phosphate esters (carboxyl groups); or indirectly as a part of calcium phosphate nanoclusters. The role of calcium on the stability of casein protein is a source of controversy in various models (Little and Holt, 2004). Calcium phosphate nanoclusters are the protein strands interlocking mediums (McMahon & Oommen, 2013) and they convert an intrinsically unstable milk system into a hermodynamically stable system (Holt, 2004) . The dynamic nature of milk and also the role of calcium is crucial in all dairy processing.

2.1.2.2.2 *Temperature*

Casein micelles are heat stable and withstand temperatures as high as sterilization for more than 24 hours and UHT process for 15-20 minutes(D. S. Horne, 2008). Casein micelles are also stable against cooling down to refrigeration temperatures. Although a limited proportion (up to 20%) of β-caseins dissociate from the micelle, mainly due to hydrophobic interactions at low temperatures. Those interactions are weak and the molecule becomes solubilized in the aqueous phase. This solubilisation is reversible upon warming up the medium and giving back the energy to activate the hydrophobic interactions (Raynal & Remeuf, 2000).

2.1.2.2.3*pH Reduction*

Casein micelles are destabilized and precipitate upon pH reduction to their isoelectric point (pI=4.6). The mechanism is defined by reduction of the overall charge over the micelle as well as colloidal calcium phosphate (CCP) dissolution. Under such conditions, the electrostatic repulsion is depleted and is not sufficient to counteract the attractive-aggregative forces(Lucey, 2008). This precipitation is temperature dependent and happens at slow enough rates below 5-8 ^{o}C to be considered negligible.

2.1.2.2.4*Ionic strength of the medium*

High ionic strength is an influential factor on casein stability (D. S. Horne, 1998; Saito, 1973). As discussed earlier in this chapter the stabilizing factors are dealing with electrostatic charges. On the other hand, the ionic equilibrium in milk is a dynamic equilibrium between different phases of solution and suspension. Therefore, any change in the ionic strength of the surrounding medium will affect the stability of casein proteins. Variations in pH and temperatures will alter the colloidal-serum equilibrium conditions and consequently ionic strength. Sodium chloride at concentrations between 0.1-0.3M was shown to increase the soluble calcium and inorganic phosphate concentrations in a casein micelle dispersion (Aoki, 1999). This evidenced the solubilisation of micellar forms of the calcium and phosphate as a result of ionic balance interruption in the medium surrounding the casein micelles and shows the potential of interactions between the ionic elements and consequently the effects on stability of caseins. A high salt content has been reported to be influential on hydration/solubilisation of casein micelles hence an increase in the stability of caseins in their native form (Dalgleish (2011); (Karlsson, Ipsen, & Ardö, 2007; Zoon, Van Vliet, & Walstra, 1989).

Another method to form a gel is by changes in the C-terminal of κ-casein. As this portion of κ-casein is glycosylated, therefore hydrophilic, it protrudes into the surrounded serum and plays a

major role in micelles stability. With removal of this layer by either enzymatic cleavage of 105Phe-Met106 peptide bond or neutralization of charge through pH reduction towards the isoelectric point, the overall charge over the micelle is decreased and if continued, will result in micelle destabilization (Fox & McSweeney, 2004). The general aspects of these two major curd making mechanisms are reviewed in more details in Sections 2.3 and 2.4.

2.2 Cheese making process

Due to the artistic aspects of cheese making that has been developed all around the world, various preparation methods exist which result in different types of cheeses. A general process-flow of cheese making is shown in Figure 2-5. Despite the differences in the procedure, there are some common steps in all cheese making practices. Namely, irreversible alteration of milk proteins to a network that entraps milk fats, forms a gel and gel manipulation into cheese curd (Lucey, 2002). Although it is difficult to classify such a diversity into comprehensive categories, scientists have categorized cheese based on their similarities (and preparation methods). An example of this classification is presented in Figure 2-6.

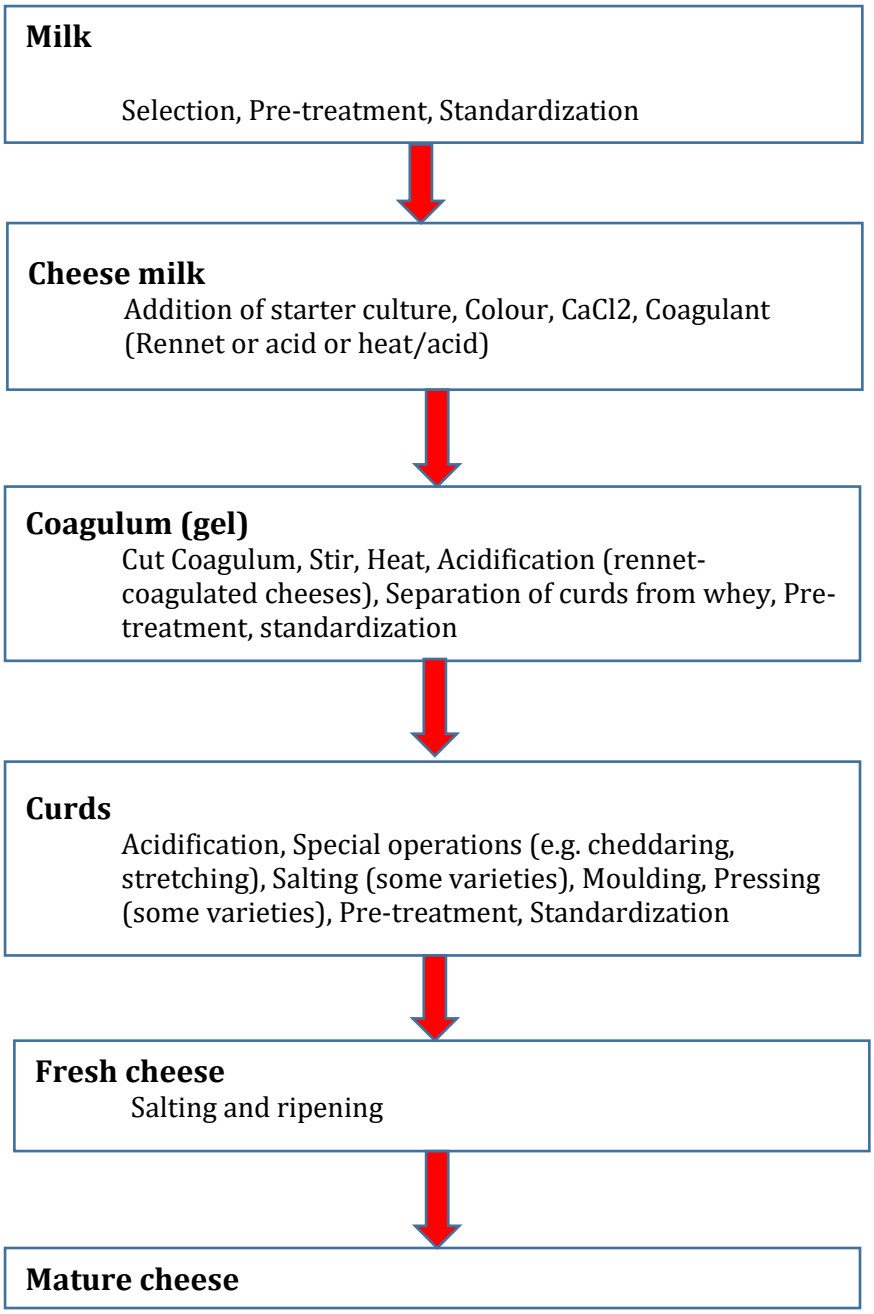


Figure 2-5. General cheese manufacturing protocol (Fox, Guinee, Cogan, & McSweeney, 2017a)

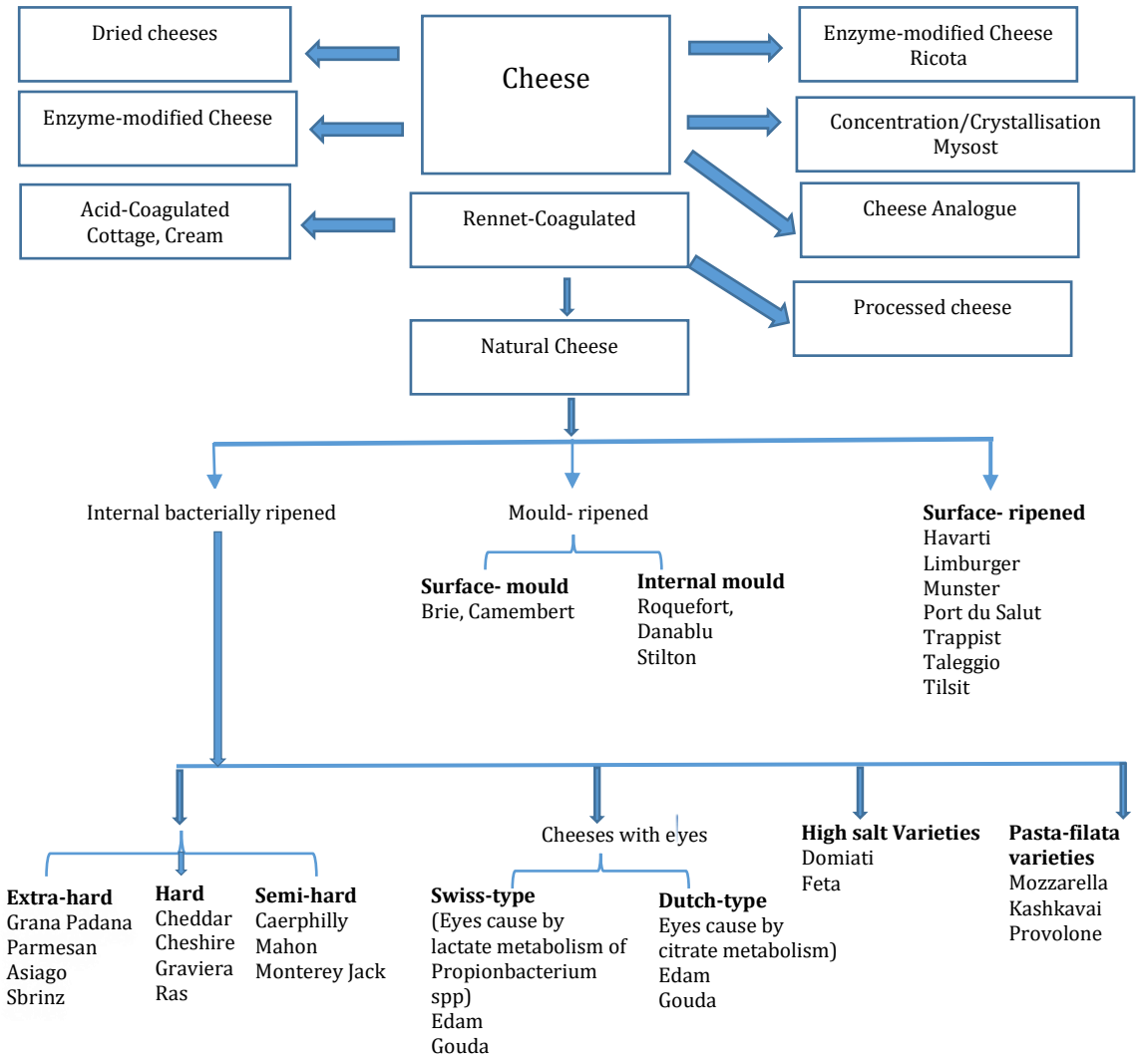


Figure 2-6 An example of cheese varieties (McSweeney, Ottagi and Fox; 2006)

2.2.1 Milk selection and pre-treatment

Almost all cheese types are characterised by a fat-in-dry matter content and fat-to-protein ratio. This can be achieved by centrifugation of milk to skim milk and cream streams followed by one or more of these methods (Patrick F Fox, Timothy P Guinee, et al., 2017a):

1) Mixing cheese milk with skim milk to decrease the fat-to-protein ratio;
2) Adding cream to increase fat-to-protein ratio;
3) Adding micellar casein from ultrafiltration membrane processing to increase protein content;
4) Adding evaporated, concentrated milk or milk powder to increase the total solid content.

In some cheese-making practices calcium chloride is added to improve milk coagulation and also provide characteristic texture in cheese curd. Cheese milk is also subjected to heat treatment in order to get rid of pathogenic bacteria and also for the consistency in the procedures and hence final product. Once the cheese milk is pre-treated, it is transferred to cheese vats. The major steps from then on can be generalized as coagulum and curd formation and de-wheying post vat steps (including maturation).

2.2.2 Coagulum formation

Regardless of the type of cheese, in any cheese making process, the casein proteins are required to be coagulated to form a gel network as the major structure which entraps fat fraction (if present) (Fox, McSweeney, Cogan, & Guinee, 2004; Lucey, Johnson, & Horne, 2003; Fox, Guinee, et al., 2017a). This can be attained by:

- Acid coagulation of caseins;
- Enzymatic coagulation of casein proteins;
- Acid-heat utilization; made using acidification to destabilize the casein micelles accompanied by heating to a temperature close to 90°C;

Acidification is able to form an acid coagulum at ambient temperature without any catalytic effect from other gel forming parameters. However, it is a normal practice to utilize acidification in addition to enzymatic coagulation. Most cheese types outlined in Figure 2.6 are produced using enzymatic coagulation (i.e. rennet); however, some important categories are made by acid coagulation.

2.2.2.1 **Acidification**

Acidification can be achieved by two different methods; direct using an inorganic acid (HCl), organic acid (lactic acid) or acidogens (glucono–delta-lactone), or indirectly by starter bacteria (Lucey & Singh, 1997, 2003).

During acidification, the pH of the medium and consequently all interactions of serum with salts, minerals, proteins, fat and lactose is changed, thus it has huge impacts on cheese manufacturing steps (Fox & McSweeney, 2013). A reduction in pH (regardless of the acidification method) improves the shelf life of the cheese and additionally contributes to the textural quality (by affecting syneresis and consistency of curd) of the final product (Walstra et al., 2006).

Starter bacteria are a group of lactic acid bacteria (LAB) added to the cheese vat to start acidification. Their main role in cheese making is to convert milk sugar (lactose) to lactic acid.

While direct acidification is used in production of some cheese types (Quarg, Cottage and UF treated Feta cheese) and easier to manipulate compared to biological acidification, it does not play any role in cheese ripening and hence is normally used in cheese where flavour development after day one of cheese making is not essential such as fresh cheeses (Fox et al., 2017). On the other hand, starter bacteria play an important role for the development of characteristic quality attributes of cheese through the activity of their proteolytic system (Beresford, Fitzsimons, Brennan, & Cogan, 2001).

2.3 Acid coagulation of milk

Acid coagulation/gelation is obtained by the decrease in pH towards the isoelectric point (pI=4.6) of casein proteins (Lucey & Singh, 2003). While above pH 6 only a very small amount of CCP is dissolved and the structural attributes of the casein micelles remain unchanged, with the reduction of pH towards pI, CCP solubilises (Dalgleish, 2011; Lucey, 2008). Most of the colloidal phosphate will be solubilized as a result of pH reduction from normal pH of milk down to pH=5.2 (Figure 2.7) and the remaining calcium will be lost from the internal casein micelles into the serum once the pH is gone further down to 4.6 (Dalgleish & Corredig, 2012).

Figure 2-7. Effect of pH reduction on the ability of micellar calcium (filled circle) and inorganic phosphate(open circle) to remain inside the micelle (Fox, Guinee, et al., 2017a).

On the other hand, as pH decreases towards the pI value of casein micelles, the caseins destabilize due to the dominance of attractive forces in the protein chains (Horne, 2008, Lucey 2016). With decrease in pH there is an increase in hydrogen ions and these shield the negative charge on the glycosylated end of the κ-casein resulting in a collapse of the hairy layer (de Kruif, 1997; Dalgleish & Corredig, 2012).

As a result of these confounded effects from the pH reduction (acidification to 4.6), sufficient destabilization (no repelling forces from outside and no integrity from inside) of the micelles happens and consequently the colloidal system rearrange, shrink and aggregate to form a three-dimensional network, coagulum (Lucey, 2008) (Fox, Guinee, et al., 2017a). This heterogeneous network is held together by covalent and protein-to-protein interactions (Dalgleish & Corredig, 2012).

2.3.1 Fermentation

2.3.1.1 Introduction

The biological activity in cheese and other dairy products which results in desirable acid production is called fermentation (Walstra et al., 2006). The fact that raw milk gets sour if left at

ambient temperature is the basis of dairy fermentation. Over time, people learnt to use some of the fermented product as the culture for next fermentation (called back slopping). When the raw material (milk) is cultured, the fermentation parameters are set in order to suit growth and development of the target bacterial varieties but prevent or retard growth and development of other undesirable varieties, which could be present in the raw material. These days same method is used to specifically make commercial starter cultures which makes it possible to carry out "controlled" or "pure" fermentation with high consistency, even at massive industrial production scales.

2.3.1.2 **Lactic acid bacteria**

Lactic acid bacteria (LAB) are a group of bacteria comprised of 12 genera of bacteria from *Streptococcaceae* family. They are called LAB because they are able to metabolize relatively huge amounts of lactic acid from carbohydrates. The LAB include *Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Lactobacillus, Enterococcus, Aerococcus, Vagococcus, Tetragenococcus, Carnobacterium, Weissella, and Oenococcus* (Ray, 2004). Four of those genera including *Lactococcus, Streptococcus, Leuconostoc* and *Lactobacillus* are used in dairy fermentations. They are characterized as gram positive, non-spore forming, and catalase negative bacteria without the ability of motion. They are anaerobic bacteria but tolerate oxygen and therefore called aerotolerant (Walstra et al., 2006).

2.3.1.2.1 *Starter selection criteria*

Selection of starter bacteria is an important step in defining cheese quality attributes and it has to suit the specifications of the final product. In most cheese making practices, more than one sub-strain of LAB is utilized. Starter selection is typically based on the following points (Miller et al., 2000; Walstra et al., 2006):

- The rate of acid production at early stages of inoculation: This rate is dependent on the inoculum volume, type of starter and temperature of the curd(Fox, Guinee, Cogan, & McSweeney, 2017);
- The extent of acidification: This affects the activity of unwanted bacteria and also cheese textural characteristics through solubilisation of CCP (de Kruif, Huppertz, Urban, & Petukhov, 2012). For most hard cheeses the final pH lays within a range of 5.0-5.3, but in soft acid coagulated cheese like Cottage and Quarg this value is close to pI of caseins *i.e.* 4.6;
- The possibility of gas production (mainly CO2) in some cheeses (e.g. Gouda, Tilsiter, Camembert and Roquefort);
- The ability of starter to withstand phage contamination; this is one of the reasons a multi-strain approach is opted in cheese industry to minimize the chances of 'no-starter activity' due to phage contamination by having different strains in the medium. Under this conditions, if one strain is phage deactivated, the other strains present in the inocula will carry on the fermentation.
- The coagulation rate as the rennet activity is dependent on pH (optimum pH for chymosin is ~6)(Horne & Banks, 2004);
- The acidification pattern: The important step of gel syneresis (rate and extent) is dependent on this pattern as it defines final moisture, composition of cheese as well as enzymatic activities (and consequently the proteolysis pattern and all quality attributes);
- Temperature profile of cheese making practice: Lucey and Singh (2003) reported differences in gel properties at different acidification temperatures.
- The amount of coagulant remaining in the curd and hence coagulant activity during later stage of cheesemaking;
- The incubation of bacteria before inoculation to the vat; during this time, they have time to reach their logarithmic growth phase and if there are more than one sub-strain they have time to adjust themselves to have a balanced growth while they are in the vat; In

some cheese types a secondary culture is also added to complement the activity of starter bacteria (Bockelmann, 2010); the impacts of this extra activity is an important parameter to be considered when applicable;

2.3.1.2.2 *Lactococcus*

The genus *Lactococcus* comprises several species, while only *Lactococcus lactis* is commonly used in the industrial dairy fermentation. These species involve three subspecies (SSP) named biovar. *lactis*, biovar. *cremoris* and biovar. *hordniae*, but only the first two are used in dairy fermentations(Kim, 2014).

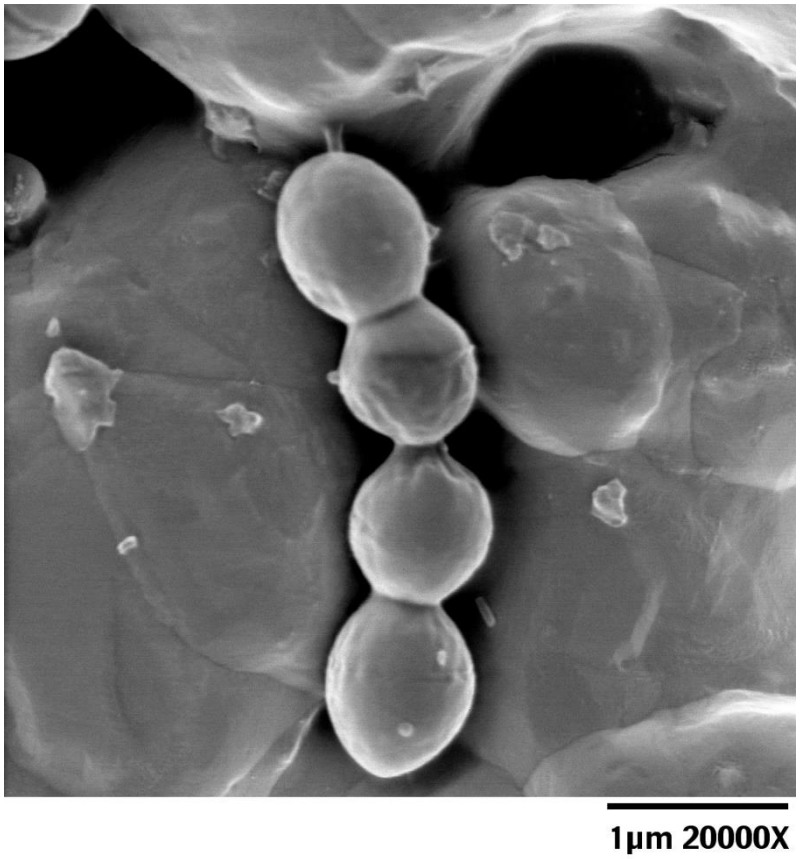


Figure 2-8 Scanning electron microscopy (SEM) of Lc. lactis organisms

The cells are ovoid with a diameter between 0.5 to 1 µm and presented in pairs or short chains (Figure 2-8). Their optimum growth temperature is around 30 °C and not able to grow at NaCl concentrations higher than 6.5% or pH 9.6. They can produce acid in a suitable broth and reduce

the pH down to 4.5. The *cremoris* subsp. is unable to grow at 40 °C and 4.5% NaCl and this is a method to separate this subspecies from the other. They are able to hydrolyse casein and this aspect of their characteristics is important in the research (Fox, 1989; Gatti et al., 2008; Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Upadhyay, McSweeney, Magboul, & Fox, 2004) as this research aimed to evaluate the influences of bacterial proteolytic system on milk-based medium during fermentations with different durations.

They are naturally present the dairy environment and raw milk (Ray, 2004). They tend to be fastidious species and normally require specific amino acids and B vitamins as their growth factors (Walstra et al., 2006).

With the growth of lactococcal bacteria, lactic acid concentration and formation of smaller sized peptides and free amino acids increases due to proteolysis (Peter Walstra et al., 2006). However, in an environment that readily contains amino acids and peptides required for their survival and growth, their proteolytic activity will significantly decrease (Walstra et al. 2005).

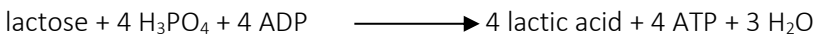
2.3.1.3 **Metabolism of lactose by LAB**

LAB require lactose as a source of organic carbon to provide their energy during fermentation. Lactose—galactopyranosyl-(1→4)-D-glucose— is a disaccharide known as milk sugar. This sugar forms about 54% of solid non-fat (SNF) portion of milk and 30% of the energy of milk.

During fermentation, initially the bacteria need to transfer lactose from the surrounded medium (milk) into their cell. There are two different methods of lactose transportation system for *lactococcal* bacteria. The first method is known as phosphoenol pyruvate phosphotransferase system (PEP-PTS). This system is characteristic for lactococci and involves different proteins and enzymes which chemically modify (phosphorylate) the sugar during the transportation procedure. This modified sugar is finally hydrolysed by phosphor-β-galactosidase (P-β-gal).

The second system is with the help of cytoplasmic proteins (permeases) which transfer the lactose without any chemical modification. The transported sugar is hydrolysed by β-galactosidase (β-gal).

Lactococci are homofermentative bacteria — only produce lactic acid from the metabolism of lactose. A homofermentative reaction of lactose fermentation can be described as below:



According to this equation, each lactose mole consumption will result in 4 moles of lactic acid. This suggests that while fermentation continues, especially at high cell populations, lactic acid is constantly produced and injected to the medium similar to an active pump of acid.

2.3.1.4 **Metabolism of proteins**

In milk, limited amounts of free essential amino acids are available that can be readily utilized by LAB, especially at high populations used during dairy fermentation. *Lactococcus lactis* strains specially need leucine, isoleucine, valine, methionine and histamine and small peptides for their growth (Flambard & Juillard, 2000; Kunji et al., 1996). This fact indicates that the lactococci need to hydrolyse large milk proteins, such as caseins, to source themselves with nitrogen source to survive (Reid, Moore, Midwinter, & Pritchard, 1991). The proteolytic system of lactic acid bacteria is briefly reviewed in Figure 2-9.

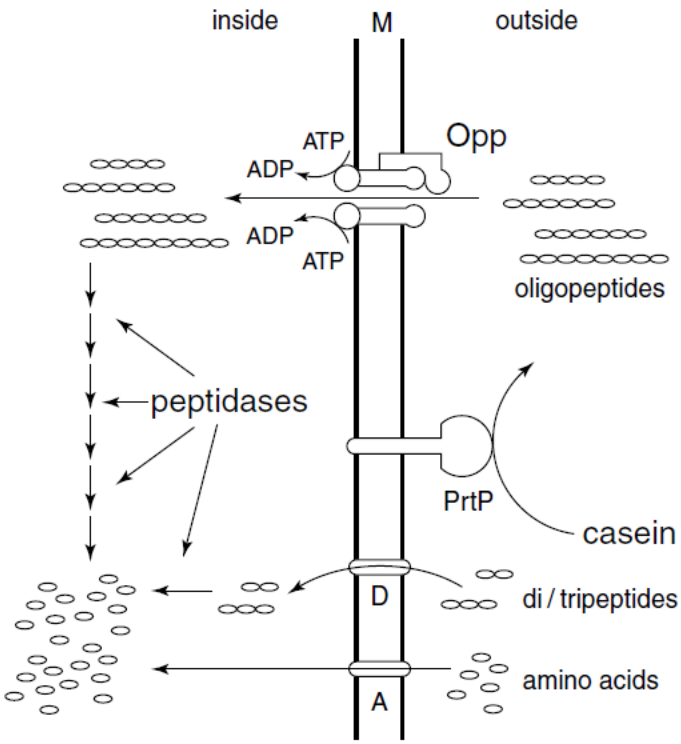


Figure 2-9: schematic of proteolytic system of Lc. lactis.

Lactococci have a membrane-anchored serine proteinase (PrtP) named lactocepin (EC 3.4.21.96) which enables them to break the casein proteins into smaller peptides. Lactocepin is a 180- to 190-kDa enzyme that belongs to the subtilisin family of serine proteases. Calcium is very important for these enzymes as they are attached to the cell via calcium ions. It has been shown that the proteolytic activity of cells will be lost if treated in calcium free buffers.

The cleavage peptide bonds of some of the lactococcal strains in different casein proteins are presented in Figures 2-10, 2-11, 2-12 and 2-13.

Figure 2-10: Cleavage sites of lactococcal lactocepins in αs1-casein (Upadhyay et al., 2004).

Figure 2-11: Cleavage sites of lactococcal lactocepins in αs2-casein (Upadhyay, McSweeney et al., 2004).

Figure 2-12: Cleavage sites of lactococcal lactocepins in β-casein (Upadhyay, McSweeney et al., 2004).

Figure 2-13. Cleavage sites of lactococcal lactocepins in κ-casein (Upadhyay, McSweeney et al., 2004).

One of classification methods of *lactococcal* proteinases is based on their specificity toward hydrolysing substrate (in our research casein). Two distinct proteinases, P_I and P_{III} proteinases are identified. P_I was identified in *LC. lactis* spp. cremoris HP and WG_2 and hydrolyses β-casein and to a lesser extent κ-caseins, while its degradation activity on α_{s1}-casein is very slow. P_{III}

proteinases are presented in *Lc. lactis* spp. *cremoris* AM1 and SK11. These proteinases breakdown β-casein in a different approach to P_I and, they are able to degrade α_{s1}-casein and κ-caseins (Law & Haandrikman, 1997). Although this classification was useful, it was not versatile enough to cover all the hydrolytic activities of those enzymes. The new method has recently been introduced based on the proteinases specificity toward the α_{s1}-casein fragment 1-23 and seven groups are identified (a to g letters) in a different nomenclature system (Reid, Moore et al. 1991; Reid, Coolbear et al. 1997; Flambard and Juillard 2000; Pillidge, Crow et al. 2003).

The produced peptides resulting from the activity of proteinase are transported into the cell across the cytoplasmic membrane using the transportation mechanisms. Subsequently, they undergo further degradation to produce amino acids (Christensen, Dudley, Pederson, & Steele, 1999; Kunji et al., 1996) (Beresford & Williams, 2004). The catabolic reactions of amino acids, however, will be the very important in flavour development of cheese (Beresford & Williams, 2004; Christensen et al., 1999; Gatti et al., 2008; Law & Haandrikman, 1997). As we aim to focus on the curd formation, these catabolic reactions remain out of the scope of our research.

2.3.2 Parameters affecting LAB during fermentation

The most important parameters are:

2.3.2.1 Lactose (energy source)

Lactose is the major carbohydrate in milk and LAB can uptake lactose to metabolize as a source of energy. Homofermentative strains convert the lactose to lactic acid with a high efficiency >90% (Powell, Broome, & Limsowtin, 2011) which acts as a coagulant of milk. It is noteworthy that lactic acid bacteria can use citrate and pyruvate metabolism as a source of energy in absence of lactose but as long as they have access to lactose, they don't tend to use those two options effectively. The enzymes to catalyse the reactions for all of these substrates are controlled by genes and environmental conditions can result in different expressions of the genes and consequently the pathways undertaken by starter cells (Walstra et al., 2006). Lack of carbohydrates or the so-called "carbohydrate starvation" would result in a prompt exponential growth, however when all the

cell reserves are consumed, the cell turn into a viable dormant culture until its demands for energy sources are provided (Sanders, Venema, & Kok, 1999).

2.3.2.2 **Source of amino acid**

LAB have a limited ability to synthetize amino acids (Flambard, 1998) and their growth depend on the available amino acids in the medium. There are limited amount of essential amino acids in milk. The starter bacteria will consume all the available free amino acids as a source of nitrogen and once this insufficient source is eliminated, they have the ability to provide themselves the nitrogen source by hydrolysing casein proteins using their comprehensive proteolytic system (Pritchard & Coolbear, 1993). The proteolytic system of some strains of LAB was shown to be media dependent (Law & Haandrikman, 1997) and hence they need an adaptation time if their source of nitrogenous compounds exposed to a dramatic change. *Lactococcus lactis*, for instance, has a specific medium to grow in lab *i.e.* M17. Milk is also a general growth medium of LAB. Regardless of the method of inocula preparation, in dairy fermentations milk and its available nutritional components would be the source of nutrients for the bacteria. The availability of amino acids in these two mediums are drastically different as in M17 the nutritional requirements of the bacteria are formulated to suffice, however, milk is designed to meet the requirements of the infant ,and not the bacteria. Hence they are not necessarily similar in terms of availability of nutrients. The readiness of proteolytic system to facilitate the bacterial cell requirements for amino acids is genome defined and medium dependant. This means the ability of the bacteria to grow in these two mediums are different. In other words if we propagate inocula on each of these growth mediums, once they are transferred to milk for fermentation, the one which was grown previously in milk has a shorter adaptation time and reaches logarithmic phase of growth in shorter time compared to M17 grown bacteria. The importance of this in our research will be elucidated in Chapter Four.

2.3.2.3 **Time**

Time is of major importance in enzymatic reaction (Wiseman, 1985). In fermentation as the bacteria are inoculated to the medium, they need a time to reach their logarithmic phase of growth. The time to reach the log phase is dependent on how favourable other growth parameters are. Depending on the size of inoculum, it takes 6-12 hours for *Lc. lactis* medium to acidify the cheese milk from natural pH=6.7 down to pH=5.2.

2.3.2.4 **Temperature**

One of the most important parameters in growth and development of bacteria is the temperature of the medium (Jay, 2012). The surrounding medium of the bacterial cells is required to have satisfactory level of thermal energy to provide the activation energy for their enzymatic activities (Frazier, Freizer, & Westhoff, 1978). From the viewpoint of heat demands for optimum growth, lactic acid bacteria can be broadly categorized into two major groups:

- Mesophilic, with optimum temperature between 30-37°C; these starters are used in cheese varieties where the curd processing temperatures do not exceed 40°C (e.g. Cheddar, Edam, Gouda, etc.).
- Thermophilic, with optimum temperature between 40-45 °C; these starters are suitable for cheese types where a high temperature is utilized at early stages of cheese making (e.g. Swiss cheeses).

An example of a mesophilic starter bacteria regeneration time dependency on temperature is presented in Figure 2-14 for *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. As shown in this Figure, the regeneration time of these widely used mesophilic bacteria(Callanan & Ross, 2004) is increasing (as a sign of unfavourable conditions) as the medium temperature lays out of their desirable range. High heat is regarded as one of the most damaging stresses for the bacteria and serves as a method to kill undesirable bacteria by denaturing their macromolecules (Smith, Dykes, Soomro, &Turner, 2010). Mesophilic *Lc. lactis* bacteria begin to be adversely affected by temperatures ≥48°C by damaging their structural membrane or ribosomal fatty acids and

denaturing bacterial proteins which bring about the malfunction of metabolic pathways (van de Guchte, Serror, Chervaux, Ehrlich, & Maguin 2002; Yousef & Courtney, 2003).

Figure 2-14. Effect of temperature on growth of Lactococcus strains; (blue line* Lc. lactis*, red and green lines* Lc. cremoris *(Fox et al., 2017)).

2.3.2.5 **Oxygen**

Lactic acid bacteria are facultative anaerobic bacteria meaning they need very small amounts of oxygen to grow (Jay, 2012). They are aero-tolerant and abundance of oxygen is not regarded as a toxin to inhibit their growth (Higuchi, Yamamoto, & Kamio, 2000). However, they don't tend to use oxygen as the terminal acceptor of electron (van de Guchte et al., 2002). Thus, when grown industrially the fermenter does not need to be aerated to supply oxygen for the metabolism of these bacteria and even sometimes nitrogen is added to prevent any aeration as a result of stirring in the fermenter. There has been evidence of differences in lactose metabolism by LAB if the fermenter is aerated (Condon, 1987).

2.3.2.6 **pH and lactic acid concentration**

The major reason of LAB utilization in dairy products is to produce acid from lactic acid metabolism. This causes significant pH reduction and makes the extracellular environment

unfavourable for other unwanted bacteria (van de Guchte et al., 2002). In cheese processing, LAB are expected to withstand these acidic conditions and sometimes high temperatures (like scalding in some cheese varieties which reaches up to 54 °C). It has been shown that they have two different response to the stress from high quantities of lactic acid. They demonstrate a general response in their stationary phase and an adaptive stronger response which occurs during their logarithmic growth phase called logarithmic acid tolerance response (LATR) Parente & Cogan, 2004; van de Guchte et al., 2002). With the exception of some species of *Lactobacillus*, *Leuconostoc* and *Oenococcus*, LAB are considered as neutrophils which means their optimum growth pH is between 5 to 9. Although it is out of their optimum pH for growth, they can easily tolerate the isoelectric pH of casein proteins (pI=4.6).

2.3.2.7 **Salt content**

Like any other bacterial cell, LAB are in constant exchange with the surrounding medium to uptake the necessary ingredients from and discharge their surplus outside the cytoplasmic membrane. In order to keep the metabolic pathways active, the intracellular conditions of the cell have to remain constant in terms of pH, ionic composition and level of available metabolites as much as possible (Csonka & Hanson, 1991). Salts and ionic compounds can disrupt the positive turgor effect between the bacterial cell and surrounding medium which allows water to pass through the semi-permeable cytoplasmic membrane and selectively passes the solutes according to the metabolic requirements (Jay, 2012). A higher salt content in the medium correlates with a lower water activity (Frazier et al., 1978) and hence the metabolic functions would be impacted depending on the water activity shift from the cell's optimum growth. Lactic acid bacteria have an adaptation ability by collecting so-called "compatible solutes" (Yousef, 2003)(including carbohydrates and amino acids) by which they keep turgor even in the presence of salt and ionic compounds and thus can survive these unfavourable conditions (Higuchi et al., 2000).

Lc. lactis is most widely used in cheese manufacture due to its ability to quiescently acidify the cheese milk, and also withstand process conditions including salt content up to 5% w/v (Smith et

al., 2010). The growth is inhibited at >6% w/v salt (Smith et al., 2010; Tripathi, Misra, & Chander, 2003) and reduced by 80% at this level while the acid production is also compromised and reduced by 50% (Tripathi et al., 2003).

2.3.3 Parameters affecting acid coagulation of milk

2.3.3.1 Type of acid

Type of acid or acidulant is a defining parameter in acidification of cheese milk. It directly influences the taste and aroma of the final products. In a study, Shehata, Lyer, Olson, & Richardson (1967) studied the effect of different acidulants on moisture, firmness and calcium levels in the final cheese. They concluded that the calcium levels were significantly higher in varieties of cheese made with different acidulants (in cheese which were acidified before renneting). They also concluded that the moisture content and firmness was acidulant dependent (Shehata et al., 1967). The acidulant used also contributes to the composition of the whey and this has to be considered in selection of suitable processing steps and equipment.

2.3.3.2 Rate of acidification

The rate of acidification is an important parameter when acidifying milk (de Kruif, 1997; Lucey & Singh, 2003). If the rate of acidification is too slow (e.g. more than 16 hours to reduce the pH form normal 6.7 to I=4.6), then the slowly-forming aggregates of casein proteins may not link together to form large strands and eventually a network. Under these conditions (which may happen for instance as a result of phage contamination) the aggregates precipitate irreversibly and will not take part in the structure of cheese. An example of this problem happens in Cottage cheese production lines and is known as ‘major sludge formation’ (Fox, Guinee, Cogan, & McSweeney, 2017).

A very rapid acidification (normally happens upon addition of inorganic acids like HCl) would also result in precipitation but there will be a high level of aggregation and fusion together to shrink and precipitate with a less hydrated and less porous structure (Lucey, 2008).The rate of

acidification defines the textural attributes of coagulum and the proteolysis pattern and this consequently would impact the quality of final product (Lucey, 2002; Lucey et al., 2003; O'Keeffe, Fox, & Daly, 1975)

In cheeses made with a mixture of acid and rennet actions an early or late acidification would result in a different pH at renneting. This parameter would also be crucial to downstream parameters of cheese making (including curd formation, syneresis and cheese yield) and more importantly the quality attributes (texture and flavour) of the final cheese (Daviau, Famelart, Pierre, Goudédranche, & Maubois, 2000; Horne & Banks, 2004; Upadhyay, McSweeney, Magboul, & Fox, 2004).

Biological activity of LAB is a reliable method for the production of acid at a desirable rate if the conditions of the growth medium (e.g. cheese vat) is kept favourable for them. The extent of acidification is also important as it is correlated with CCP solubilisation. If the coagulum is being produced by acidification, and the pH reduction is high enough (i.e. approaching pI of caseins) then most of the CCP will be solubilized.

The overall procedure of acid coagulated cheese and related products is presented in Figure 2-15.

Figure 2-15. Flow chart for production of acid coagulated cheeses and related products (Fox et al., 2017).

2.4 Enzymatic coagulation of milk

2.4.1 Introduction

Almost 75% of cheese is made by enzymatic coagulation. In this method the κ-casein plays the key role(Horne & Banks, 2004; Lucey et al., 2003). The spatial arrangement of casein micelles in normal conditions of milk is such that the concentration of κ-casein is higher at the surface of the micelle than the interior parts of it and this spatial arrangement is the source of two different types of stabilizing mechanisms named steric repulsion and electrostatic repulsion (section 2.2). κ-casein is hydrolysed by a group of enzymes found in rennet known as chymosin (or its recently developed replacers). These enzymes break down the peptide bond between Phenylalanine 105 and Methionine 106 of the 169 peptide chain in κ-casein protein (Jacob, Jaros, & Rohm, 2011;

Stepaniak, 2004). Rennet hydrolyses the highly electronegative portion of the κ-casein; hence the electrostatic repulsive forces between micelles are reduced (Lucey et al., 2003) and at the same time the stabilizing steric repulsion is also disrupted. In the presence of sufficient calcium to neutralise the remaining electronegative charges of the glycosylated parts of intact κ-casein in the depleted micelles, the zeta potential of micelles approaches zero (from -10/-20 to -5/-7 mV as a result of CMP cleavage only), hydrophobic attractive forces dominate, resulting in the formation of a casein gel-coagulum (Patrick F Fox et al., 2004) (Figure 2-16).

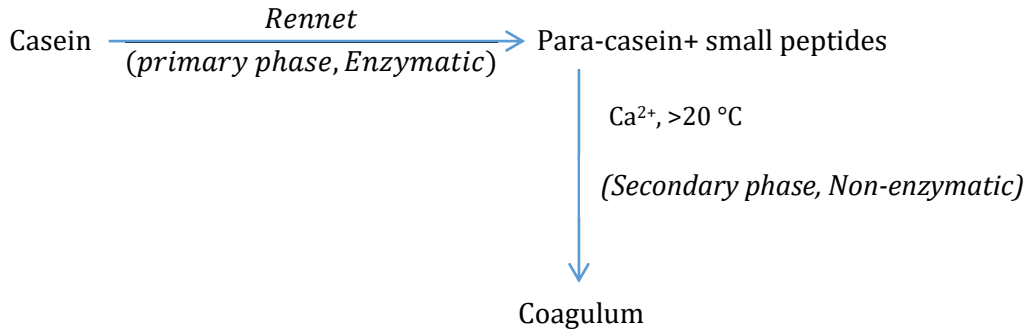


Figure 2-16. Process flow of enzymatic coagulation of milk (Fox et al., 2017).

Figure 2-17. Schematic representation of enzymatic coagulation of milk. (a) Casein micelles with intact extended CMP layer exposed to chymosin enzyme; (b) Partially CMP-cleaved caseins: (c) Aggregating micelles with no 'hairy' layer (Fox et al., 2017).

The overall steps and schematic of rennet coagulation of milk are presented in Figures 2-17 and 2-18. As shown in Figure 2-17, rennet coagulation is a two-step procedure. The first step is the

enzymatic hydrolysis of milk proteins to reduce the repulsive surface charge, while the second step includes the aggregation of those destabilized proteins.

Figure 2-18. Enzymatic coagulation of milk. First step- caseinomacropeptide (back springe shaped parts on the surface part (a)) is being removed from intact micelle surface as a result of rennet action. Micelles tend to come closer (Dalgleish & Corredig, 2012).

2.4.2 Primary phase (Enzymatic hydrolysis of κ-casein)

The first step in enzymatic coagulation of milk is the specific hydrolysis of peptide bond Phenyl alanine 105 and Methionine 106 of the 169 peptide chain in κ-casein protein (Jacob et al., 2011). During this step, the CMP, which is a polyelectrolyte rich layer of κ-casein (peptide 105-169), is removed as a result of the rennet action (Horne & Banks, 2004). This would result in an increase in diffusion coefficient of caseins as the polyelectrolytes are the major source of the stabilizing steric repulsion effect (see section 2.2) (de Kruif, 1999). The hydrolysed CMP diffuses away from the micelle (now called para casein) but at early stages of this enzymatic reaction, there would not be any significant changes in the rheological properties of cheese milk. There has been reports of a decrease in cheese milk viscosity but no gelation is observed until enough stabilizing layers

are removed (Dalgleish & Corredig, 2012). This has been reported to be when about 80% of them are cleaved (Tuinier & De Kruif, 2002). Dalgleish reported that micelles will start to aggregate when 85% of the 'hairy' layer is removed (Dalgleish, 1983)and this has been reported to be the level of hydrolysis accounting for 60% of visual rennet coagulation time (RCT) (Fox, Guinee, Cogan, & McSweeney, 2017b).The intrinsic and medium parameters that affect the primary phase of rennet coagulation of milk are briefly reviewed here:

2.4.2.1 **Type of coagulant**

In order to do the enzymatic coagulation of milk, a number of coagulants are being used. Chymosin, from aspartic proteinase category, is derived from young calf abomasum and is the most commonly used proteolytic enzyme in cheese making (Bennett & Johnston, 2004). Due to increased demand for the enzyme in cheese making, some replacers have been considered. Pepsin and some fungi-based (*Rhizomucor miehei* or *Cryphonectria*) as well as some plant-based enzymes are the major chymosin replacers (Crabbe, 2004). The type of coagulant can affect the rate of hydrolysis (Lawrence, Creamer, & Gilles, 1987) and the final product can be affected by the source of the aspartic coagulant. Some replacers do excessive proteolysis which result in adverse effects including fat loss in the serum stream as well as defected textural attributes of the curd (Grag and Johri, 1994).

The amount of retained rennet is proportional to the original amount added (Fox et al., 2017) and play an important role in cheese functional properties as the coagulant activity has been reported to be continued during cheese ripening (Hurley, O'Driscoll, Kelly, & McSweeney, 1999; Lawrence et al., 1987). Ohmiya & Sato (1970) reported the level of retained rennet in cheese curd (depending on cheese variety and characteristics of coagulant) to be between 0-15% of total rennet added to vat and this was recently reported to be up to 30% (see section 2.6). The result of this activity would result in a different proteolysis pattern and reveal new peptide profile and textural and flavour influences thereof. Gel strength was also reported to be strongly influenced by the type of rennet used (Bansal, Fox, & McSweeney, 2009; Fox et al., 2017a) as per calf

chymosin which acts quickly to apply a quick increase in gel strength compared to microbial rennets.

Garnot, Molle and Piot (1987) reported a lower rennet residual activity from *Rhizomucor meihei* retained in cheese compared to chymosin. The ratio of enzyme to protein is reported to be an important parameter in coagulation (Bansal, Fox, et al., 2009). Both the ration of rennet to protein and the residual activity will impact the extent of proteolysis in cheese during processing and ripening.

2.4.2.2 **Heat treatment history and temperature of the reaction**

Heating milk at temperatures close to short pasteurization of milk (72 °C) is reported to affect the coagulation time of milk. The adverse effect of heating on primary phase of coagulation arises from the fact that whey proteins, especially β-lactoglubolin, are heat labile and denature at temperatures above 64 °C. The extent of denaturation is temperature dependent and will increase as the heating temperature increases. This denatured protein will then make a complex with κ-casein either by disulphide bonds or hydrophobic reactions (Anema, 2008). The impact of this undesirable effect can be so high that sometimes the heated milk loses its ability to coagulate due to inaccessibility of the substrate sites for enzyme. Kannan and Jenness (1961) reported that β-lactoglubolin during heat treatment at 85-90 °C for 30 min, formed a complex with caseins which resulted in inhibition of rennet clotting activity.

Like any other enzymatic activity, there is an optimum temperature for the reaction. This parameter like almost every other influential parameter is cofounded and is affected by both pH and type of rennet. The optimum temperature for rennet coagulation of milk in normal cheese practices (i.e. pH 6.6 down to 6.4) is 45-48 °C and the optimum for the primary phase lays in this range(Fox et al., 2017b). It is noteworthy due to technological aspects of cheese making (starter bacteria optimum activity, rate of acidification control, network development and gel strength,

control of shrinkage and syneresis, etc.) the renneting step is performed at temperatures around 32 °C.

2.4.2.3 **Milk composition**

Ultra filtration has been successfully utilized to increase the protein and fat content of cheese milk to produce some soft cheese varieties (including Feta, Quarg, Cream and Cottage) (Waungana, Singh & Bennett, 1998). Kosikowski (1986) reported a 1.8x concentration as the limit for making acceptable quality Cheddar cheese.

2.4.2.4 **pH**

The optimum pH for chymosin in bovine milk for primary phase of rennet coagulation at both 4 and 30 °C is reported to be ~6.0(Esteves, Lucey, Wang & Pires, 2001; Waungana et al.,1998; Fox et al., 2017).

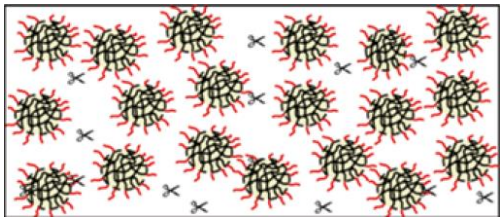
2.4.2.5 **Ionic strength**

The effects of ionic strength on rennet coagulation starts from the primary phase. Both chymosin and substrate are negatively charged and a balanced charge interaction exist for the action of the enzyme. If the ionic strength increases too much, enzyme attachment to the substrate is reduced due to screening of charges (Daviau et al., 2000). An increase in NaCl content of the casein dispersion (in water or salt solution) was reported to decrease both primary and secondary phases of rennet coagulation (Famelart, 1999).

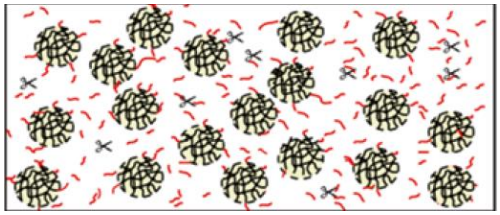
2.4.3 Secondary phase (Aggregation and gel formation)

The second and final step in curd formation of rennet induced gels of casein protein is rather complicated. Once 85% of the stabilizing CMP is removed, this secondary phase is triggered in which the so-called para-caseins are destabilized enough to allow the micelles to come closer to each other (Figure 19-b) and even remain beside each other when they collide to form strands (Figure 19-c) of proteins (Green, 1993). These small aggregates then join to each other in a three-dimensional network (Figure 19-d) to eventually form the cheese coagulum (Figure 19-e) (Fox et

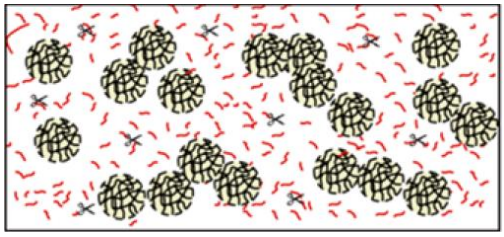
al., 2017; Lucey, 2008; Dalgleish, 1983). The presence of calcium ion is crucial in this secondary phase, as it acts as 'bridges' between the aggregating micelles by reducing the net negative charge over the neighbouring micelles (Dalgleish, 2012).



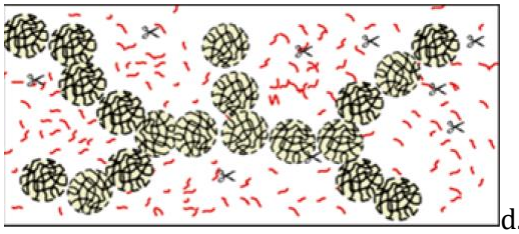
a. Intact casein protein upon addition of rennet (depicted as scissors);



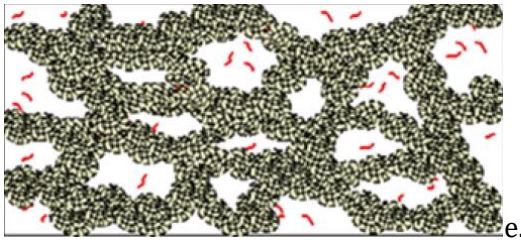
b. Some CMP (red layers) hydrolysed by rennet;



c. Onset of gelation, para-caseins are forming aggregates;



d. Gel network is forming from Para-casein aggregates;



e. Cheese coagulum.

Figure 2-19. Schematic representation of cheese curd formation from milk (a-e) (Fox et al., 2017).

Various parameters are involved in this phase and all of those parameters should be optimized (as they are mostly cofounded) in order to have consistent and reproducible results from a cheese making practice.

2.4.4 Factors affecting gel formation

As presented in the process flow (section 2.16) calcium ions and temperature are the two main parameters. Beside these factors, there are other important parameters as follows:

2.4.4.1 Temperature

The temperature dependency of the secondary step of rennet coagulation is markedly higher than the primary step and it is reported that under 18 °C, bovine milk does not coagulate unless calcium levels of cheese milk are increased (Fox et al., 2017).

2.4.4.2 Calcium

Although calcium ions have no effect on the primary phase of rennet coagulation (if the pH is kept constant) (Van Hooydonk, 1986), they play a major role in the secondary phase.

Cleavage of the GMP from the κ-caseins would result in a reduced net negative charge and thus a reduction in repulsive forces. This consequently would result in micellar destabilization in the cheesemilk and so they can come closer to neighbouring micelles. Depending on temperature, pH and ionic strength of the medium as well as the extent of GMP removal, there would be some of those stabilizing effects retained on the micelles which prevents them to come close enough to aggregate. In presence of enough calcium ions (Ca^{2+}) to neutralize the remainder of negative charge, the micelles can come close enough to aggregate (Dalgleish, 1983; Udabage, 2001). Zoon et al. (1988) reported that a lower CCP content in cheese milk had resulted in a longer rennet coagulation time (RCT), than if the Ca^{2+} was kept constant. Addition of CCP can result in a shorter RCT and improved gel strength (Zoon, 1988; Udabage, 2001).

In rennet coagulation, if the pH is kept constant, then a reduced CCP level would result in a longer RCT and different quality attributes (decreased firmness and increased meltability) (Choi, Horne, Johnson, & Lucey, 2008).

The equilibrium between the soluble and colloidal phase of calcium can be manipulated to define the properties of curd and final cheese in both acid and acid-rennet coagulations (Wang, Zhang, Luo, Guo, Zeng & Ren, 2011; Lucey, 2005). This equilibrium will be influential even at later stages of cheese making (will be discussed later in detail) (Hassan, Johnson, & Lucey, 2004). This important equilibrium itself is defined by pH. This is another reason all of the defining cheese quality parameters are confounded.

2.4.4.3 **pH**

As mentioned under primary step of coagulation the optimum pH for that phase was ~6, however when it comes to secondary phase this shifts to lower pH values with the confounded effects of a lower pH and solubility of CCP. This increasing effect continues down to pH 5.1-5.3 (Van Hooydonk, 1986). It is reported that the rennet coagulation of milk is not very efficient at pH values lower than 5.0 (Nájera, de Renobales & Barron, 2003).

2.4.4.4 **Salt and ionic strength**

Karlsson *et al.* reported a shield effect for the charges on casein micelles as a result of NaCl presence in cheese milk (Karlsson et al., 2007). This shield effect is important as it can affect the calcium equilibrium and all the effects thereof (see 2.4.4.4).

2.4.4.5 **Protein concentration of cheese-milk**

Increasing the casein concentration, via ultra-filtration (UF), has been found to have minimal impact (8%) on gelation bahaviour of cheesemilk at low casein concentration factors (2.21x) (Zoon, 1988), but has a larger effect at higher concentration factors (7x) (Bech, 1993) (Figure 2-20).

2.5 Post coagulation steps in cheese making

Referring to Figure 2.16, once the cheese milk is coagulated, it undergoes other steps to finalize the production procedure. The major purpose of these treatments is to facilitate whey removal from the gel (syneresis) and reach the final product moisture content with characteristic attributes. These steps can be divided into cutting, cooking, stirring, further acidification by bacterial activity, and whey drainage (Bennett & Johnston, 2004; Fox et al., 2017). Each of these steps have their own impact on the pattern and extent of syneresis.

Figure 2-20. RCT (T_0) as a function of protein concentration at 32 °C (square) and 40 °C (diamond) (Bech, 1993).

Coagulum cutting is the start of whey drainage. The effect of cutting on de-wheying arises from the selection of the appropriate gap between the cutting blades. Considering all other parameters equal, a faster and higher extent of de-wheying can be achieved by selecting a smaller mesh size (Fox et al., 2017). The higher the extent of three-dimensional network of coagulum rupture, the more available surface for whey to expel out and consequently the higher the extent of syneresis. This is adjusted according the desired type of cheese being made and its moisture content.

Curd cooking is an important step to control the activity of starter bacteria, metabolism of lactose, curd shrinkage and syneresis (Fox et al., 2004; Castillo, 2006). The applied temperature depends on the type of cheese, moisture content of final cheese and type of starter culture utilized. In high moisture cheese varieties, the cooking temperature is as low as the renneting temperature (e.g. camembert with 31 °C) however in lower moisture varieties higher temperatures (as high as 52-55 °C in parmesan and Emmental) are used (Fox et al., 2017).

pH and rate of acidification are also important parameters in controlling syneresis. Generally, a lower pH enhances the rate of drainage which is a very common parameter in curd quality assessment (Walstra, 1985; Castillo, 2006). Castilo *et al.* (2006) reported a decreased syneresis rate as a result of a faster acidification rate, presumably by inhibitory effect of this higher rate on network rearrangement ability of proteins during whey expulsion.

The moisture content of the cheese is defined at this stage and this consequently would control the:

- Extent of starter bacterial activity during the ripening;
- The extent of residual coagulant activity in the cheese;
- The salting process and its cofounded effects on pH, enzymatic activity, calcium ion activity
- The extent of lactose content of curd (Castillo, 2006) and therefore the pH of curd and cheese, subsequently cheese texture and flavour.

2.6 Cheese Ripening

During the ripening phase, final flavour and texture of the cheese is developed (Upadhyay et al., 2004). This complicated process comprises of several biochemical changes including (Fox et al., 2004):

- Glycolysis of residual lactose and catabolism of lactate;
- Catabolism of citrate;

- Lipolysis and metabolism of fatty acids;
- Proteolysis and catabolism of amino acids.

Among these processes, the most important in formation of characteristic quality attributes of cheese is proteolysis (Fox, 1989; Fox, Guinee, Cogan, & McSweeney, 2017; McSweeney, 2004; Shalabi & Fox, 1987; Sousa, Ardö, & McSweeney, 2001; Upadhyay et al., 2004). Proteolysis is mainly responsible for the hydrolysis of casein matrix, thus affecting the nature of peptides in cheese and these changes will consequently influence all textural parameters of the cheese. As a result of this, a new pattern of carboxylic acid and amino groups would be revealed and this consequently would result in a different water activity (a_w)(McSweeney, 2004). The breakdown of proteins also results in the formation of smaller sized peptides and free amino acids. The latter is required for further biochemical pathways during ripening that affect the cheese flavour (Christensen et al., 1999; Sousa et al., 2001).

2.6.1 Proteolysis during cheese ripening

The main components in cheese making practices are the nitrogenous compounds (Table 2.1). Around 95% of these compounds are found in the protein fraction of which 80% is comprised of casein protein and the rest are serum or whey proteins.

The enzymes involved in the proteolysis of cheese originate from six different sources which include the coagulant, starter Lactic acid bacteria (LAB), milk, non-starter lactic acid bacteria (NSLAB), secondary starters (e.g. in blue cheeses) and exogenous enzymes that may be added to accelerate ripening (McSweeney, 2004). In this review the emphasis will be on the activity of the starter bacteria's proteolytic systems, however the importance of other sources is also briefly reviewed. Although, proteolysis is present all the way through the cheese milk preparation (intrinsic milk proteinases) and processing (bacterial proteolytic system and rennet activity), the proteolytic activities related to cheese ripening takes place after finishing the manufacturing operations at day one of cheese making.

2.6.1.1 **Coagulant residue**

An important source of proteolysis in cheese is the added coagulant or rennet that remains in the curd after whey drainage. Up to 30% of rennet activity may remain in the cheese curd depending on various factors including the cooking temperature, pH at whey drainage, extent of acidification prior to rennet addition, moisture level, casein micelle size, ratio of different caseins, ionic strength of milk, cheese age and coagulating enzyme type (Wilkinson & Kilcawley, 2005).

Rennet is highly sensitive to pH and temperature profiles that are commonly used in cheese making processes (Larsson, Andrén, Geurts, de Roos, & Walstra, 1997). For example, rennet activity during ripening is limited in well-cooked cheese varieties such as Emmental and Swiss types, as it has been denatured extensively (Hayes, 2002), while in cheeses cooked at lower temperatures such as Gouda with similar initial pH, a considerable rennet activity is reported (Wilkinson & Kilcawley, 2005).

Rennet activity has been reported to affect texture, especially cheese softening and flavour of cheese (Bansal, Fox, et al., 2009).

2.6.1.2 **Starter Lactic acid bacteria (LAB)**

A significant percentage of starter bacteria added to cheese vat contribute to the curd and cheese as they are entrapped in the interface of protein and aqueous phase and also the periphery of fat globule (Wilkinson & Kilcawley, 2005). Proteolytic activity of lactic acid bacteria is a key factor in the development of texture and flavour during ripening in most types of cheeses (Beresford & Williams, 2004; Sousa et al., 2001). They have a comprehensive proteolytic system which enables them to extract their required amino acids from the proteins in their environment. This complicated system includes extracellular and intracellular proteinase and peptidases. A schematic of this system is shown in Figure 2-21 (McSweeney, 2004; Sousa et al., 2001).

Extracellular proteinase enzymes present in Lactic acid bacteria start to degrade the cheese-milk protein immediately after their introduction into the process. On the other hand, their

intracellular enzymes are released in the cheese matrix after the bacterial cell is lysed due to environmental conditions such as nutrient deficiency.

As illustrated in the Figure 2-21, there is a transfer system for oligopeptides, di- or tri-peptides and amino acids from the cell wall into the cell. In the case of large proteins, such as caseins, it is necessary for them to be broken down by the proteinase PrtP, which is located outside the cell. Inside the cell, there are peptidases which catalyse the hydrolysis of peptides to make smaller peptides and finally the required amino acids.

Figure 2-21- Summary of the proteolytic system of Lactococcus (McSweeney, 2004)

Factors affecting the proteolytic activity of lactic acid bacteria are reviewed here in more detail:

Time

Apart from the active enzymes present in the medium, during the cheese making process some conditions become unfavourable for the bacteria (e.g. salt addition, a_w reduction) and would result in cell lysis, and hence release of some new enzymes. The amount of casein breakdown and produced peptides and amino acids depends on the duration of the process.

Temperature

Proteolysis in cheese is an enzymatic reaction that is temperature-dependent. Most cheese varieties are coagulated at 30-37 °C and cooked at higher temperatures (37-54 °C). Thus, the activity of the present bacteria is affected by alterations in temperature (Beresford & Williams, 2004). LAB cells are considered to be sensitive to temperature changes. A small change from 38 to 40 °C has been reported to induce thermolytic cell lysis in *Lc. lactis* strains (Lortal & Chapot-Chartier, 2005).

Lactic acid bacteria biomass concentration

The main goal for the addition of starter LAB at initial stages of cheese making is to produce lactic acid by fermentation of lactose, but they contribute to other biochemical pathways involved in cheese ripening (Kunji et al., 1996). LAB are considered to have the highest population of microorganisms in cheese, and reported to reach to more than 10^8 $cfug^{-1}$ in young cheeses during the first production day, therefore, such a huge number of microbial biomass has the potential to take part in bio-catalytic pathways and affect the cheese matrix rapidly (Christensen et al., 1999).

Cell lysis

For a long time it was considered that the starter bacteria contribution in cheese making is restricted to the viable cells and their activity on the cheese-milk lactose, therefore production of lactic acid. However, based on current knowledge it can be concluded that the starter cell autolysis plays an important role in cheese making by the release of the intracellular enzymes in to the cheese curd matrix (Valence, Richoux, Thierry, Palva, & Lortal, 1998). The cells are assumed to be lysed as a result of the environmentally induced lysogenic phage activity or autolysis due to the defects in the cell wall synthesis system (Beresford & Williams, 2004).

Autolysis occurs generally under unfavourable environmental conditions such as nutrient starvation and results in the inhibition of peptidoglycan synthesis. Under such conditions, peptidoglycan that are essential for the structural integrity of the cell wall are hydrolysed causing the cell autolyse and release its intracellular contents (Lortal & Chapot-Chartier, 2005).

Intentional controlled cell lysis of starter LAB by for instance simultaneous incorporation of the starter bacteria with bacteriocin (to facilitate the cell lysis) producing strains has been suggested as an effective accelerated method to develop flavour during cheese ripening (Morgan, Ross, & Hill, 1997; Ruyter, Kuipers, Meijer, & Vos, 1997).

2.6.1.3 **Proteolytic activity of the secondary starter**

In contrast to the primary starter cultures, secondary starters are not involved in acid production before curd production. The objective of their utilization is to incorporate them in cheese ripening while they grow mainly on the surface of cheese. The secondary and adjunct cultures that may be utilised includes yeasts (e.g. *Geotrichum candidum*, *Debaryomyces hansenii*), moulds (e.g. *Penicillum camemberti*, *P. roqueforti*) and bacteria (e.g. *Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Propionibacterium* sp. and heterofermentative lactobacilli) (Chamba & Irlinger, 2004).

Although the proteolytic activity of these microorganisms is different, they are all capable of applying specific flavour, texture and colour characteristics in the cheese. The produced amino acids (end metabolic product) act as the substrate required for catabolic reactions. The products of these metabolic-catabolic reactions contribute to the specific characteristics of each cheese type.

2.6.1.4 **Proteolytic enzymes of milk**

Milk contains two main indigenous proteinase systems derived from the blood. Cathepsine is responsible for defence against invasive micro-organisms and plasmin is responsible for dissolving fibrin blood clots (Kelly, O'Flaherty, & Fox, 2006).

Plasmin is specific for β-casein, α_{s2}-casein and to a lesser extent α_{s1}-casein. β-casein is its primary substrate. The plasmin system is relatively heat stable (compared to other sources of proteolysis); thus its hydrolytic activity is more pronounced in cheeses that are cooked at higher

temperatures. This may be caused by the heat inactivation of inhibitors of plasminogen activators which will result in a higher conversion of plasminogen to plasmin.

Cathepsin D on the other hand, is mostly active on Phe105-106Met peptide bond of κ-casein, but its concentration in milk (about 4 μg/mL) is not high enough to be considered as a coagulant. It is reported that this enzyme can resist pasteurization temperatures (72 °C for15 s), and therefore it may take part in ripening of cheeses that are made from pasteurized milk (Sausa, Ardo, & McSweeney, 2001).

2.6.1.5 **Exogenous proteolytic enzymes**

These proteolytic enzymes are added at different steps of cheese making normally while the cheese curd is forming. Their presence increases the rate proteolysis of cheese-milk proteins and the process is called “Accelerated Cheese Ripening”. The type of enzymes used depends on the cheese type. These enzymes are specific at cleaving particular peptide bonds which provides substrate for further peptidolytic or catabolic reactions.

2.6.2 Evaluating the proteolysis progress in cheese

2.6.2.1 **Introduction**

Cheese ripening involves a series of complicated reactions which finally result in the characteristic texture, flavour and aroma of the ripened cheese (Urbach, 1993). Proteolysis is a major biochemical reaction during ripening. As proteolysis involves the breakdown of proteins and peptides, the resultant products can be used to determine the extent of ripening progress (Mallatou, 2004).

Casein proteins do not have a tertiary and quaternary structures and even its secondary structure is different from α-helix and β-sheets of globular proteins (See 2.2). Thus during cheese-making, the primary structure of casein proteins (the backbone of protein) undergoes significant changes as well as its secondary structures (*i.e.* hydrophobic/ hydrophilic balance). The newly revealed peptide and amino acids are expected to differ in hydrophobicity/ hydrophilicity as well as

solubility in different solvents. Extraction of samples with different levels of hydrolysis and consequent quantification of nitrogenous compound in each extractant is an efficient measure for the extent of proteolysis.

Peptide profiling is an effective method to evaluate proteolysis. This technique uses the differences between peptide characteristics, namely length of their chain, molecular weight, side R-groups and hydrophobicity, to differentiate and evaluate them. A classical fractionation scheme of milk nitrogenous compounds is presented in Figure 2-22.

Figure 2-22. Summary of common methods used to assess proteolysis in cheese during ripening. [Analytical techniques are highlighted in bold (Sousa et al., 2001)]

2.6.2.2 **pH 4.6 fractionation**

This fractionation is based on the fact that the isoelectric point of casein proteins is around pH 4.6. At this pH, whey proteins remain soluble in the serum while casein proteins flocculate and precipitate gradually or more rapidly by centrifugal force. Although casein proteins are insoluble, it doesn't necessarily mean that their hydrolytic products (large peptides, small peptides and amino acids) are also insoluble. Depending on the characteristics of the peptides (chain length,

size, nature of amino acids, hydrophobic/hydrophilic balance, etc.) they can be soluble or insoluble in different solvents at various pH values.

In this method, a dilute acid is added to decrease the pH to obtain two distinct fractions. One is the pH 4.6 insoluble (casein proteins) and soluble fraction (regarded as serum). The serum contains whey proteins, non-protein nitrogen, ionic fractions and trace elements, lactose residue and the –potentially- smaller peptides and amino acids produced during the fermentation, depending on the extent of hydrolysis.

Although casein proteins have been removed during the previous step of fractionation scheme, the remainder of proteins and peptides are still present in pH 4.6 soluble fraction. To evaluate these components, we need to further discriminate the nitrogenous contents. Aqueous solutions of ethanol (mostly 70%) have widely been used to fractionate peptides in cheese industry (Bansal, Piraino, & McSweeney, 2009).

2.6.2.3 **Trichloroacetic acid (TCA) fractionation**

TCA is generally regarded as a classical protein/peptide precipitating chemical. A various range of its concentrations have been used (from 2% to 12%) depending on the degree of fractionation targeted (Polychroniadou, Michaelidou, & Paschaloudis, 1999). The logic behind its concentration selection is the larger the size of target proteins or peptide to precipitate, the lower the concentration of TCA to be used. In cheese industry, this method has been widely used to precipitate smaller peptides from water soluble extract or pH 4.6 soluble fractions. It has been reported that the peptides smaller than 22 residues to precipitate with this extractant. It was also reported that the peptides with less than 7 amino acid residues were precipitated with 12%TCA. The extraction results of 12% TCA was reported to be similar to 70%Ethanol (McSweeney & Fox, 1997). In the case of ripened cheese, the LAB proteolytic system is reported to have a higher contribution in 12% TCA soluble nitrogen fraction (Bansal, Piraino, et al., 2009). The hazardous nature of TCA should be considered for peptide extraction as it is difficult to get rid of it to obtain purified peptides.

2.6.2.4 **Phosphotungstic acid fractionation**

Dodecatungstophosphoric acid (commonly named Phosphotungstic acid (PTA)) is regarded as an efficient protein precipitant with high discrimination. Different concentrations of PTA have been used for fractionations, however, 5% PTA is most widely used (Bansal, Piraino, et al., 2009). It is shown that only free amino acids and small peptides (molecular weight less than 600 Da) are soluble in 5% PTA. Therefore, it is regarded to be an efficient method for highly hydrolysed proteins. In the cheese industry this factor is useful in secondary proteolysis of cheese proteins which is regarded to be mainly as a result of starter and non-starter bacterial enzymes.

2.6.2.5 **Soluble Nitrogen Indices**

As the protein undergoes cleavage new nitrogen sites become free. The solubility of newly revealed parts and their affinity toward different chemicals varies depending on the size of the resultant peptide chains and the side groups of the amino acids, as well as the extent of the enzymatic activity and cleavage thereof.

The water soluble nitrogen fraction is used to determine the amount of water soluble polar volatiles that affect cheese flavour. This fraction includes peptides, amino acids, mineral salts, lactose, lactic acid and a vast variety of volatile compounds (Taborda, Molina, Martínez-Castro, Ramos, & Amigo, 2002).

Different types of solvents including 5% NaCl, 2, 5, 7 and 12% Trichloroacetic acid, 50 and 70%Ethanol, and 0.85% Picric acid are used to extract nitrogen compounds generated during proteolysis include (Reville & Fox, 1978). Also, phosphotungstic acid (PTA) with H_2SO_4 (5%) is used that extracts only free amino acids and peptides smaller than 600 Da (L. M. L. Nollet, 2009). Water-soluble peptides are characteristic of particular varieties of cheese and related to the specificity of the proteinases and peptidases of its microflora. The peptides present in the ethanol-soluble fraction of the pH 4.6-soluble extract contribute to cheese flavour as they are smaller sized chained (Poveda et al., 2006).

As the proteolysis progresses during ripening, the amount of soluble nitrogen (SN) increases. pH 4.6-Soluble Nitrogen are suitable representatives for the extent of casein breakdown.

2.6.2.6 **Free amino acid evaluation**

The characterisation of proteolysis in cheese can be defined by the formation of free amino acids (Creamer, 1970). The breakdown of protein chain will continue by the proteolytic enzymes and this at some point result in formation of individual amino acids. Free amino acid formation was reported to be in correlation with cheese flavour and off-flavour (Are Hugo Pripp, Skeie, Isaksson, Borge, & Sørhaug, 2006). The concentration of free amino acid in cheese is mainly related to the activity of LAB proteolytic system (Fox et al., 2017).

Chromatographic methods have been used widely to identify the level of free amino acids (Nollet & Toldrá, 2009). The advantages of this method were mentioned to be its high reproducibility of the results and the ability to work with small samples sizes.

2.6.2.7 **Polyacrylamide gel electrophoresis**

Polyacrylamide gel electrophoresis (PAGE) provides a versatile and high resolution method for fractionation of molecules based on their size, conformation and net charge in the electrophoresis gels. Properties, such as molecular sieving, and the ability to use casein standards simultaneously, thus, detecting small changes in the specific casein fractions are the advantages of this technique (Ledford, O'Sullivan, & Nath, 1966).

In this technique, after staining and de-staining steps, visible bonds of different proteins and peptides can be detected. Those bands can be compared with the standard band which is being run in the gel simultaneously, and finally they can be determined.

2.6.2.8 **High Performance Liquid Chromatography**

High performance liquid chromatography (HPLC) is used to efficiently to separate different chemical components based on their molecular weight and physicochemical characteristics.

Although the different techniques for this method are used, but Reverse-phase HPLC (RP-HPLC) is most commonly used for peptide profiling (Bansal, Piraino, et al., 2009).

In this method, extraction of the desired fraction of milk/cheese is carried out using a solvent or special pH extracts required. The results from this methods are accurate and highly repeatable thus providing a better judgment about the cheese matrix composition (Parente, Patel, Caldeo, Piraino, & McSweeney, 2012). Commonly the pH 4.6 extracts fractionation is used to obtain chromatographic data on peptides (Nollet & Toldrá, 2009).

2.6.2.9 **Capillary electrophoresis**

Capillary electrophoresis (CE) is an accurate method for peptide profiling in cheese. It is normally performed in a buffer filled capillary which starts from a source vial and ends in a destination vial, based on the ionic charges of the peptides and proteins — electro-osmotic flow affinity. This method is faster compared to the conventional Electrophoresis and coupling it with in-line UV and UV-Visible detectors provides more accurate information. It is a suitable method for peptide profiling of cheese and accurate results are obtainable on primary proteolysis of caseins (Otte, Ardoe, Weimer, & Soerensen, 1999).

2.6.2.10 **Mass Spectrometry peptide profiling**

Although chromatographic and electrophoretic methods are able to provide valuable information about the proteolysis products, the use of mass spectrometric defining peptides produced during proteolysis is emerging during recent years (Piraino et al., 2007). The advantage of this method is the shorter analysis time compared to other methods. An elegant mass spectrometry technique named quadrupole time-of-flight (Q-ToF), has been recently developed and is being used widely to characterize the peptides in food materials analysis (Alomirah, Alli, & Konishi, 2000). This method reported to be capable to characterize the proteolysis in cheese (Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Piraino et al., 2007). It was also used to characterize the proteolysis in yoghurt resulting from LAB (Fedele, Seraglia, Battistotti, Pinelli, & Traldi, 1999).

2.7 Development and assessment of quality attributes and functional properties of cheese during ripening

2.7.1 Introduction

The main quality attributes of cheese are its texture and flavour. Texture collectively describes the specific sensory perception of cheese (Everett & Auty, 2008) and normally deals with characteristics such as flowability, stretchability, hardness, softness, meltability, spreadability of the cheese. These attributes are known as functional properties of cheese (Gunasekaran, 2002).

Functional properties of cheese are important for both the consumer and producer. For example in dishes containing cheese that are cooked at high temperature, such as pizza and pasta, the softness, flowability , meltability, strechability of the cheese determines the quality of the final food product (Richoux, Aubert, Roset, & Kerjean, 2009). On the other hand, the producers use functional properties of cheese for quality control purposes.

In most cheese types ripening is the most important step in the development of texture and flavour despite its costliness, variety and complexity of effective factors (Everett & Auty, 2008; Sousa & McSweeney, 2001; Upadhyay et al., 2004; Wang et al., 2011).

Rheology is the science of flow and deformation study of a material (Gunasekaran & Mehmet, 2002; Tunick, 2000). Rheological properties of food material can be related to their microstructure and macrostructure, composition as well as its physicochemical specifications (O'Callaghan & Guinee, 2004). Considering these, all of the parameters reviewed so far in this chapter including the structural arrangements and the parameters involved in their rearrangements, characteristics of cheese coagulum and curd formation, degree of hydrolysis, and the interactions of casein micelles influenced by CCP solubilisation, pH, salts and moisture content are all defining parameters. The age of cheese and extent of ripening can also affect these parameters and therefore the textural properties undergo an ongoing change up to the consumer table. Textural attributes of a food material are also of the same importance as its rheological

characteristics. These terms are used interchangeably in food research, however the textural attributes are a destructive test wherein a defined force is applied to the sample resulting in loss of structural integrity (Tunick, 2000).

2.7.2 Cheese as a viscoelastic material

As explained before (cheese coagulation) cheese curd is a matrix of interconnected and overlapping strands (O'Callaghan & Guinee, 2004) of casein micelles which had initially been aggregated and clustered to form a gel in a three dimensional network(Lucey et al., 2003). This network is considered as viscoelastic material meaning it shows both viscous and elastic behaviour. In other words, it behaves somewhere between an ideal elastic (Hookean; where the applied energy is fully stored in the sample) and an ideal viscous (Newtonian; in which all the energy is dissipated) material (O'Callaghan & Guinee, 2004). The viscoelastic behaviour of the cheese gel can provide information on its microstructure state. (Tunick, 2000; O'Callaghan & Guinee, 2004; Lucey et al., 2003; van Vliet, Lakemond, & Visschers, 2004). There is a range where the stress applied to the sample is linear with the resultant response (O'Callaghan & Guinee, 2004). This range with such a linear response is known as linear viscoelastic (LVE) range (Figure 2-23). Within LVE the viscoelastic responses of sample are linear regardless of the extent of strain and stress (Gunasekaran, 2002) and lays within 0.01-0.05% of strain for most cheeses (Walstra, 1982).

There are two categories of experiments to evaluate the viscoelastic characteristics of milk gels and cheese; large strain and small strain tests. Large strain tests impose a higher deformation in the structure of the specimen while in small strain tests the parameters are selected in a way that the specimen can retain (mostly) its structure by rearrangement of the compositional /structural interactions (Everett & Auty, 2008). In this study the small amplitude (strain) oscillatory rheology was utilized.

Figure 2-23 LVE limit of cheese at a strain sweep test with a constant frequency (Gunasekaran, 2002).

2.7.3 Dynamic rheometry

Knowing cheese as a viscoelastic material, an approach to assess its rheological properties is to measure both elastic modulus (G') and viscous modulus (G") (and also the ratio of viscous to elastic moduli (G"/G') which is known as phase angle (tan δ)) of sample over a period of time (Lucey et al., 2003). One of the methods to assess these characteristic rheological properties is dynamic low- amplitude oscillatory rheometry (DLAOR). In this method a continuous low-amplitude sinusoidal torsion stress or deformation is applied on the sample and correspondent strain or stress are monitored, respectively (Van Vliet, 1991). The selection of parameters in this method is based on applying extremely small strains (less than 0.05%) to the sample in order not to cause permanent structural damage to it. Therefore, this method is an appropriate method to evaluate the gel formation step of cheese.

In order to collect information on gel formation quality of cheese and the strength of gel network three types of experiments can be approached:

- Frequency sweep (at a constant temperature)
- Temperature sweep (at a constant frequency)
- Amplitude sweep (at constant temperature and frequency)

In order to have accurate results, initially a strain sweep is performed (at a constant frequency) to define the LVE range where the moduli will only be a function of time, not the magnitude of stress or strain. Knowing this limit, a strain is selected from the range and a frequency sweep at constant temperature and strain is performed (for accuracy of the results within three orders of magnitude e.g. 1 to 100 rad/s) (Tunick, 2000). Viscous (G") and elastic (G') moduli are recorded to evaluate the behaviour of specimen. An example of frequency sweep test for Mozzarella cheese is shown in Figure 2-24.

Figure 2-24. Frequency sweep test for Mozzarella cheese. Trends of G', G" and complex viscosity (η*) are presented (Tunick, 2000).

The frequency dependence of dynamic moduli provides information on the nature of interactions within the specimen and gives insight to the strength of interactions which hold the network together (Tunick, 2010).

2.7.4 Large-scale deformation rheometry

Another approach to evaluate the textural attribute of cheese is to apply a strain to cheese sample that is large enough to break down the network of gel, curd or cheese (Gunasekaran & Mehmet, 2002). These tests usually result in visible fracture (Fox et al., 2017). This happens when the strain lies outside of the LVE range of cheese sample (~0.1-0.9 for compression and much larger than 1 for shear deformation). The main reason to conduct this test is to mimic the behaviour of cheese during further processing (shredding, dicing, slicing, portioning) or eventually sensory characteristic during eating (mastication) (O'Callaghan & Guinee, 2004). It also gives insights to the manufacturers of the food material who are taking benefits from functional properties of cheese as an ingredient in other food systems. In order to gather information about large strain rheological behaviour of cheese, some empirical and fundamental methods have been developed. These include penetration, compression, cutting, curd tension, bending and creep tests. Among these the compression test is the most commonly used in cheese (Gunasekaran, 2002; O'Callaghan & Guinee, 2004).

2.7.4.1 Uniaxial (linear) compression

Due to simplicity of sample preparation and its versatility over different cheese varieties, and also the ability to mimic the chewing compression by molar teeth of consumers, this method is the most common large strain test in cheese assessments (O'Callaghan & Guinee, 2004). In this method a linear deforming stress is applied by a linear moving plunger to a sample (prepared in rectangular or cylindrical shape) which is placed on top of a fixed plate (parallel to the moving plunger) up to a pre-defined percentage of sample's original height (Figure 2-25). A force-strain response is continuously recorded.

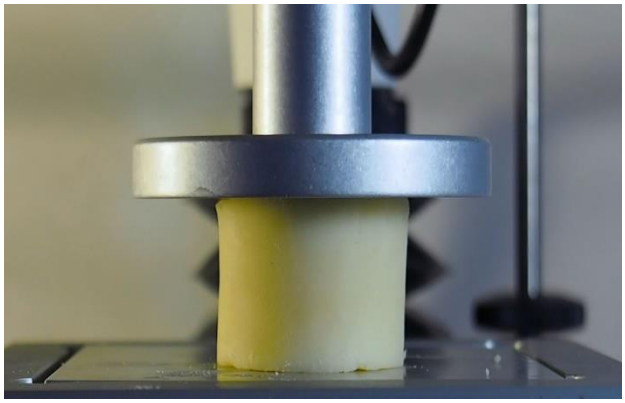


Figure 2-25. A cylindrical cheese sample between two parallel plates of a uniaxial compression machine.

Texture profile analysis (TPA) is a method to assess the large strain textural properties of cheese (Gunasekaran, 2002). In this method a two-stoke compression is applied to roughly simulate the real mastication of cheese samples and give a better instrumental measurement for the sample (Tunick, 2000). The crosshead approaches the sample at a defined speed, until it reaches the specimen and triggers the force-time recording. The speed of compression is also defined and not necessarily the same as crosshead speed before it touches the sample. It applies the force (the area is known) and compressed the sample down to a predetermined strain (e.g. 0.75). The load is the removed with the same rate as load exertion. This is repeated in two so-called bites. A force-time curve of a TPA analysis is presented in Figure 2.26 and the textural attributes which can be derived from the curve is presented in Table 2.3.

Figure 2-26. A typical texture profile analysis curve (Tunick, 2000).

Table 2-3. Functional properties derived from TPA curve and their method of calculation.

Term	Definition	Obtained from	Calculation (and units) using Figure 1
Fracturability	Force with which food fractures	Force at first significant break (if any) in curve	Height of F (N)
Hardness	Force needed to attain a given deformation	Maximum force during fist compression cycle	Height of H (N)
Adhesiveness	Work needed to overcome attractive force between food and other surface	Force area of negative peak (if any) following first peak	Area of A (J)
Cohesiveness	Strength of food's internal bonds	Ratio of positive force area of second peak to that of first peak	Ratio of A2 area to A1 area (none)
Springiness	Rate at which deformed food returns to original condition after removal of force	Height specimen recovers between end of first compression cycle and start of second	Length of S (mm)
Gumminess	Force needed to disintegrate a *semisolid* food to a state ready for swallowing	Product of hardness and cohesiveness (N)	
Chewiness	Work needed to masticate a *solid* food to a state ready for swallowing	Product of hardness, cohesiveness, and springiness (J)	

A significant correlation between some sensory attributes and instrumental evaluation by TPA has been observed in the literature (O'Callaghan & Guinee, 2004), however this has been a source of controversy (Tunick, 2000).

2.7.5 Factors affecting functional properties of cheese

Different cheese varieties —briefly categorized in section 2.1— are obtained using various cheese-making practices. The basic steps for the production of most cheeses is presented in Figure 2.27.

Figure 2-27: Basic steps in cheese manufacturing (Bennett & Johnston, 2004).

These steps have to be carefully taken in order to result in physicochemical and microbiological reactions during cheese making including size reduction, dehydration, acid production, pH changes, ionic strength changes and enzymatic reactions. The extent of these reactions affect the characteristics of the produced cheese and thus various types of cheeses are obtained.

Differences in cheese functional properties are related to several parameters including cheese milk composition, type of coagulant, heat treatment history of cheese-milk, type of starter bacteria, acid production rate, pH at renneting and mineral concentration especially of Calcium (Lucey et al., 2003).

The main parameters affecting the functional properties of cheese include:

2.7.5.1 **Milk composition**

The fat, protein and other compositional characteristics of milk changes during lactation period. These changes are very important in cheese texture and flavour. Furthermore, milk is a source for proteolytic enzymes i.e. plasmin and cathepsin. These enzymes affect the hydrolysis of milk proteins during cheese making (Buchin et al., 1998).

UF concentrated cheese milk is an example of compositional effects of milk on cheese quality attributes (explained before in this chapter). Waungana and fellow researchers (1998) assessed the small oscillatory responses of gel formation attributes as a result of UF-increased casein content of cheese-milk at two different pH values (natural pH and 6.50) (Figure 2-28). In both pH values an increased level of casein concentration resulted in a higher storage modulus (G') of the gel Also fat content of cheese milk is an important parameter in defining the meltability of cheese (Rüegg, Eberhard, Popplewell, & Peleg, 1991).

Walstra and van Vliet (1982) reported the impact of an increase in the ratio of moisture to protein on rigidity of the cheese curd. They observed a weakening effect of curd rigidity when the moisture was increased in the above mentioned ratio, which consequently affected other functional properties of cheese (e.g. softness, shreddability and meltability)(Walstra, 1982).

Figure 2-28. Storage modulus (G') as a function of time during Renneting normal pH of milk (A) skim milk (open circle), 2X UF concentrate (open square) or 3X UF concentrate (open triangle), and at pH=6.50 (B) skim milk (filled circle), 2X UF concentrate (filled square) or 3X UF concentrate (filled triangle) printed from (Waungana et al., 1998).

2.7.5.2 **Quantity and type of coagulant**

See section 2.4.2.1.

2.7.5.3 **Heat treatment history of the cheese-milk**

Cheese quality is strongly affected by heat treatment of the cheese milk as shown in Figure 2-29 (Guinee et al., 1998) and Figure 2-30. In a very general classification scheme cheeses can be grouped in raw-milk cheeses and pasteurized-milk cheese (McSweeney, Ottogalli, & Fox, 2004). Heat treatment of cheese-milk is important mainly because of the heat sensitivity of whey proteins. These proteins can be denatured by pasteurization temperatures(Singh, 2007) (see section 2.1) and therefore affect the coagulation of casein protein. The mechanism of this reaction arising from the fact that at higher temperature the extent of denaturation of serum proteins will

increase and consequently these altered proteins will make complexes with casein proteins. The presence of these complexes would reduce the accessibility of network forming interactive sites on the micelles and therefore the extent of gel-firming pattern would be lessened.

In the current work which is focussed on understanding the importance of long fermentations by starter cultures on the subsequent curd formation of the cheese milk it is necessary to choose a pasteurisation method that has a minimal impact on the gelation. Therefore a heat treatment with the lowest temperature is likely to be optimal.

Figure 2-29: Effect of temperature on gel formation of milk (Guinee et al., 1998).

Figure 2-30. Storage modulus (A) and loss tangent (B) of Cheddar cheese at different ripening times (circle: 2days, square: 14 days and triangle 1 month) affected by temperature (J. A. Lucey et al., 2003).

2.7.5.4 **Type of starter**

Different starter cultures are being used in cheese industry. The enzymatic systems of these starters vary resulting in the different patterns of enzymatic activities (Law & Haandrikman, 1997; Tan, Poolman, & Konings, 1993). These differences are initiated during lactose fermentation and continue in the catabolic enzymatic activities during cheese ripening. It is thus necessary to choose bacteria for this study that have adequate catabolic enzyme activity. The peptide profiles of two different mediums (MPC85 and sodium caseinate) following pH-stat long term fermentations using five *Lc. lactis* sub-species have been assessed in our preliminary experiments and the results are provided in Chapter Four of this thesis.

2.7.5.5 **Acid production rate**

The rate of acid production affects the extent of protein hydrolysis in cheese (O'Keeffe et al., 1975). For example, higher rates of acid production increase the rate of coagulant activity during coagulation. It is well established in the current literature that the rate of acid production is influential on CCP dissolution and therefore micellar rearrangements tendencies (Patrick F Fox, Timothy P Guinee, et al., 2017a) which eventually defines the textural attributes of cheese.

Acid production rate is also important in defining the extent of coagulant retention in cheese curd during manufacture. A higher acidification rate subsequently would result in a higher coagulant residue in the rest of process which may affect the proteolysis during ripening and the final product quality (Feeney, Guinee, & Fox, 2002; Patrick F Fox, Timothy P Guinee, et al., 2017a; Guinee, Feeney, Auty, & Fox, 2002; Peter Walstra et al., 2006).

It also determines the mineral content of cheese (as discussed before) and the consequences thereof. Lucey (2004)evaluated the effect of rate of acidification considering Horne's model of casein stability (2.1.2.1) and concluded a decreasing amount of CCP as a result of acid development would increases the phosphoserine groups' (see section 2.1) electrostatic repulsion. This effect eventually resulted in a weakened cheese structure and higher meltability of cheese (if other parameters are constant) (Lucey et al., 2003). Here is also another confounded effects of parameters contributing in cheese quality.

Thus, the rate of acid production is required to be monitored to reach constant functional properties in different production batches of cheese.

2.7.5.6 **pH**

pH value especially in the initial stages of ripening along with the ratio of bound casein-moisture has been reported to be the primary factor determining texture of cheese (Lawrence *et al.*, 1987) (Figure 2-31).

Changes in pH values affect moisture content, CCP solubility, ionic strength, water binding capacity of protein and degree of protein hydrolysis (Guinee et al., 2002; Marchesseau, 1997;

Creamer & Olson, 1982). These parameters affect the functional properties of the resultant cheese.

Figure 2-31. Elastic modulus (G') development in renneted milk (18% protein w/w). Open circle pH 6.67, filled circle pH 6.55, open square pH 6.45, filled square pH 6.3, open triangle pH 6.15 and filled triangle pH 6.0 (Fox et al., 2017).

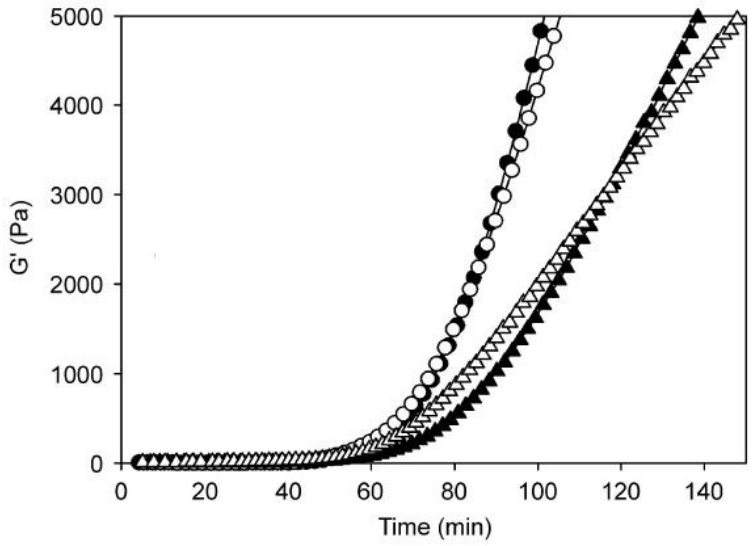


Figure 2-32. Effect of pH on elastic modulus of renneted UF-concentrated milk. Filled circle: pH 6.22, open circle: pH 5.83, filled triangle: pH 5.51 and open: pH 5.29. Reprinted from (López, 1998).

Lopez (1980) reported that the rate of structural rearrangements of cheese gel are dependent on pH during gel formation (López, Lomholt, & Qvist 1998) (Figure 2-32). Deformation at fracture of Gouda and Emmental cheese is reported to be influenced mainly by pH and the amount of

calcium associated to caseins (J. A. Lucey et al., 2003). As we discussed earlier in this chapter, these rearrangements were also dependent on calcium equilibrium. Considering these, it can be concluded that the parameters of cheese making, from gel formation to curd processing and final cheese at day one are all cofounded.

2.7.5.7 **Calcium**

As shown previously (Section 2.1), calcium is a fundamental part of the casein micelle (colloidal) and milk serum (soluble). The balance between these two colloidal and soluble fractions is of high importance. Calcium tendency toward one of those phases is dependent on various parameters. pH seems to have direct effects on solubility of calcium (Watkinson, Coker, Crawford, Dodds, Johnston, McKenna, & White 2001). So, it can be concluded that all of those parameters involved in cheese pH can strongly affect calcium and therefore its roles in cheese texture. (Guinee et al., 2002).

2.7.5.8 **NaCl**

NaCl addition is regarded to be influential on rheological properties of cheese as it increased the ionic strength (and the effects thereof discussed before). Zoon *et al.* (1989) studied the impact on NaCl addition on rheological properties of skim milk gels in a range of 50-300mM. Higher G' values were reported as a result of NaCl addition up to 100mM. When the addition exceeded 100mM, the effect was less significant, while the RCT was retarded at more than 100mM. They reported a 150mM of NaCl to be the upper limit for gel formation at 30 °C. Beyond this limit (500-1500 mM) a decrease in curd firmness was observed (Gouda, 1985).

Guinee (2004) reported an increase in cheese firmness of some studied chasees and fracture stress (including Camembert, Cheddar, Feta) as a result of increase in salt in moisture (S/M) within a range of 0.4-12% (w/w) (Guinee, 2004). However, he concluded this might be due to concomitant effects from other parameters including pH effects.

2.7.6 Confocal laser microscopy of gel microstructure

The structure of cheese and curd is principally defined by the spatial arrangement of their major component (protein, fat, carbohydrate and minerals)(Everett, 2007).

Microscopic methods can facilitate the assessment of microstructure by the power of magnification to reveal the structure at larger scales. Confocal laser scanning microscopy (CLSM) has widely been used to evaluate the microstructure of food (Auty, Twomwy, Guinee, & Mulvihill, 2001). There are various advantages for this method over conventional optical light microscopy including a higher resolution and the ability to change the focal point to image different sections of the specimen.

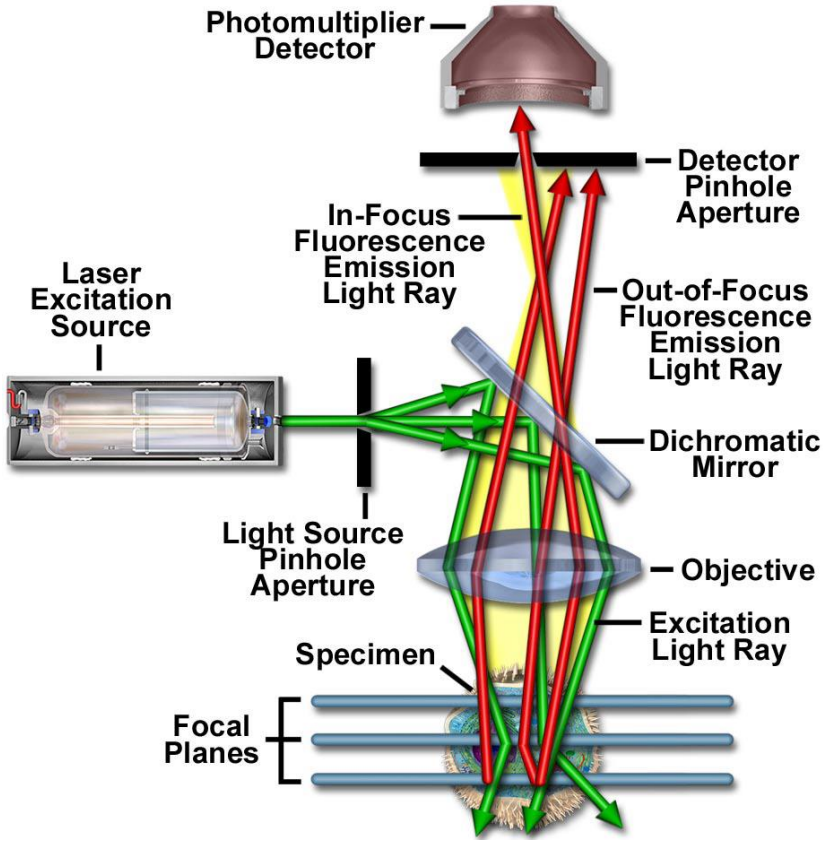


Figure 2-33. Schematic of confocal laser scanning microscope (Claxton, Fellers et al. 2006)

This particular aspect is highly important as it can compensate the adverse effects of sample preparation by offering images from different layers of sample to have access to internal layers. A schematic of the basis of CSLM is presented in Figure 2-33.

2.8 Conclusion

So far we have reviewed the casein proteins and the models to explain their stability in milk. The stabilizing parameters and their impact during cheese processing were also reviewed. Most of the parameters have confounded effects, with pH being one of the most influential factors to rule out others at significant levels.

The importance of bacterial activity in cheese processing and ripening was also reviewed. The importance of their biochemical activities were noted with an emphasis on their proteolytic activities. The importance of proteolytic activities from different sources in cheese texture and flavour were also reviewed. We also have gone through the methods for analysis of the extent of proteolysis.

Possible approaches for assessment of curd rheological and cheese textural attributes have also been considered.

While the importance of starter LAB in cheese ripening is addressed (to some extent) in the literature, the confounded effects from other cheese making contributors have made it impossible to assess the merely effects of starter LAB. On the other hand, an inevitable pH alteration happens during cheesemaking which consequently influences other parameters (including CCP solubility and enzymatic activities) confoundedly. Hence this field of study has become even more complicated. There is a gap in the literature to address the merely effects of starter LAB on the development of cheese structure development in absence of other contributors. If the study of these effects can avoid the effects of pH fluctuations, the interpretation of results can be performed more accurately.

Based on these, an approach was decided to find answers for research questions.

2.9 Research questions

- Does long term bacterial fermentation of the cheese milk under defined conditions (pH-stat, controlled acid production rate, controlled temperature and controlled conductivity) have a significant effect on casein proteins and their ability to form a rennet-induced gel?
- How would the selected strain (or strains) of LAB change the peptide profile of the casein proteins?
- How will the curd formation of a sample obtained from pH-stat long fermentation be affected?
- Is it possible to make a cheese curd from the long fermented samples under pH-stat conditions?
- Is there any difference between the textural attributes of normal cheese and the cheese produced under long fermentation conditions?

2.10 Experimental approach

In order to answer the research questions, the following experimental approaches were chosen based on the literature review:

- To determine an appropriate milk-based medium that would enable the production of a range of cheese-milks with varying fermentation times while maintaining the same final composition;
- To select an appropriate bacteria strain (or strains) to conduct the experiments based on an ability to overcome unwanted parameters (e.g. growth of Bacillus), and to achieve acceptable proteolytic activity;
- To develop an appropriate fermentation method for the long term fermentation of the milk-base medium at a controlled, constant pH;
- To characterise the influence of bacterial proteolytic activity on the treated milk-based medium;
- To evaluate the rennet gelation characteristics of the treated medium;
- To evaluate the textural and structural attributes of the final cheese product.

Finding a method to perform pH-Stat long term (PSLT) fermentations was considered to be of high importance. The uncontrolled bacterial activity would result in pH change and coagulation of milk based material. We decided to keep the pH constant by pH-stat method, however, in order to do so an alkali was required. Addition of the alkali to the longer fermented medium was expected to be higher than shorter time trials, and this consequently would change the formulation. It was also expected to increase the concentration of Na in the form of lactate causing another difference in the trials. It was eventually decided on a materials and methods (will be described in chapter 3) to equalize the samples with different duration of fermentation so as to be able to assess the proteolytic activity of bacteria.

Maintaining the pH constant at the end of fermentations were considered to be achieved through lactose elimination and starter deactivation.

The proper fractionation scheme of fermented medium would enable us to assess the proteolysis. Some well-developed protocols were decided to follow. Different methods (HPLC, MS,) were approached to assess the peptide profile of the samples. It was decided to evaluate the formation of free amino acids in ripened cheese as an important marker of proteolysis.

Appropriate rheological methods to assess the gel formation quality of treated sample was regarded to be of high importance. Well-established DLAOR method was considered as an accurate method to do so. The textural attributes of final cheese were also decided to be evaluated by texture profile analysis method which is the most commonly used method for cheese analysis.

Chapter Three Materials and method

3.1 Development of a model system

3.1.1 Introduction

In an attempt to evaluate the impact of proteolysis by starter bacteria in cheese, a model system was designed. In this system, long term fermentation was carried out using LAB prior to curd formation. This was done due to the fact that the medium during curd formation is liquid thus, the effective parameters (such as pH, presence of other enzymes, temperature, and salt) are easier to control.

The effect of lactic acid bacteria present in the milk environment (Section 2.5.1) were considered as an interference and their contribution to proteolysis was to be inhibited. On the other hand, pH, lactose content, time, temperature, oxygen content and microbial population affect the properties of the final fermented cheese product (Section 2.5.1.2.1). In order to get accurate and repeatable results from the activity of LAB, the effect of these parameters also had to be controlled.

The medium was formulated first and lactic acid bacteria were then added. A key consideration was that in a normal milk system, the pH of milk decreases with fermentation time as the lactose is converted to lactic acid. Thus the final milk produced under normal fermentation conditions would have a range of pHs and therefore ionic calcium concentrations as a function of fermentation time, in addition peptide profile resulting from bacterial mediated proteolysis would differ. Thus, it is desirable to maintain pH stat conditions throughout all of the fermentation times considered. In order to maintain pH-stat conditions, the lactic acid produced by the bacteria needed to be neutralized. In the present study, pH-stat conditions were maintained by continuously adding NaOH to neutralize the lactic acid produced by the bacteria. Under pH-stat conditions, the bacteria were able to continue fermentation for the various durations used in the present study. Considering the fact that LAB need protein for their growth

and development (see 2.4), they were expected to have different pattern of protein hydrolysis. The pattern of proteolysis as a function of fermentation time was monitored and the impact of the resulting peptide profile of the cheese milk on curd formation was characterised

3.1.2 Fermenter design

Figure 3-1 The pH-stat fermentation apparatus consisting a fermenter (left), online measuring unit (centre) and computer (right) to record and process the data.

A pH-stat system was optimised to run the fermentations (Figure 3.1). Using this fermenter, the conditions for bacterial growth such as pH, Lactose concentration, time and temperature were optimised. To be able to study the effect of specific bacterial activity on the protein base of cheese milk, especially casein proteins, the variations in these parameters were minimised. The system consisted of a fermentation tank (2 litre) mounted on the fermentation system (Figure 3.2).

A pH probe (A162, Silamid®, Shott® AG, SI analytics, Germany) (Figure 3.3) was utilized to check the pH inside the fermenter tank (±0.005) and report it to a computer. This digital signal was also sent to a peristaltic pump to inject NaOH (from NaOH reservoir) to the fermenter tank once the pH dropped down to the minimum pH set on the system.

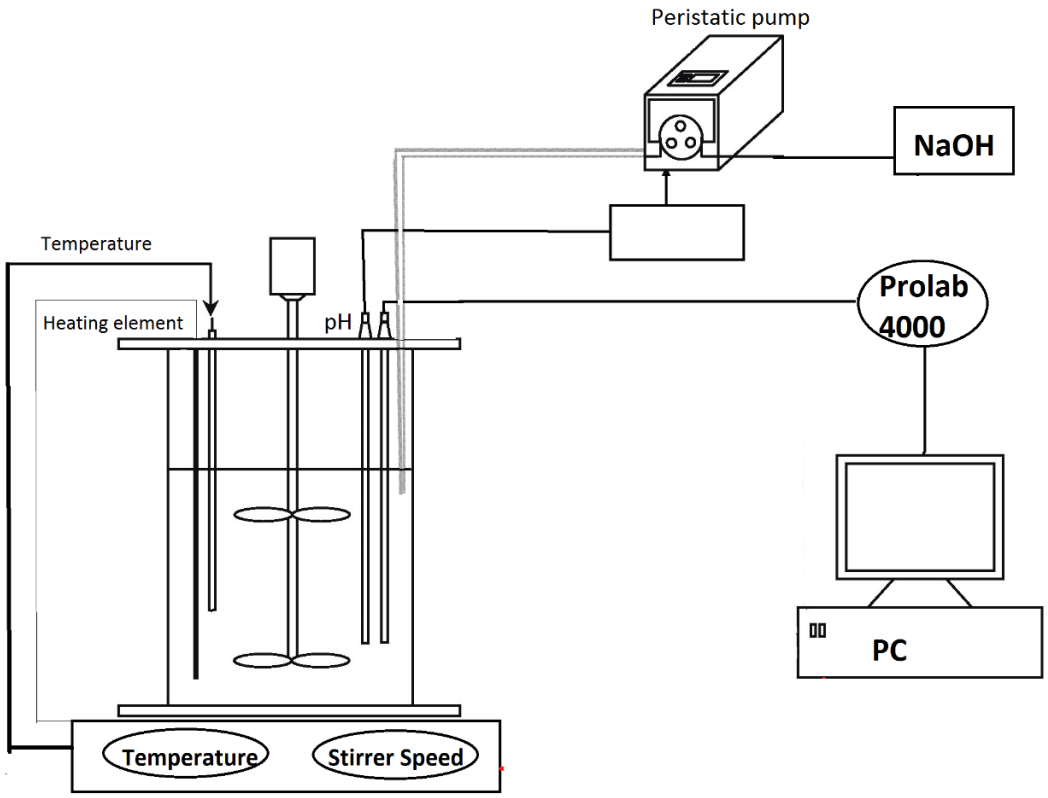


Figure 3-2. Schematic design of the pH-start Fermentation system

Figure 3-3.Schott Prolab 4000 measurement system,

3.1.3 Medium selection

The duration of fermentation by bacteria depends on the amount of available lactose.

The strategy chosen for this work was to select a casein base medium which had minimal lactose concentration so that a specific quantity of lactose could be added to control fermentation. A dairy

based powder was required to provide a casein based colloid with water to assess the effects of bacterial fermentation on proteolysis.

The medium was suggested to be fat-free mainly because the presence of fat would add on the complexity of the cheese milk constituents. This would have consequently made the result interpretation complicated as the fat fraction of cheese milk plays an important role in cheese structure.

Skim milk powder contains a high amount of lactose (about 50%), thus process control would be difficult when a short fermentation time was targeted. Another alternative was caseinate. Casein is precipitated from skim milk. The skim milk may be acidified to produce acid casein or treated with an enzyme, resulting in the rennet casein. The precipitated casein curd is separated from the whey, washed and dried. Water-soluble derivatives of acid caseins, produced by reaction with alkalis, are called caseinates. If the caseinate is produced by the addition of NaOH, the final product is Sodium Caseinate.

Initially, calcium caseinate was used as a medium to provide casein protein because the casein is arranged in a colloidally stable micelle-like form and the product contains no lactose. However, when fermented, this medium did not form a gel on addition of rennet (Figure 3.4) and thus was considered unsuitable for assessing the functional and structural properties. Further preliminary work suggested that MPC85 retains the native micelle structure and has a low lactose (4%). MPC85 was found to be able to form a suitable rennet gel.

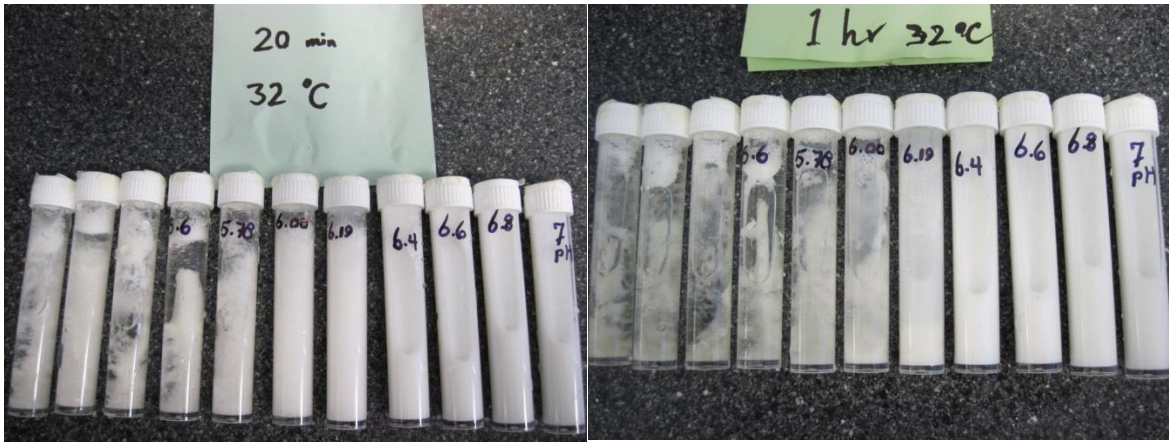


Figure 3-4 Poor gel formation quality of the experimented medium (calcium caseinate) at different pH values (pH from 5 to 7 with 2 values increments from left to right). The pH range was selected to be cover target pH-stat value (i.e. pH=6)

3.1.3.1 **Preparation of medium for long fermentation**

Although addition of sodium azide has been reported to inactivate bacteria already present in the milk-based medium, our findings showed that this chemical was not effective to prevent the growth and development of the gram positive starter bacteria (LAB are gram positive; see section 2.4)(Lichstein & Soule, 1944). Thus, the mediums were pasteurized at 62.5 °C for 30 min. This time/temperature combination was used to have minimal effects on the structure of casein (referring to section 2.2). In particular, this combination of heat treatment was chosen as it is below the denaturation temperature of whey protein.

3.1.3.2 **Why 5% MPC85 4850?**

The formulation was decided to be 5% MPC concentration according to the preliminary experiments. When a medium of 10% concentration was used for the fermentation, the final concentration of casein in the medium after fermentation (addition of NaOH) was still too high to compare the results with any practical, real world fermented medium. The protein concentration of milk is around 3.2-3.3% with a casein content of around 2.8- 3%. We decided to have our final casein content in the medium closer to cheese-milk of industrial cheese making. It is noteworthy

that in some cheese making processes, that UF technology has made it easy to remove some of the non-protein non-fat components. This would result in higher protein contents and is becoming very common practice in some cheese types. However, the majority of cheeses are made from cheese milk with a casein content close to 3%. We designed our experiments in order to have a casein content close to 3% (Equation 1)

[5% MPC x 81% (protein content) x 80% (casein content)] =3.2% (Eq. 4.1)

$$\frac{3.2 \text{ x } 1800}{(1800+100)} = 3.03$$

After addition of NaOH (or lactate in the shorter fermentations to have the same dilution), this ratio would be ~3%.

3.1.4 Lactose adjustment

Considering all the optimum conditions (temperature, pH, water activity, nitrogen source, etc.) for growth of LAB, the lactose content would be a restricting parameter in a continuing fermentation. Hence in order to continue the fermentation (regardless of pH-stat conditions or not), the fermenter content must be formulated to ensure an adequate amount of lactose is presented for bacteria. In preliminary experiments, different lactose contents were utilized and the required amount for the desirable duration was defined by trial and error.

Each fermentation trial was continued until the lactose content was fully utilised. At this point (Lactose elimination point, LEP), no further changes in pH took place and the pH-time graph remained steady and unchanged.

The peristaltic pump was set to start when the pH reached a pH 0.05 value lower than the set point. This means the end point of all of the trials were within 0.05 pH units of the set point. The final pH of all samples was then adjusted using NaOH in order to have consistent pH values at the end of all trials. The pump speed was adjusted in a way, that in combination with the mixing speed

inside the fermenter, the pH value did not exceed the set point as it was slow enough not to inject too much NaOH when the pump was working.

As the lactose content of MPC85 was only 4%, it was easy to adjust our fermentation trials durations. For all of the fermentation durations considered, we needed to add some lactose to the fermenter to supply the bacteria with enough carbon source. The amounts required for each fermentation and the added lactose is reported in Tables 4.2 and 4.3.

3.1.5 pH adjustment

Lactose solutions have minimal electrical conductivity while lactic acid, due to the presence of ions, has a high level of electrical conductivity: thus the conversion of lactose to lactic acid can be effectively monitored using conductivity. The NaOH, continuously added to the medium to keep the pH constant, also increases the conductivity. As elaborated previously, pH is a defining parameter in cheese making and its variations can easily affect all other aspects. In the present study, the aim was use a constant pH value in order to prevent the consequence fluctuations explained before.

It should be noted that the pH history of casein proteins is an important parameter. Their natural pH is ideal for their native form, however, exposure to variation in pH affects their stability. In order to obtain a suitable range of pH to carry for the fermentation trials, the following aspects were considered:

1. The target pH should be as low as possible to eliminate the possibility of bacillus growth in our fermenter. Their presence and activity could result in parallel proteolytic activity which can make our results difficult to interpret.
2. The pH should be as close to native pH as possible to eliminate the possibility of pH induced changes in the casein micelle structure.
3. The solubility of CCP and other minerals is pH dependent (Figure 3-5). This can be directly (by dissolution of structural ions) and indirectly (by changing ionic balances and ionic strength)

4. The pKa value of lactic acid is about 3.9; our operational pH was chosen to be higher than this value and therefore most of the lactic acid would exist in its dissociated form of it salt: lactate.

Figure 3-5 Solubility of citrate, ionic magnesium, inorganic phosphate and ionic calcium as a function of pH (Walstra et al. 2005).

Based on these considerations, pH 6.00 ± 0.05 was selected to be the target pH for our pH-stat fermentation trials. At this pH, the gel formation characteristics of the casein micelles still similar to natural pH (explained in Chapter 2). As discussed before in Chapter 2, most of rheological parameters of the fermented milk down to pH~5.8 are still relatively similar to the natural pH of milk. This was important as we wanted to correlate the results of our long fermentation to the gel formation quality of the fermented milk. Therefore, it seems necessary to have the side effects of the treatments (fermentation) as moderate as possible in this regard so as to be able to judge on the effect of the treatment itself. For instance, here we want to evaluate the effect of long term fermentation on curd formation and if the pH at which the fermentation happens to be a turning point in curd formation, we would not be able to judge on the effects of our treatment.

3.1.6 Oxygen removal

The oxygen level of the reactor was kept as low as possible and this was important as the fermenting culture used in this study (*Lc. lactis*) belong to an anaerobic genus. Thus it was very

important to help the target bacteria dominate the environment. A particular problem in this system was the need to mix the system (to disperse the NaOH upon addition) coupled with the high foaming properties of milk proteins which could lead to the entrainment of headspace air into the medium.

In the preliminary experiments to mitigate oxygen entrainment the headspace of the fermentation vessel was flushed continuously with N2 gas to remove the oxygen. However, a comparison of fermenter data from the N2 flushed system to the non-flushed system indicated that there was no significant difference. Therefore, for the remainder of the work N2 gas was not used mostly over concerns of health and safety considerations for our long experiments in which the odourless nitrogen gas had to be injected the whole night to the fermenter.

3.1.7 NaOH addition and concentration

NaOH 1N was utilized to maintain the pH-stat conditions. Once the fermentation is triggered by the addition of lactic acid bacteria to the fermenter, a continuous reduction in pH was expected. The speed of this reduction was very low at the beginning, as this is the time the bacteria are adapting themselves with the new environment. The reason for this slower speed is the fact that it takes a while for the bacteria -even if all environmental conditions are favourable for them- to pass the lag phase and reach their logarithmic growth phase. The nutrient that limits bacterial growth is lactose and thus the endpoint of this process as mentioned above is the elimination of lactose. Upon NaOH addition, there was the risk of micro-domains of high pH depending on the concentration of the NaOH, nozzle size and stirring speed of the fermenter. The more concentrated the NaOH, the bigger the impact of these concentrated domains would be on micellar form of caseins. As explained in chapter 2 (section 2.2), these extreme pH ranges can disrupt the micelle and if this happens frequently (at each injection which may be a few hundred times at our longer fermentations), there will be a considerable amount of casein affected as a result of this ‘spot clotting’. The slow speed of alkali addition and the low concentration of alkali resulted in satisfactory results.

3.1.8 Adjusting the ionic strength and conductivity

During the fermentation, lactic acid bacteria produce lactic acid and the pH-stat system adds the required amount of NaOH to neutralize it (producing sodium lactate). Consequently, the samples with longer duration of fermentation will have higher amounts of lactate compared to the samples with shorter fermentation times. As the ionic strength is an important parameter in protein/peptide behaviour (see Section 2.1), similar values were required between the samples that were fermented for various periods. In order to maximize the similarity of samples and consequently analysis reliability we found it necessary to adjust all the samples' conductivity to a predetermined value. This value was determined through a series of preliminary experiments and the framework of those experiments is briefly presented.

Sodium lactate salt is a by-product of neutralization when lactic acid and sodium hydroxide are the reaction substrates. Thus, sodium lactate was used to adjust these parameters. Using this method, sodium lactate content was correlated with NaOH consumption. A spreadsheet was designed to calculate the required amount of sodium lactate.

One of the particular aspects of this study was to evaluate the effect of long-term pH-stat fermentation on our target proteins. As fermentation goes on, the lactose is converted to lactic acid and this metabolic pathway provides energy for our starter bacteria. To keep the pH constant, we needed to neutralize the acid at the same pace as it is produced. In this study we used NaOH 1N to attain this goal. It is obvious that the longer the fermentation period, the higher the conductivity of fermenter contents would be. In samples with different conductivity (confounded with ionic strength) the casein micelles stability would be different, and hence the behaviour of them during any downstream step (either physicochemical analysis or processing) would be different.

As sodium lactate salt is product of neutralization when lactic acid and sodium hydroxide are the reaction substrates, this was used to adjust the conductivity value to mimic the reaction happening inside the fermenter. Thus, the longest fermentation time and its sodium lactate

content according to NaOH consumption was considered as the highest quantity. The amount of sodium lactate required to adjust the conductivity values of samples at different (shorter) fermentation times (compared to the longest one) were calculated accordingly. However, with the addition of commercial concentration sodium lactate, the pH increased significantly (toward pH 12). Although lactate is a salt, its aqueous solutions show alkaline pH ranges. To avoid unwanted increases in pH a solution of Na-lactate was made in the lab with a pH identical to the fermented pH. It included a mixture of NaOH 1N with concentrated lactic acid to make a pH 6 solution. When this solution was used to adjust the conductivity of the fermented milks, the pH of the samples was remained unchanged. This conductivity adjustment process simulates the same reaction which is going on inside the fermenter (*i.e.* lactic acid production from bacteria and added alkali).

3.2 Materials

3.2.1 Bacterial culture

Five different strains of *Lc. lactis (*two lactis strains: *Lc. lactis* spp*. lactis* 347 and ML8) and four cremoris strains *Lc. lactis* spp*. cremoris* 2338, 2448 and HP) were selected for the study. These bacteria were prepared by sub-culturing from Fonterra (Fonterra Research Centre, Palmerston North, New Zealand) frozen stock. These were delivered from Fonterra microbiology in two sets of 2 mL vials of RSM and M17 broth. The preparation of inoculum from these vials are presented in 4.3.1.

3.2.2 Milk protein concentrate (MPC85)

Milk Protein concentrate (MPC 85) was obtained from Fonterra (Fonterra, New Zealand). This material can be produced with different specifications for different target uses. The digits 85 indicate the protein content of it (roughly). The proportion of whey protein to casein in this powder in not altered from the original milk. Like many other dairy powders, the solubility of this

powder can be adjusted by inclusion of some steps in the production procedure. The MPC85 4850 was selected as it contains the closest calcium content to natural milk. The importance of calcium on cheese structure is reviewed in detail in chapter 2.

3.2.3 Skim milk powder

Low heat skim milk powder (LHSMP) for bacterial propagation and also model development were obtained from Fonterra (Fonterra, New Zealand).

3.2.4 Sodium caseinate

Sodium caseinate Alante 190 was obtained from Tatua (Tatua, New Zealand).

Table 3-1. Compositional analysis for MPC85 4850 (Fonterra, product bulletin 071)

Component	**Quantity**
Protein (N x 6.38) (g/100g)	81.5
Moisture (g/100g)	5.7
Fat (g/100g)	1.5
Total Carbohydrate (g/100g)	4.5
Ash (g/100g)	6.8
Inhibitory substances (IU/mL)	<0.005
Sodium (mg/100g)	80
Calcium (mg/100g)	2130

3.2.5 Rennet

Double strength calf rennet, contained 580 IMCU/mL, was obtained from Renco (Renco, Eltham, New Zealand). For daily uses, it was prepared in 1:10 dilution using Milli-Q water (Millipore Corporation, Bedford, USA) and the diluted portion was not used for after 12 Hours.

3.2.6 Lactose

Pharmaceutical grade HMS pasteurized Lactose powder was used for lactose adjustments (Fonterra New Zealand)

3.2.7 Chemicals

All of the chemicals used in this research were of analytical grades provided from well-known chemical supplying companies.

3.3 Methods

3.3.1 Powder rehydration

The MPC85 powders were rehydrated into a colloidal solution. Considering the final concentration of the solution based on dry weight, the required water (Milli-Q water) was freshly acquired and the required volume was transferred into a sterilized stainless steel beaker. An overhead stirrer with propeller (4 blade, 2 cm radius) mounted on top of the beaker while sitting in a temperature controlled water bath was used to mix the solution. The temperature was set at 61 °C and the water was stirred at less than 4000 rpm which was found to be enough to make a vortex in our operational volumes. Once the temperature of water reached 60 °C, pre-weighed MPC85 powder was added to the vortex slowly to prevent any big clump formation. Once all the powder was added to the water, the rotational speed was decreased to 1000 rpm. The position of propeller was always as close to the bottom of the beaker as possible. This was important to minimise oxygen entrainment into the solution as a result of propeller mixing in a protein rich medium. The selection of temperature was based on the fact that MPC85 or other high protein MPCs are hard to rehydrate at lower temperatures unless their formulation is altered during their manufacturing process (Crowley et al., 2015). The MPC85 used in these experiments was a standard non-modified MPC85.

The stirring step was continued for 1 hour and the water bath set point was then changed to 32 °C. The water in the water bath was gradually replaced, over about 3 minutes, by cold water until a 32 °C was reached. On cooling the rotation speed was decreased to 400 rpm and the stirring was kept on for one more hour before the medium was aseptically transferred to the fermenter.

3.3.2 Fermenter sanitization

In order to sterilize the fermenter, the fermentation tank was pre-washed. Then all of the parts except for probes and sensors were re-installed and the openings sealed by aluminium foil. The fermenter was then placed in the autoclave chamber to be autoclaved at 121°C for 30 minutes following by cooling time down to 69 °C inside the chamber. It was then removed from autoclave chamber and left for further cooling at ambient temperature.

3.3.3 Bacterial stock preparation

A mixture of *Lc. lactis* subsp. l*actis* 2338 and *Lc. lactis* subsp. *cremoris* HP were chosen based on preliminary experiments. This selection of *Lc. lactis* subsp. *cremoris* 2338 was based on its ability to produce acid and thus decrease the fermenter's pH fairly fast while *Lc. lactis* subsp. *cremoris* HP was chosen due to its high proteolytic abilities arising from its PI type cell envelope proteinase (Lambie, Altermann, Leahy, & Kelly, 2014).

In the course of starter preparation, several sub-culturing steps were required. The methods outlined below were utilized to prepare the inocula.

3.3.4 Inoculum propagation

M17 agar is the most commonly used medium for selective growth of *Lactococcus*. This medium is formulated to suit the growth and development requirements of these bacteria (Terzaghi & Sandine, 1975). Although this medium was used for enumeration purposes, it was decided not to use it in propagation stages for inoculum preparation. The reason for this decision was to make sure that the bacteria are not being exposed to a significant difference in terms of nutrients availability from propagation medium to experimental reactor medium.

Most of the functions of metabolic pathways are plasmid expressed in LAB. The history of life cycle of bacteria is considered to be important to provide the necessary enzymes and co-factors for metabolic pathways. LAB grow perfectly, as expected, on their growth selective medium (with balanced quantities of proteins, peptides and amino acids as nitrogen source), but the abundance of nutrients at an optimum level for this bacteria could be a negative factor in our experiments as our milk based medium is not so perfect for them. This difference may have suppressed the growth of bacteria and prolonged the lag phase (and even shock them where the difference is bigger).

The fact that nutritional requirements of the bacteria in the fermenter has to be supplied from MPC85 (milk based medium) on one hand, and the preference of similarity between the mediums at both propagation and final fermentation stages on the other hand, guided us to utilize RSM as our propagation medium. By doing so, the bacteria did not face any significant nutritional differences and will adapt smoothly and quickly during their lag phase.

3.3.4.1 **M17 Agar method**

The M17 medium is a specific medium for *lactococcal* bacteria in microbiological laboratories. In order to prepare this medium to grow the bacteria, the manufacturer's instruction was followed. For one litre of broth medium, 37.25 gram of M17 medium was added to 950 mL of distilled water in a 3 litre vessel and this mixture was heated to boil and clarify at this boiling without leaving any visible clotted particle in it. This was then transferred to the Durham bottles and sterilized by autoclaving.

In parallel a metal micro-filter was also mounted on a liquid filter set and sterilized to be used for microfiltration of lactose to be added to M17 broth. A 10% lactose solution (50 mL) was prepared to be filtered. The sterilized filter was connected to a vacuum in order to accelerate the filtration of lactose solution.

The sterilized lactose solution was added to the sterilized M17 broth (the temperature of the broth had to be lower than 50 °C) and this was then ready to be used for bacterial propagation. When an agar (solid) medium was required, it was added at a concentration of 15 grams per litre to the broth before sterilization. This medium (M17 agar) was used in Petri dishes for both bacterial incubation as well as colony forming unit (cfu) count.

3.3.4.2 **RSM method**

During the fermentation trials, the starter bacteria will be growing in a MPC85 solution (see 3.1.3), a milk-based product containing the same proportion of milk proteins as raw milk with about 3.4% protein content. On the other hand, the inoculum (to be added as starter) can be prepared by two major methods: M17 broth/agar or milk based RSM. M17 agar is the specific growth medium for *Lc. Lactis*. We decided to grow the starter culture in a milk based medium in order to minimise the lag phase once inoculated into the MPC85 cheese milk system: in other words, we aimed to have inocula with the appropriate enzymatic system to be able to grow and develop in MPC. A higher similarity between RSM and MPC in terms of available nutrients and growth factors was expected. The production of proteinases and peptidases in *Lc. Lactis* is strain and media dependent (Bruinenberg, Vos, & De Vos, 1992; Mierau et al., 1996). It was shown that if a particular nutrient that is already in a form that can be readily absorbed by the bacteria is abundantly available then this results in a suppression of enzymes involved in breaking down any non-absorbable complex forms and vice versa. Thus when casein hydrolysates (containing absorbable peptides and amino acids) were added to growth medium, the expression of required enzymes (proteinase and peptidases) to convert intact casein to those peptides and amino acids were supressed 5 to 150-fold (Guédon, Renault, Ehrlich, & Delorme, 2001).

M17 broth medium vials, supplied by Fonterra were sterically cultured into RSM medium. Petri dishes were filled with 15 mL of M17 agar and inoculated from the vial by the streaking method. The petri dishes were kept at 30 °C for 48 hours. A single colony grown on the Petri-dish was then transferred to a 10 mL vial of M17 broth and kept at 30 °C for 48 hours. This fully grown turbid

vial was then used as the source of fresh bacteria to be transferred to a slant M17 agar. The slants were kept in the incubation room at 30 °C for 48 hours. These slant cultures were used to harvest the bacteria and inoculate sterile 25mL bottles filled under sterile conditions with 10 mL sterile 10% RSM (w/w) (sterilized at 115 °C for 15 min) The bottles were incubated at 30 °C for 13 hours and then topped up with 10 mL of sterilized cold (4 °C) 10% RSM (w/w) and kept in the freezer (-80 °C) to be used as starter culture inoculum into the fermenter for our fermentation trials.

3.3.4.3 **Cell extract method**

During starter preparations, inoculated medium was incubated for long periods (12-14 hours) and this duration was similar to the duration of the longer fermentations in the main study – thus it was expected that similar casein peptide profiles would be present in the inocula. As we intended to evaluate the effects from bacterial activity on milk proteins during the fermentation trials any post fermentation peptide profiling and gel formation study would possess an artefact – a portion of peptides from the initial inocula that would be identical to the profiles of the longest fermentations studied. Therefore, an attempt was made to remove this artefact.

A modified method to discard the non-bacterial ingredients from inocula was followed (Pripp, Rehman, McSweeney, Sørhaug, & Fox, 2000) as below:

1. Subculture (2%) from frozen vial into 10 mL of 10% sterile RSM;
2. Incubate it for 13 hours until a soft coagulum is formed;
3. Add 10 mL sterile RSM to increase the pH from ~4.7 to 5.8;
4. Freeze it;
5. Take 1 frozen bottle of each of starter *Lc. lactis* spp. *cremoris* 2338 and HP;
6. Inoculate it to 1 litre of 10% sterile RSM (2.5% v/v) and incubate it at 30°C for 13 hours;
7. Take it out of incubator and clarify the medium with 60mL/L of trisodium citrate (25%w/v);
8. Adjust the pH to 7.0 using 10M NaOH;

9. Harvest the cells by centrifugation at 8000 g for 15 min at 4°C;
10. Wash it twice using ¼ strength Ringer's solution (Standard: 6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 0.2g of sodium bicarbonate is dissolved in one litre of distilled water);
11. Disperse the washed cells in 250 mL of Ringer's solution and freeze it, make 10 vials and freeze these vials.

One frozen vial of each *Lc. lactis* spp. *cremoris* 2338 and HP (overall two vials) is added to one fermenter containing 1600 mL of MPC85 5%.

Once these cell extracts were used in fermentations similar results were observed.

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3.3.4.4 **Bacterial count**

In order to evaluate the bacterial status in the fermenter the bacterial population numbers were evaluated. There are different methods of bacterial cell count among which two methods are being utilized more frequently; standard plate count method and spectrophotometric method.

In this research the plate count method for *lactococcal* bacteria developed by Terzaghi and Sandine (1975) was used. In this method, the viable bacterial cells are enumerated via a sequential series of dilutions to reach a plate count of 25-250 colony forming units (cfu). The actual number of bacteria in the original sample is calculated by multiplying the plate count by the dilution factor. The procedure can be elucidated as following steps:

- Peptone water preparation for serial dilution (Figure 3-6);
- M17 agar for selective *Lactococcal* growth;
- Hygienic sampling from fermenter;
- Dilution using peptone water,
- Spreading the diluted sample aseptically on the agar;
- Incubation at 30 °C for 48 hours;
- Counting the colonies in the proper range (plates with 25-250 colonies were considered);
- Calculation of the viable cells according to the colonies as each bacterial cell is considered as a colony forming unit (cfu);

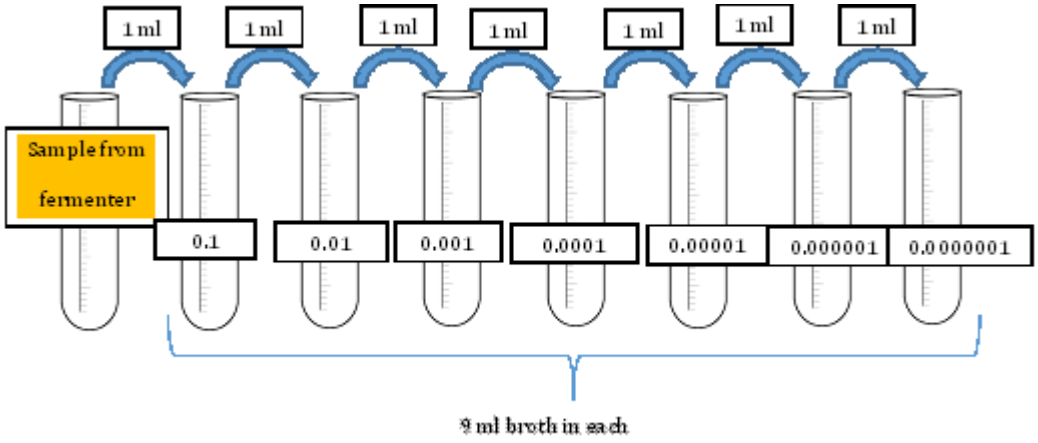

Figure 3-6 preparation for serial dilution and CFU count.

3.3.4.5 Inoculum defrost

In order to maintain the consistency among the samples, all the waiting times, delays and adaptation times for the sample were kept constant. The bacteria need time to change from lag phase to exponential growth phase and this can vary depending on the nutrients availability and environmental conditions. In order to keep the consistency in this research we followed a uniform procedure as below:

The inoculums were taken out of the -80°C and transferred to a container of 20°C water. The water level was just below the point where our bottles (most of the time two of 25 mL bottles; one for each strain) tended to start floating. After 10 minutes, the outer layers of frozen material inside the bottle was melted and soft enough to drop out of the bottle neck. By doing this we managed to have consistent melting time for the inocula regardless of ambient temperature changes over seasons.

3.3.5 Bacillus contamination assessment

The fermented medium was assessed at the end of each fermentation in order to evaluate the contamination with Bacillus bacteria inside the fermenter.

A solution of 0.2% EDTA was prepared and the pH of this solution was adjusted to 12.2 using NaOH. A 0.5 ml aliquot of fermented medium was added to 4.5 ml of EDTA to have 10% concentration of fermented medium. This sample was then transferred onto a slide and checked

under Phase Contrast Microscope to track the presence of spores of Bacillus. This method is a rapid and does not require the staining and other time consuming laboratory steps. Under the microscope, the spores could be distinguished (if present) as the phase-bright.

3.3.6 Nitrogen fractionation

Once the treatment was complete, in order to judge the effects of bacterial activity on the protein during the treatment, the protein part was fractionated into different sub-fractions to study the effects of treatments. By proper fractionation scheme selection, it was possible to track the changes occurring in the medium. The fractionation of the samples were done according to the method of Sousa *et al.* (2001) (Figure 3.7).

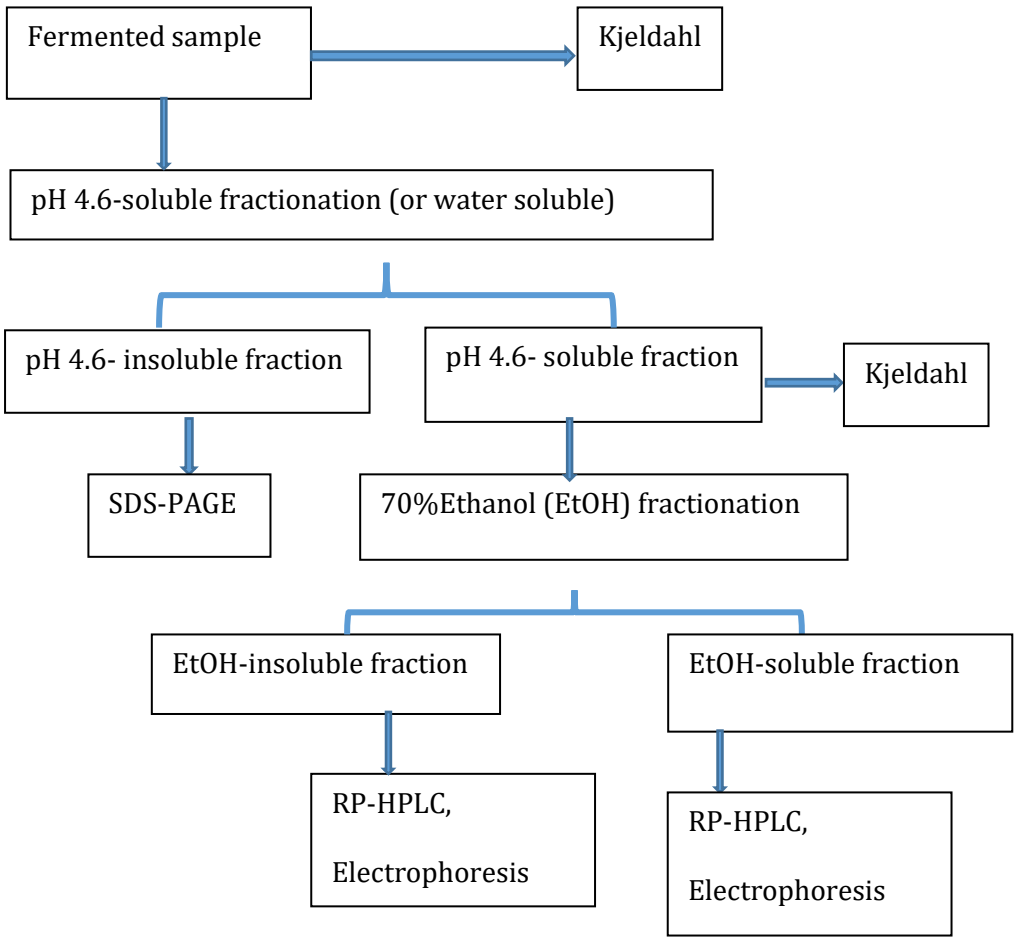

Figure 3-7 Fractionation for nitrogenous compounds of samples(Sousa et al., 2001)

3.3.6.1 **pH-4.6 solubility fractionation**

Once the fermentation is terminated, the samples' formulation were adjusted (Section 3-1 and 3-2), pasteurized and cooled, and an aliquot of the sample was taken for nitrogenous compound fractionation.

For pH-4.6 fractionation, the pH value for samples was adjusted to 4.6 using 1N HCl. This was done under high rotation speed of magnet stirrer while a top mounted pH- probe alongside a temperature sensor was mounted on top of the container to have a real time pH reading from the decreasing pH. This was then poured into centrifuge bottles and were left to equilibrate at room temperature (typically between18-25 °C) for 30 minutes. The pH was re-adjusted if required, then the sample was heated in a water bath and held at 40 °C for 1 hour. Subsequently, the sample was centrifuged at 3000 g for 30 min at 4 °C. The supernatant (pH-4.6-soluble fraction) was filtered through glass wool and Whatman No. 113 filter paper and was then kept frozen for nitrogen analysis using the Kjeldahl method.

pH 4.6 soluble (very similar to Water soluble nitrogen fraction) is used to determine the amount of water soluble polar volatiles that affect cheese flavour. This fraction includes peptides, amino acids, mineral salts, lactose, lactic acid and a vast variety of volatile compounds (Taborda et al., 2002).

3.3.6.2 **70%Ethanol solubility fractionation**

In order to separate EtOH-soluble (smaller, hydrophilic peptides) and EtOH-insoluble (larger, hydrophobic peptides) fractions, the pH-4.6-soluble fraction was fractionated using 70%Ethanol. A further fractionation was carried out on the soluble fraction from the previous fractionation at pH-4.6. The soluble fraction was mixed with 70%Ethanol (30 volume parts of pH-4.6 soluble and 70 parts of ethanol) mixed under gentle agitation by rotating the container for one minute. The mixture was kept for 30 min at room temperature; then centrifuged at 3000 g for 30 min at 20 °C. The supernatant (EtOH soluble peptides) was filtered through Whatman No. 1 filter paper. EtOH was removed by rotary evaporator (R-215, BÜCHI Labortechnik AG, Flawil , Switzerland) under

80 millibars of vacuum at 40 °C under 50 rpm rotary speed. Once the evaporation was complete, the remainder was transferred to plastic Eppendorf containers and kept frozen at -18 °C for further assessment. The pellet remaining on the filter paper (containing EtOH-insoluble peptides) was dispersed in distilled water and kept frozen at (-18 °C) for the further analysis.

3.3.7 Total Nitrogen content (Kjeldahl method)

Thoroughly mixed sample (1 g) was weighted and placed in a digestion tube. Two catalyser tabs (Kjeltabs; each containing 3.5g K_2SO_4 and 0.0035g Se) were added to each sample, then 15 mL of concentrated sulphuric acid (H_2SO_4) added to the tube. The digestion took place for 45 minutes at 420 °C until the tube became clear and left for a further 10 minute to ensure a complete digestion; The contents of each tube were then diluted with distilled water.

For each sample's distillation and titration, 25 mL of 4% boric acid solution was added to a conical flask; and the resulting the ammonium borate was then titrated against 0.1N HCl;

The nitrogen content of the sample was calculated using a crude protein index of 6.38 and is reported as nitrogen percentage in sample (Equation 1):

$$\%Nitrogen = \frac{(Normality\ of\ HCl \times ml\ HCl\ used) \times 14 \times 100}{wheight\ of\ sample\ in\ grams \times 1000} \qquad \text{Eq. 1}$$

Nitrogen contents of both pH 4.6 soluble and insoluble fractions are presented as a ratio of total nitrogen.

3.3.8 Cheese making

A novel method for cheese making was developed to produce cheese from fermented mediums in small laboratory scale (Figure 3-8).

Cheese-milk was prepared as below:

- a 5% MPC85 4850 solution was prepared (vortex condition addition, 60 °C followed by one hour at this temperature under constant stirring);
- the solution cooled down to 32 °C;
- lactose was added to the solution where required (explained in lactose content adjustment the solution was inoculated by two vials of starter cultures (refer section 3.2.1);
- the prepared solution was added to a sterilized fermenter tank with 2.5 litres capacity (New Brunswick, Germany);
- the fermentation was conducted under pH-stat conditions at pH 5.95 using a Bioflow fermenter (Bioflow 110, New Brunswick, Germany);
- the parameters were recorded using Biocommand software (BioCommand Lite, New Brunswick Scientific C., Inc.);

The fermentation was stopped once the pH remained unchanged for at least 10 minutes and the sample was taken at this step for microbiological experiments (*Lc.* count). At this step the lactate content was adjusted be identical to the lactate content of the sample that had undergone the longest fermentation time and left one hour to reach equilibrium. At this step the fermenter was emptied into a sanitized 4 litre stainless steel beaker at 32.5 °C water bath. A gentle stirring (150 rpm) was applied to ensure temperature uniformity. After 5 minutes, 350 µl of Australian Double Strength rennet (1/10 diluted) was added and after 30 second of stirring the renneted cheese milk was left for one hour uninterrupted. Figure 3.8 depicts the cheese making steps followed from renneting.

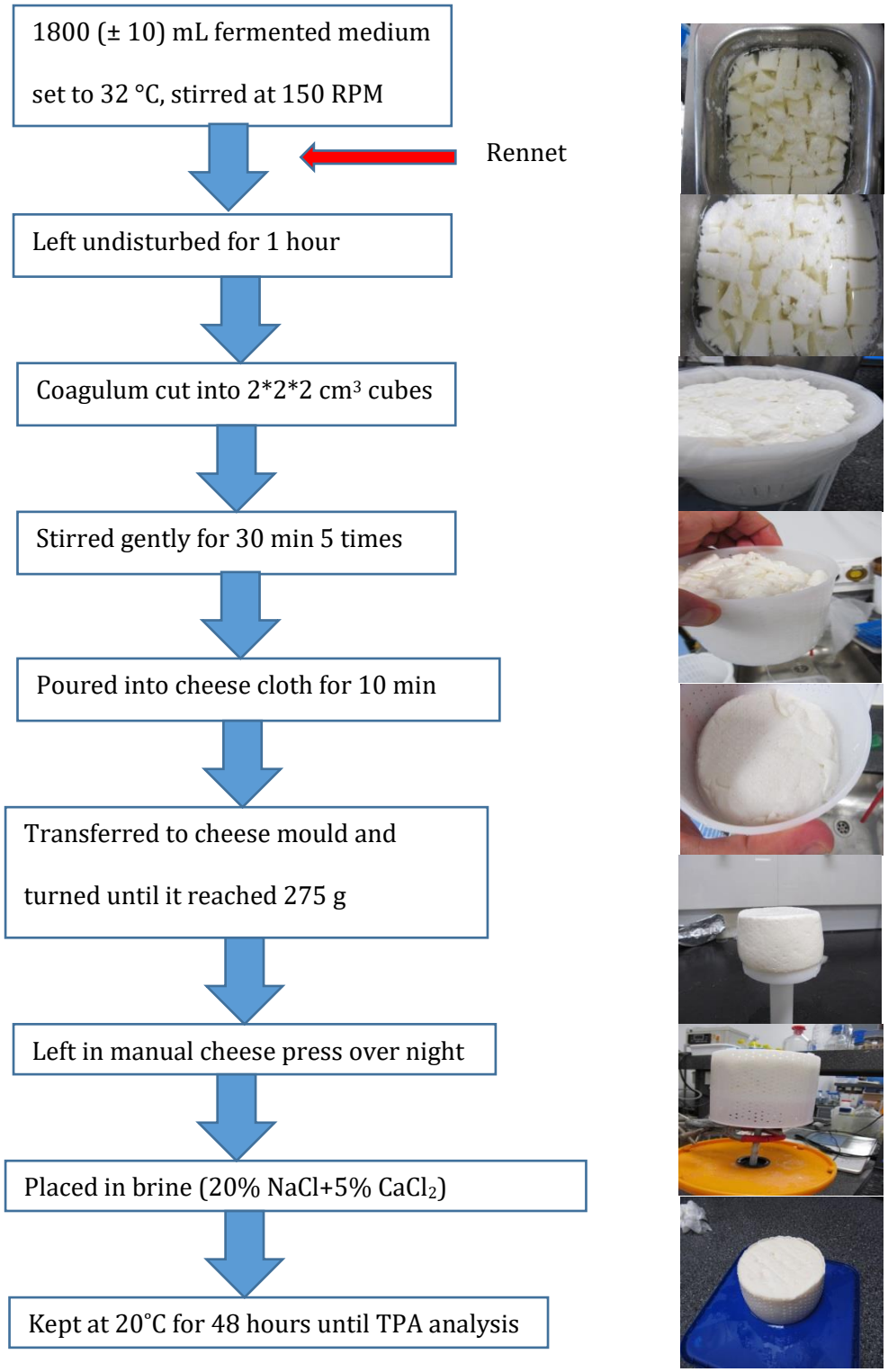

Figure 3-8 Cheese making process used for the present study.

3.3.9 Moisture content

The moisture content of the samples was measured by oven drying method. In this method samples were pre-weighed into in aluminium containers. Prior to use the containers were dried in an oven to reach a constant weight. After one hour of cooling to room temperature the container was placed on the digital scale and weighed (X_1). The containers with samples were then placed in the oven (the same conditions as before) and after drying overnight (15 hours) transferred to a desiccator to cool down to ambient temperature. After one hour, the containers were re-weighed (X_2) and the moisture content was calculated (Eq. 2)

$$\text{Moisture content}(\%) = \frac{X1-X2}{X1} * 100 \qquad \text{Equation 2}$$

3.3.10 Conductivity measurements

All conductivity measurements were carried out using a Schott Prolab 4000 measurement system equipped with a conductivity probe LF413T(-ID) with an integrated temperature sensor with stability control to ensure the reproducibility of the measuring signals. The temperature reproducibility of the probe was better than 0.002°C and reference T was 25°C. The probe was calibrated on a monthly basis using producer's calibration liquid (0.01mol/L KCL)

3.3.11 HPLC

The fractionated samples were analysed by HPLC. The absorption values (mAU) of the eluted components were recorded online using EZChrom software (EZChrom Elite, Scientific Software; Agilent Technologies). A valley to valley integration gave the most consistent results during preliminary analysis. The solvents A and B compositions were such that the hydrophilic components would be expected to elute earlier while the hydrophobic components would appear later on (considering our column specifications).

HPLC determination was based on Bergamini *et al.* (2006) who evaluated the peptide profile of semi hard cheese as a result of probiotic bacterial activity. In this method, the 70%Ethanol soluble

and insoluble fractions were selected for HPLC analysis. The insoluble parts were dispersed in 2 mL of Milli-Q water and mixed well using a vortex mixer (Grant Bio PV-1, England). Both fractions were filtered through 0.45 µm cellulose sterile syringe filters (Minsart®, Sartorius) and transferred into HPLC bottles (1 mL) for analysis.

The HPLC system was an Agilent 1200 (Agilent Technologies, Palo Alto, CA, USA) system with auto sampler and in-line degasser with quart pump. The column used was an Agilent C18 column (300SB-C18 Analytical HPLC Col 4.6 x 250). The injection volume was 40 µl. Detection was performed at 214nm and the column was kept at 40 °C. The gradient started from 100% of solvent A (H_2O:trifluoroacetic acid (TFA) 1000:1.1, v/v) and 0% of solvent B (acetonitrile:H_2O:TFA 600:400:1, v/v) was generated 10 min after injection. The proportion of solvent B was increased by 1% min-1 (80 min), 20% min-1 (1 min), 0%min-1 (4 min), and then returned to starting conditions, during 1 min. the last setting conditions were kept up for 10 minutes and the column was prepared for the next

3.3.12 Free amino acid

Free amino acid assessment was done at a commercial laboratory. Cheese samples were made according to the method mentioned in this chapter and vacuum packed and kept in a 4 °C chiller for 12 months. The cheeses were then sent to the laboratory, and freeze dried to evaluate the free amino acid content by chromatography method (AOAC 994.12).

3.3.13 Quadrupole time-of flight (Q-ToF) Mass Spectrometry

Quadrupole Time-of-Flight (Q-ToF) mass spectrometry experiments were carried out using an Agilent 6520 (Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS) QToF 6520.

The freeze dried samples of ethanol soluble fraction were assessed for their peptide profile. A 1% solution of freeze dried powder was prepared according to the method of Piraino et al. (2007) with some modification. The sample which we used started form a freeze dried powder of EtOH70 soluble fraction, while they started the sample preparation using freeze dried pH4.6 soluble

fraction followed by EtOH 70% fractionation in a liquid state. This solution was desalted using Thermo Scientific™ Zeba™ Spin Desalting Columns. Aliquots of 200 µl were then ultrafiltered using Vivaspin 500 (GE Healthcare Bio-Science AB, Uppsala, Sweden) at 15000xg for 30 minutes. The prepared samples were analysed by the Agilent 6520 LC/Ms system in duplicates and on average 90 scans were carried out on each sample run. The results were acquired by MassHunter Workstation Software (© Agilent Technologies, Inc. 2011) and then integrated and analysed using OpenChrom software (OpenChrom Community edition 1.1.0).

3.3.14 Rheological Experiments

3.3.14.1 **Dynamic rheology**

Sample cheeses were assessed considering their flow/deformation properties. Curd formation was evaluated utilizing an MCR 302, Anton Paar Rheometer (Anton Paar Germany GmbH).

The measurements were done in three steps;

- Constant Frequency and Strain. In the first step a constant frequency constant strain measurement was set. The applied strain was within the LVR limit of the sample and was set to be 0.5% and the frequency of applying this strain to the sample was 1Hz. During this step 360 measuring points were measured with the length of each duration to be 30 seconds. This time was selected based on the preliminary experiments in which a plateau was reach within this time for all of our treatments.
- Frequency sweep with constant Strain. A frequency sweep was carried out at the end of the first step (constant frequency and strain). During this step a 3 magnitude of frequency was considered (explained in chapter 2). Frequencies from 0.01 to 10 were applied while the strain level kept constant at 0.5%.
- The Amplitude sweep with constant frequency; at this step a range of different strains were applied (from 0.01-100%) at a constant frequency of 1Hz. This applies small to high amplitude deformation forces to the specimen.

3.3.14.1.1 *Gelation time*

Once the cheese milk is formulated, a coagulant is added to form a curd. In our experiments 20 microliters of 1/10 diluted rennet was added to 100 mL of the sample and mixed well by turning the container gently (5 times). An aliquot of 20 mL of this was pipetted to the cup of the Rheometer measuring system. In this study a CC27 measuring system (a concentric cylinder measuring system set consisting of a measuring bob and a measuring cup) and then the system set to measuring position (CC27-SN23944). The time taken from rennet addition until the start of measurement was taken into account in the final results. The gelation time was considered as the time where G' exceeded G" (Karlsson et al., 2007).

3.3.14.2 **Static Rheology**

As reviewed in chapter one, static rheometry is a versatile method to assess cheese textural attributes. One of the most utilized methods for cheese textural evaluation is uniaxial compression of cheese (Gunasekaran & Mehmet, 2002).

3.3.14.2.1 *TPA*

The texture profile analysis (TPA) method is an instrumental texture assessment by static rheometry principles in which a double bite compression condition is applied to the sample to a predetermined extent of strain. This has been designed to imitate the compressing action of molar teeth during food mastication of food materials in their viscoelastic range.

The experiments were carried out using a texture analyser (TA-XT HD, Stable Micro Systems, UK) (Figure 3-9a). A cylindrical sampling device with diameter of 25 mm was utilized to take sample from cheese block (Figure 3-9b). The height of samples were 30 mm.

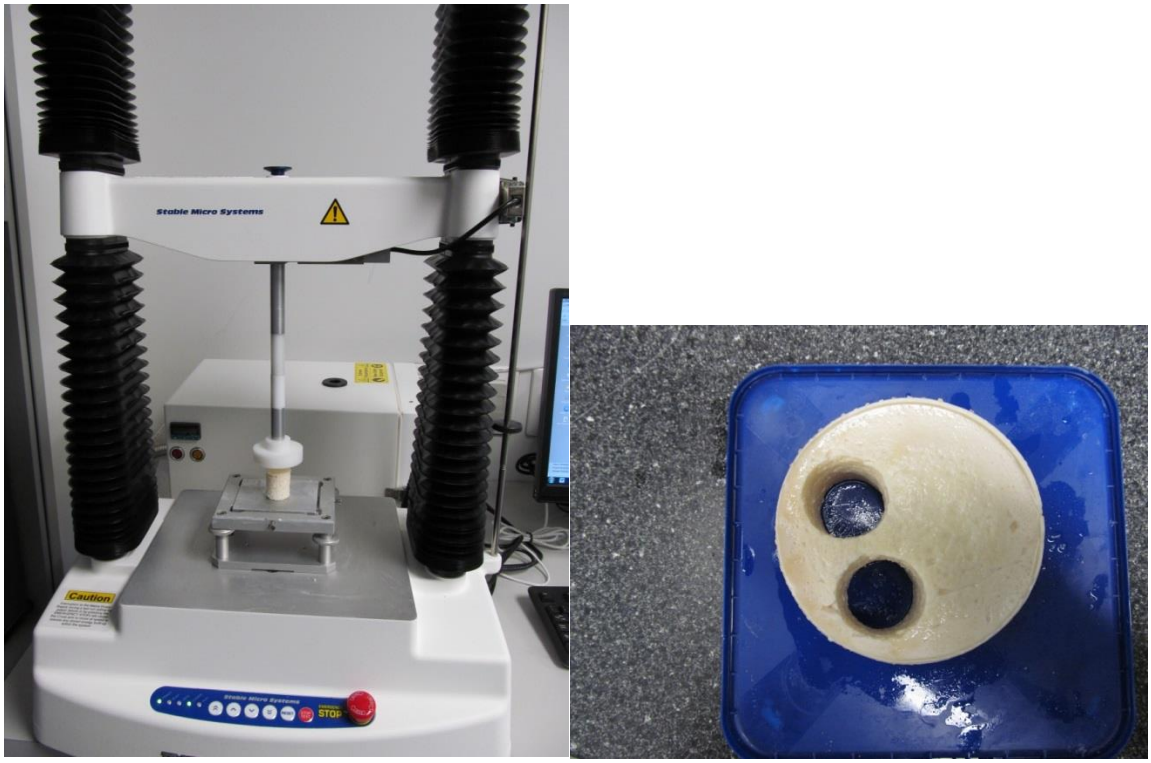

Figure 3-9.a- TA-XT HD Texture analyser, Stable Micro Systems- experimental setup for static cheese compression studies, b) an example of sampled cheese

The test conditions were chosen based on the machine designer's recommendation for cheese and is presented in Table 3-2.

Table 3-2 Test conditions used for TPA analysis of the cheese samples

Test type	Double bite compression
Pre-test speed	1 mm/sec
Test speed	2 mm/sec
Post-test speed	2 mm/sec
Target mode	strain
Strain	70%
Trigger type	Auto (Force)
Trigger force	5.0 g

Probe	61 mm

A macro designed by the same company was used to analyse the graph of each test. A sample graph of the test is presented in Figure 3-10. Hardness, adhesiveness, resilience, cohesion, springiness, gumminess, Chewiness were the rheological attributes derived from the graphs.

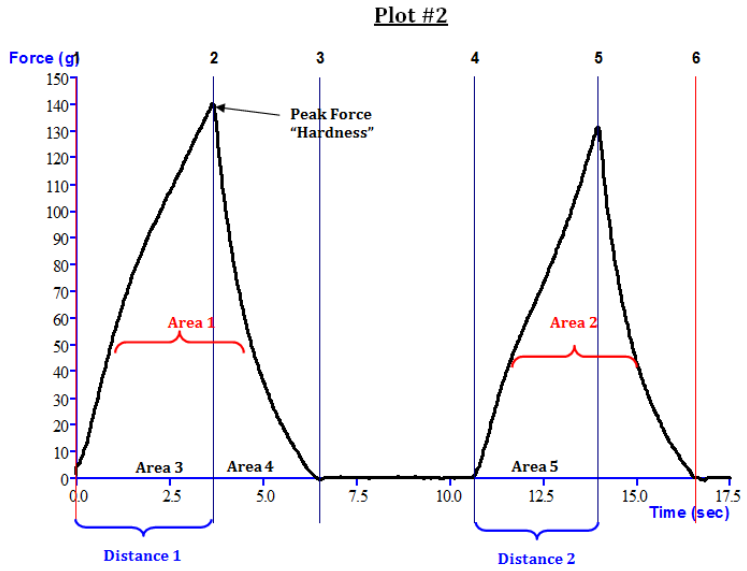

Figure 3-10. An example of the TPA graph obtained from the cheese samples.

3.3.15 Confocal Laser Scanning Microscopy (cheese microstructure)

Cheese samples were prepared as explained in earlier in this chapter. One sample was flash frozen by direct immersion of the sample in liquid nitrogen and kept frozen (-18 °C) until the analysis was carried out. One hour before the analysis of sample, the cheese was taken out of freezing conditions and kept in an ice–water incubator, then for one hour at ambient temperature (around 20°C).

A sharp scalpel blade was utilized to sample a thin specimen. This step has to be done as accurate as possible to avoid the creation of an artefact in the results. The specimen was the placed on a concave slide, stained with 2% fast green FCF (two droplets) and a coverslip was place on top of the dyed sample and left for 30 minutes at room temperature. The excess dye was removed using a lint free tissue and the slide was placed under the objective lens of microscope for examination.

The samples were then imaged using the Leica DM RBE laser scanning microscope LS 510 (Leica Lasertechnik GmbH, Heidelberg, Germany). An Ar/Kr mixed gas laser was used at an excitation wavelength of 568 nm. Images were scanned at 10–15 mm below the coverslip. The fluorescence collected from the sample was an average of eight scans and the imaging were done in duplicates.

3.3.16 Sampling for next steps

Upon finishing of the fermentation trial (lactose elimination and no further pH fluctuation), the fermented medium was heated for 30 minutes at 62.5 °C (LTLT). At the end of fermentation trial, the heat-treated and then cooled samples were checked for any possible minor fluctuations in pH and (if required) the pH was readjusted using 2M lactic acid and NaOH 1N.

One of the major difficulties of this research was arising from the conclusion made about post fermentation steps. It was decided not to keep the sample in the fridge, even for short time. The reason behind such a decision was the fact that there was, in the preliminary experiments, a lack of consistency in rheological properties during curd formation when the fermented sample was kept overnight at the fridge. Therefore, once the fermentation was finishing, all of the sampling for next steps were carried out within minutes of completion of the fermentation.

Samples were taken for:

- Chemical assessment (see Chapter 5);
- Fractionation by acid and also ethanol (see Chapter 5);
- Rheological properties of curd formation (see Chapter 6);
- Cheese making to evaluate textural properties and microstructure of final cheese (see Chapter 6).

3.3.17 Analysis of the results

The experiments of this study were carried out in replicates. In order to analyse and compare the differences between the mean values of the results, a one-way ANOVA was used (significant level

$p<0.05$) preceded by a normality test. A Tukey-test post ANOVA was performed for significant differences of ANOVA to determine the real significant difference.

Area under the chromatograms were integrated and compared with the HPLC results. To process the HPLC individual peak comparison visual matching of the peaks was carried out.

Pearson's correlation test was utilized to assess the correlations between the results of different parameters. The analyses were performed utilizing software SAS 9.3 (SAS Institute Inc. 2011. Base SAS® 9.3, Cary, NC: SAS Institute Inc.)

A least squares optimization technique was utilized on modelling data. The model coefficients were adjusted by Microsoft Excel Solver Utility (Microsoft Office package, 2013), to minimize the sum of squared residuals of each data set between the experimental and model prediction.

Chapter Four Fermentation

4.1 Introduction

Apart from some exceptions, specific species of microorganisms have to be utilized during cheese-making (Parente & Cogan, 2004). As explained earlier in chapter two, most of these bacteria belong to a group named lactic acid bacteria (LAB). The major purpose of using these bacteria is to produce lactic acid as a result of a biological activity called fermentation (Peter Walstra et al., 2006). The produced acid plays a role in characteristic flavour development of cheese. On the other hand, it naturally serves as a preservative by decreasing the pH of the medium and inhibiting the growth of unwanted microorganisms. The taxonomy and diversity of the LAB, their lactose fermentation and protein metabolism are reviewed in detail in Section 2.3. In the present chapter, the basis of strain selection, the extent of each fermentation trial and control parameters involved, and the results of our modified fermentation experiments (pH-stat conditions, NaOH added to maintain the pH at a predetermined constant value and changes in conductivity) are presented.

4.2 Bacterial starter selection

In the present research, five different strains of *Lc. lactis* were kindly provided by Fonterra Research and Development Centre (FRDC, Palmerston North, New Zealand) (see 3.2.1). A set of experiments were carried out using both MPC85 and caseinate mediums. For both of these mediums, the fermenter was inoculated by one individual strain and adequate lactose content was included in the formulation in order to keep the fermentation going for 20 hours (based on preliminary experiments). The pH reduction and conductivity increase were recorded online. These preliminary experiments were carried out to decide on the most favourable bacterium for the final experiments. The experiments were carried out with two replicates and the average results are presented in Figure 4-1.

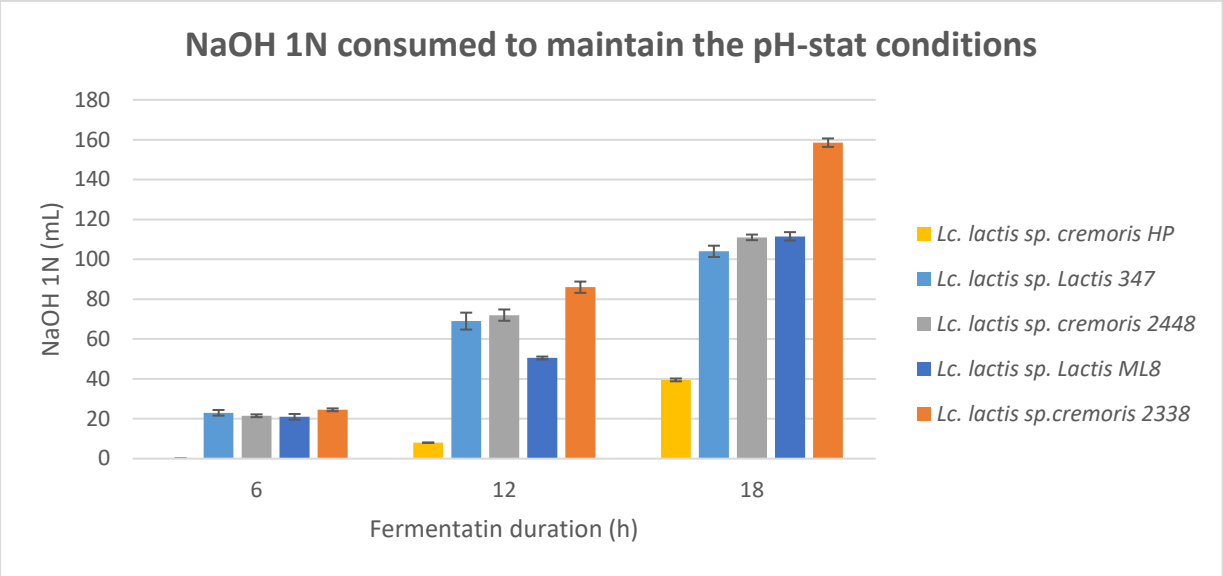

Figure 4-1. NaOH consumed (mL, n=2) to keep the pH-stat condition of MPC85 fermentation at different intervals (6, 12 and 18 hours) using different strains of LAB. The missing data at 6 hour category indicates a zero value. Error bars show mean (n=2) ±S.D.

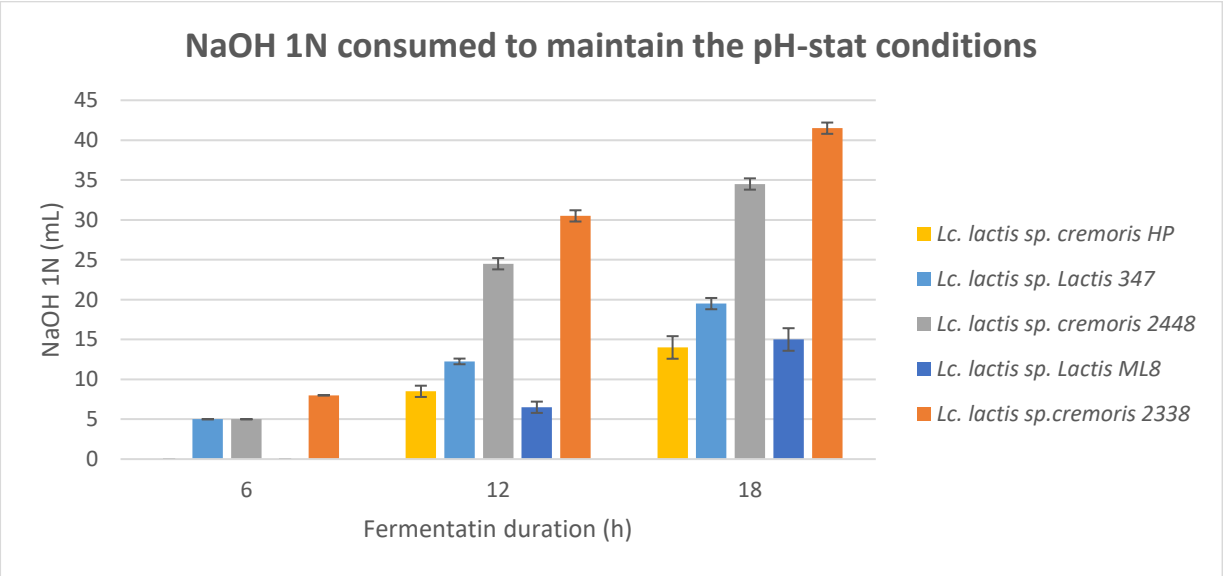

Figure 4-2.NaOH consumed (mL, n=2) to keep the pH-stat condition of sodium caseinate at different intervals (6, 12 and 18 hours) using different strains of LAB. The missing data at 6 hour category indicates a zero value. Error bars show mean (n=2) ±S.D.

The data showed that a higher conversion of lactose to lactic acid is possible when the lactose is fermented in a MPC85 solution compared to a caseinate solution. This was possibly due to a more balanced nutritional composition in MPC85 than caseinate. The mineral content of MPC85 is much closer to milk and it is used as a reference for highly nutritious medium for bacterial activity. Another possible reason for the lower activity of the bacteria in caseinate could be the fact that LAB cell-envelope proteinases (lactocepines; refer to chapter one- LAB proteolytic system) require calcium to attach to the cell. It was shown previously that LAB would lose their proteolytic activity when they were treated with a calcium-free buffer. Although it would have been ideal to use a casein-based medium (without the presence of other milk components) to assess the bacterial effect on only casein, the lower activity of LAB in caseinate as well as the lower quality of the formed gel (Section 3.1) were the major reasons that we decided to use MPC85 as our fermenting medium.

On the other hand, our results showed that *LC. lactis* sp. *cremoris* 2338 had the highest rate of fermentation (when using increasing NaOH consumption as a measure of higher lactic acid production) within a pre-determined fermentation period compared to the other investigated strains (as presented in figure 4.1 and 4.2) under similar conditions (time, temperature, nutritional supply by milk-based mediums). This aspect of *LC. lactis* sp. *cremoris* 2338 was promising for our study as we wanted to perform long fermentation at pH 6.00. It is noteworthy that pH 6.00 is considered a high pH compared to fermented dairy product where their low pH is generally less than 5.5. *LC. lactis* sp. *cremoris* 2338 acidifies the medium quickly enough to overcome the unwanted flora (e.g. Bacillus if present) and could potentially interfere with the proteolytic activity of the target bacteria. Bacillus bacterial vegetative activity is significantly decreased by pH reduction from normal milk pH values (*i.e.* ~6.7) to pH 6.0 (Puhan & Irvine, 1973; Wong, Chen, & Chen, 1988) and if the pH is rapidly decreased to this value, their activity in the fermenter becomes negligible (Mikolajcik, Kearney, & Kristofferson, 1973).

In this study, we wanted to assess the effects from LAB before renneting on curd formation and textural attributes of the subsequent curd and cheese. The proteolytic activity of the bacterial

mass was important in this study, as it is known to affect cheese texture and flavour during normal maturation of cheese. In our bacterial selection scheme, we were looking for a strain with an acceptable proteolytic activity. *Lc. lactis* sp. *cremoris* HP is a strain with considerable proteolytic activity. This microorganism, which was first extracted and categorized in New Zealand dairy industry, is comparatively more active on proteins because of its PI type cell-envelope proteinase which are capable of hydrolysing casein at a high rate (Lambie et al., 2014). In conclusion, a mixture of *LC. lactis* sp. *cremoris* 2338 and *Lc. lactis* sp. *cremoris* HP were selected (to be added at an equal volume) for our long fermentation pH-stat experiments.

4.3 Bacterial preparation by cell extract method

During our preliminary experiments, we were using cultured 10% RSM (w/w) as starter inoculum. Lactose content of RSM is very high (lactose accounts for almost 50% (w/w) of skim milk powder composition whilst only 4% in MPC85). We designed our experiments so that with the termination of the fermentation trial when the lactose was eliminated. The lactose present in the inoculum during the starter propagation can reduce accuracy when it is added to the fermenter. On the other hand, the nitrogenous compounds in RSM are used by the bacteria during propagation and thus can interrupt our experimental results (where the nitrogenous compounds and the effects of treatments on them were investigated). Hence, it was decided to discard any non-bacterial compound from the starter inoculum. Thus cell-extraction method was used to remove all the non-bacterial components from the inoculum, and the retentate was living bacterial cells. The details of this method is presented in 3.3.3.3. In order to be able to connect the results of our cell extracts with the previous results from 10% RSM (w/w) inocula; the washed cells were re-dispersed in the same amount of peptone water to have approximately the same cell numbers in each vial and the same volume to be added to the fermenter as starter medium.

Table 4-1.[1]Mean fermentation duration and NaOH consumed to maintain pH-stat conditions for MPC85 5% with no lactose added inoculated by cell extract starters and 10% RSM.

Treatment	Added lactose (g)	Fermentation after first pH 6 (min)	NaOH consumed (mL)
Cell Extract	0	120±7.09	5±0.29
10% RSM	0	150±12.85	7±0.29

[1] Mean±S.D. (n=3).

The concentration of cell extract inocula were adjusted to be the same as 10% RSM (in terms of bacterial content). The peptone water was added to the cells up to the same amount of 10% RSM (w/w) volume. This ensured that the concentration of protein inside the fermenter would not change as a result of not adding the nonbacterial part of inoculum (Table 4-1 and Figure 4-3).

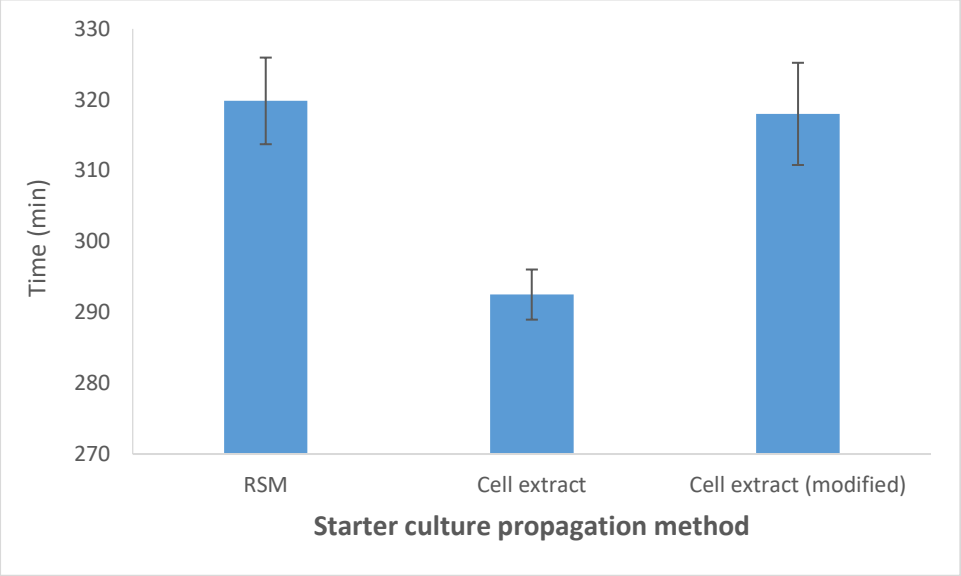

Figure 4-3 Time required for bacteria to acidify the medium down to pH 6 for the first time after inoculation. For 10% RSM, cell extract and cell extract (modified) n=6, 2 and 3, respectively. Error bars show standard deviation.

The importance of rate of acidification and its effects on relevant aspects of casein structure and curd and also cheese making quality has been reviewed in 2.3.3.2. Our result showed that the cell extract inoculum had no significant difference ($P<0.05$) compared to non-cell extracted inoculum in the traditional 10% RSM (w/w) medium in terms of bacterial activity.

4.4 Fermentation System

4.4.1 Our designed system pH-stat

A detailed review on the designed system for pH-stat fermentation system linked with online recorder is presented in Chapter Three.

One of the most valuable conclusions which was made using the recorded graphs of this system was being able to determine the duration of the fermentation trials for a better experimental design. When an abundant amount of lactose was provided to continue the fermentation for a long time (longer than 48 hours), the rate of NaOH pumped to the system (at later stages of such long runs) was dramatically lower compared to the earlier stages. Also the frequency of NaOH addition was significantly lower than earlier stages. This was connected to a lower activity of starter bacteria and therefore lower lactic acid production.

Three major reasons were considered to be the source of this lower bacterial activity in the later stage of fermentation:

- Higher ionic content and lactate content;
- Presence of higher amounts of inhibitory compounds as a result of bacterial activity on proteins (Mullan, 2006).
- Possibility of salting out at higher sodium contents despite the adjustment of the length of fermentation trials to be out of the range casein salting out zone. The concentration of sodium in the milk base medium (*0.056M*) was well below the concentration required to salt out casein proteins (0.5 M) (Ho & Waugh, 1965; Strange, Van Hekken, & Holsinger, 1994). It is noteworthy that pH 6 (our pH-stat value) was reported to lie within the optimum pH range in terms of casein solubility in NaCl solutions (See chapter Two). Based on these considerations, the experiments for final trials were designed so that the likelihood of salting out was negligible even for the very long trial.

4.4.2 Brunswick BioFlo 110 system

As a larger fermentation tank was needed for cheese making at a later stage of the experiments, for this purpose a New Brunswick bio-flow system with a 2.5 litre tank (Figure 4-4) was used. All of the formulation adjustments were based on the designed system explained previously. The only differences were in the higher accuracy of the pumps. The pH variation in this system was minimal (+/- 0.01 pH unit).

Figure 4-4. New Brunswick BioFlo 110 fermentation system (fermenter tanks, attachments and PLC).

This system was not used from the beginning due to access limitations. As a considerable number of fermentation runs had been carried out to develop our unique pH-stat system, it was not possible to repeat all these runs on the New Brunswick fermenter.

4.5 Formulating the milk-based medium for fermentation

The design of the formulation was based on the available media. The rationale of medium selection is described in Chapter Three.

Once we decided on using the MPC85, we had to readjust the formulation to suit our requirements. The medium was adjusted in terms of lactose content and also casein concentration. In this section the basis of these adjustments are presented.

4.6 Length of fermentation

According to our experiments, if the fermenter is formulated to 5% of MPC, the inherent lactose content of dried powder (4%; according to manufacturer's fact sheet) will be enough to run the fermenter for only two hours (from the time pH dropped to 6, under our fermentation conditions) before it is eliminated. Thus, based on a set of preliminary experiments the amount of lactose required to continue the fermentation for a predetermined duration was calculated.

Table 4-2. Lactose content inside the fermenter to adjust the duration of fermentation trials.

Fermentation treatment	Lactose added (g)	Lactose content (%)[1]	Target fermentation duration (h) after first pH 6
Non fermented	0	3.2	0
Short fermented	3.4	6.4	~4
Medium fermented	6.8	10	~6.5
Long fermented	10	13.2	~10

1- Based on dry matter MPC, Added lactose (g)+ MPC lactose content (g)

Table (4-3) shows the relation between the lactose addition and length of the fermentation with the NaOH required to keep the pH-stat condition. Based on the discussion presented so far in this chapter, it was eventually decided on the maximum lactose content, which was equivalent to (and defining for) maximum duration of our fermentations.

Table 4-3. Mean values of fermentation duration after first pH 6 and NaOH consumed to maintain the pH-stat conditions based on the initial added lactose to the fermenter.

Added Lactose (g)	fermentation duration (h) after first drop to pH=6	NaOH consumed (mL)
0	2.5	7
3.2	4.2±0.14	42.5±0.71
6.8	7.075±0.11	68±2.5
10	10.45±0.07	95.5±0.71
12.5	12.18±0.03	106±1.41
14.4	13.415±0.12	114±1.41
24	20.58±0.11	138.5±0.71

28.8	22.83±0.24	160.5±0.71
36	30.25±0.35	185±4.24
52.8	58±2.12	239.5±1.41

[1]n=2

The correlation between the between the lactose content in the fermenter, fermentation duration after first pH6 and NaOH 1N consumed to maintain the pH-stat conditions are shown in Figure 4.5 and Table 4.4.

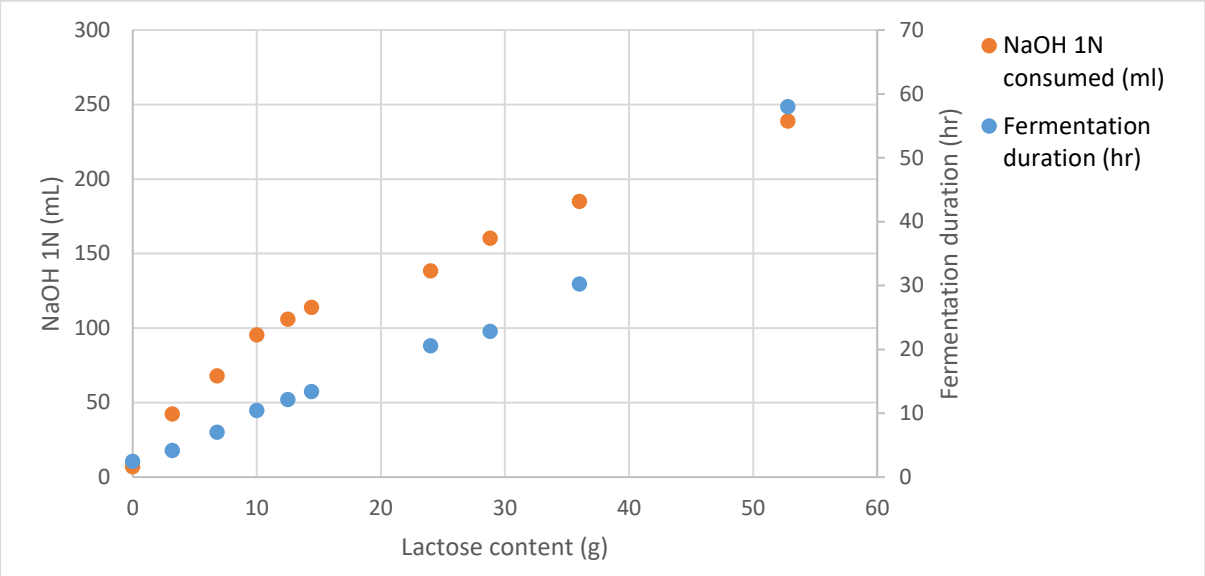

Figure 4-5. Correlation between lactose content and duration of fermentation (minutes) and NaOH consumed to maintain the pH-stat conditions

Table 4-4. Correlation coefficients between the lactose content in the fermenter, fermentation duration after first pH6 and NaOH 1N consumed to maintain the pH-stat conditions.

	Lactose content (g)	*fermentation duration after pH6*	*NaOH 1N consumed (mL)*
Lactose content (g)	1		
fermentation duration after pH6	0.977318922	1	
NaOH 1N consumed (mL)	0.974516724	0.931654062	1

4.7 pH range

The range in which pH alteration was happening during fermentation was dependent on the pump speed, NaOH concentration, stirring speed inside the fermenter tank and volume and buffering capacity of fermenting medium (which were all kept constant for consistency among the trials). On average, the pH cycles using our pump speed and NaOH concentration was 0.05 pH units (±0.05).

It is noteworthy that in a modern fermenter this range can be managed to be very small due to the higher sensitivity and improved performance of pumps. The importance of having a small variation range is arising from the fact that casein micelles are vulnerable to the pH of the surrounding medium. As explained before in chapter Two, the structure of micelles change once the pH is decreased to 5.8 or lower. Under such conditions we could not have differentiated the effects of bacterial activity on micelles. On the other hand, if a higher range of pH value was chosen the possibility of growth of unwanted microorganisms would have been higher. An example of a typical pH graph with a focus on pH range is presented in Figure 4-6.

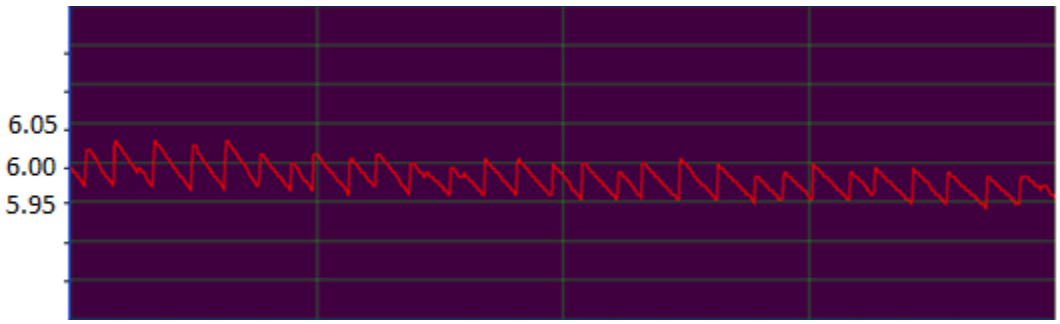

Figure 4-6. pH range in which the pH changes from lowest pH when the set point is reached to the highest degage when the pump stops to inject NaOH. X axis is time of fermentation at a selected range.

4.8 Fermentation trials

Once the fermenting medium was rehydrated, cooled to 32°C, lactose adjusted and defrosted starter bacteria added, it was poured into the fermentation tanks under sterile conditions. The

stirring speed was set to 200 rpm. The temperature was set at 32.5°C as the optimal temperature for growth of the LAB bacteria. The NaOH addition by the peristaltic pump was optimized through a set of preliminary experiments in order to maintain the pH range. An example of pH graphs for SF and MF PSLT fermentations are presented in Figure 4-7 and Figure 4-8.

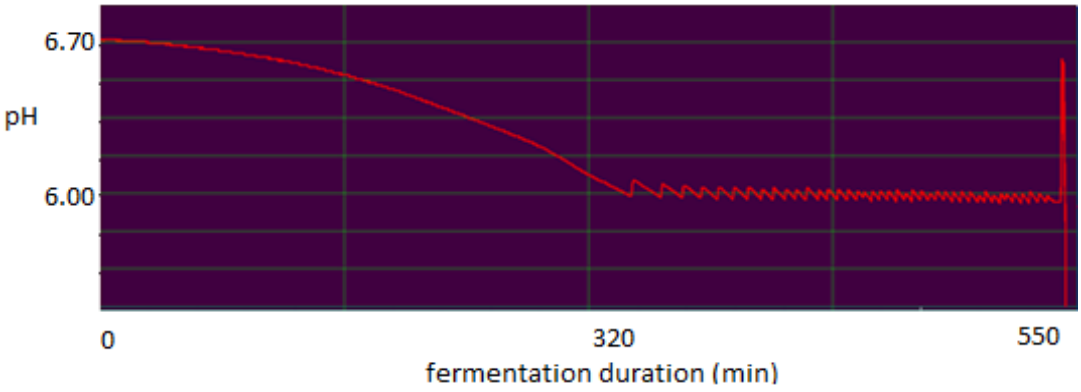

Figure 4-7. An example of pH graph of fermentation trial for SF (3.85hours of fermentation after first pH=6).

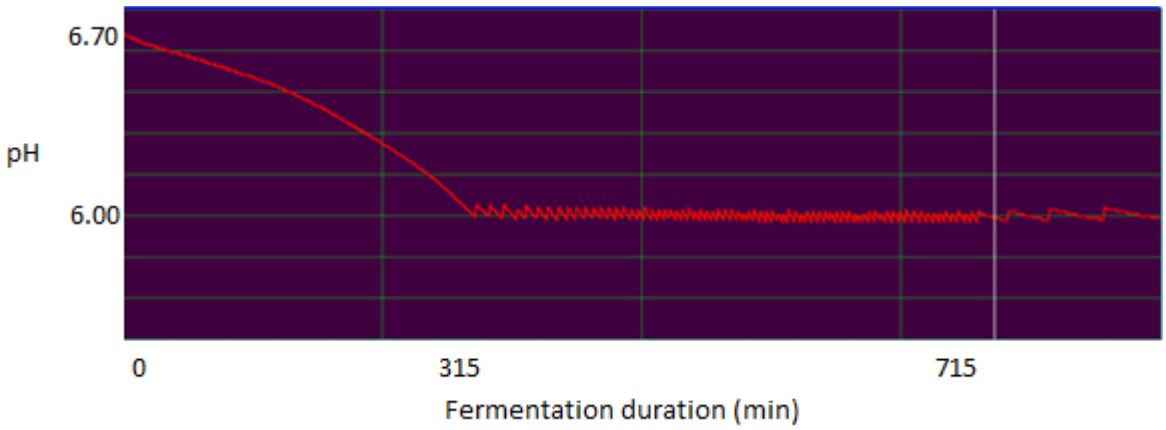

Figure 4-8. An example of pH graph of fermentation trial for MF (6.5 hours of fermentation after first pH=6).

As shown in Table 4-4, the initial quantity of lactose content had no significant ($P<0.05$) effect on the adaptation time (Time for first pH drop to 6.0) and growth rate of *Lactococcus Lactis* strain. This is important for the fermentations as it was the only independent parameter which was different among our trials.

Table 4-5. Fermentation duration at pH 6 and quantity of NaOH consumed to maintain the pH-stat conditions until the end of the fermentation. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

	NF	SF	MF	LF
Lactose added (g)	0	3.4	6.8	10
Time for first pH 6 (min)	320±0[a]	318±7.64[a]	315±7.07[a]	322±16.07[a]
Fermentation duration (min)	0	551.7±27.59[a]	708.75±16.52[b]	922±19.66[c]
NaOH consumed (mL)	0	42±1[a]	67.5±1[b]	94.25±0.96[c]

1 Mean values (±S.D.), (n=3). NF: non–fermented medium, SF: short-fermented medium, MF: medium-fermented medium and LF: long-fermented medium. Within rows, mean values followed by different superscripts are significantly different (P<0.05).

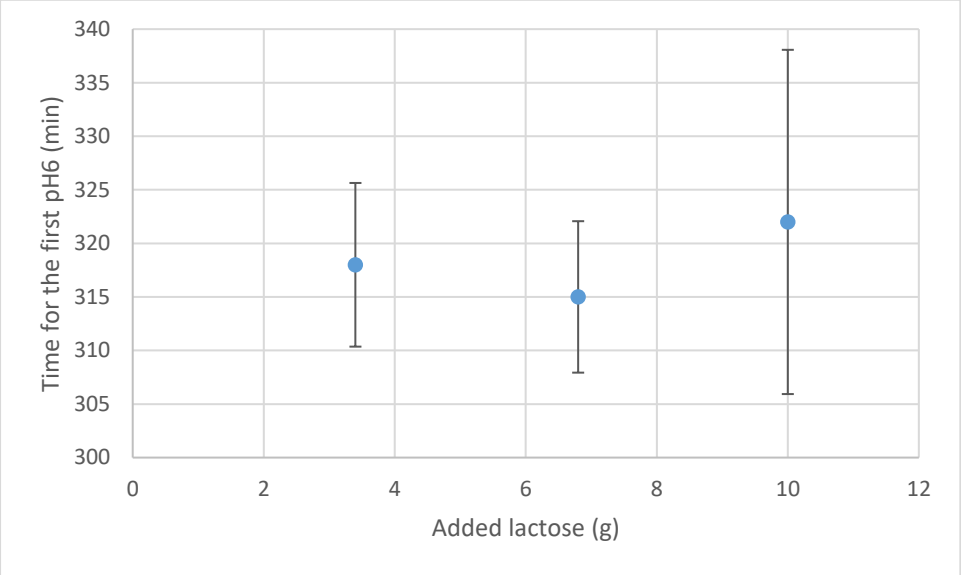

Figure 4-9. Effect of added lactose on time required for first pH 6.00 in PSLT fermentation trials.

This results were also important as the rate of acidification in the initial parts (common in all of the fermentations with different duration) were evaluated to be similar. In other words, the presence of higher initial lactose content did not show to increase the rate of acidification of LAB. It is noteworthy that the rate of acidification in very long (for fermentations longer than 20 hours, results are not shown) fermentation were slowing down significantly. This was possibly related to the inhibitory effects of high sodium – lactate content in the medium. However, the selection of treatments was designed is a way to avoid that range.

4.9 Lactose elimination point (LEP)

With the lactose scarcity in the media, bacterial activity is lost. This was monitored through the pH graph. An example of the pH graph showing this feature is presented in Figure 4-10.

When lactose is about to finish in the reactor, the cycle duration doesn't change but suddenly stops when there is no more lactose in the medium.

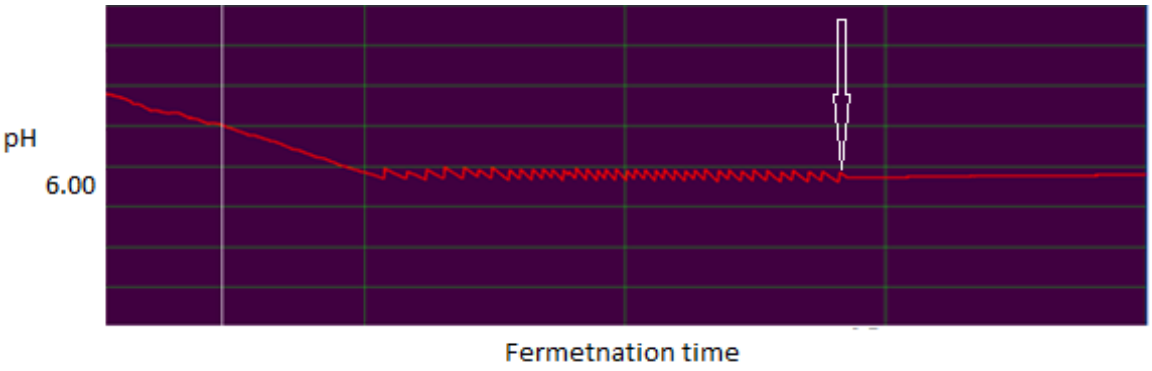

Figure 4-10. Lactose elimination point in a pH-stat fermentation. Arrow indicates LEP.

As clearly shown in the graph, the length of the cycles of the pH-stat system -as a measure of acid production ability of the bacteria- is not affected by the fact that 'the available lactose is about to be finished'. The length of the last cycle in all of the graphs was assessed and equal to the average length of neighbouring cycles. It is important to note that the length of each final cycle before LEP was unique for that lactose content. As it was concluded earlier in this chapter, the length of the cycles is not necessarily the same and was dependent on the length of the fermentation and all of the other parameters involved in the fermentation.

4.10 Non fermented control trial

Our control sample was designed to be the same conditions as all treated sample with zero LAB activity. In order to attain this goal, we needed to have similar (independent parameters) formulation in this sample. MPC85 was prepared according to the method explained in chapter three (materials and methods). Following reconstitution of medium, it was cooled down to 32 °C

and the pH was reduced from around 6.70 to 6.00 over a 6-hour period using 2M lactic acid. The acid used was chosen to be lactic acid to have similar conditions as lactic fermentation by the starter bacteria. The concentration of acid had to be low enough to prevent spot clotting of casein micelles upon addition without the necessity of ultra-high mixing speed. Due to the protein content of the medium, the likelihood of foam formation during high rotational speeds of mixing is high. Thus, 2M lactic acid was added at 250 rpm over a period of 6 hours until the pH reached 6.0. Once the pH reached 6, sodium lactate (pH 6) was added in order to adjust the conductivity of medium to an amount that resembled the longest fermentation trial. The mixing continued for a further 30 minutes and pH was readjusted if necessary.

4.11 Bacterial enumeration

In order to enumerate the Lactococcal bacteria, M17 medium plate count methods were considered. A detailed review of the method is presented in chapter three, materials and methods. By doing serial dilution under sterile conditions, the number of bacteria per mL of inoculate were counted. A plate with colony count of 25-250 (Figure 4-11) was selected for bacterial enumeration.

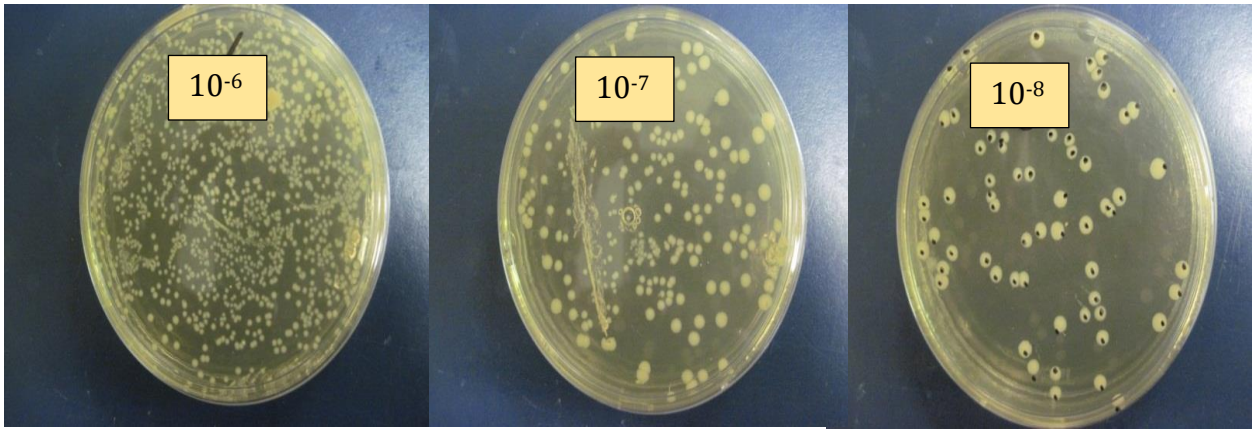

Figure 4-11. An example of plate count results from three dilutions. Dilution 10^{-8} was selected to report the cfu/mL result of the experiment as the colony number is between 25-250.

In order to have an estimation of the number of bacteria inoculated into the fermenter, bottles of starter inoculum were assessed to enumerate their colony forming unit. The results of strains 2338 and HP are shown in Figure 4-12.

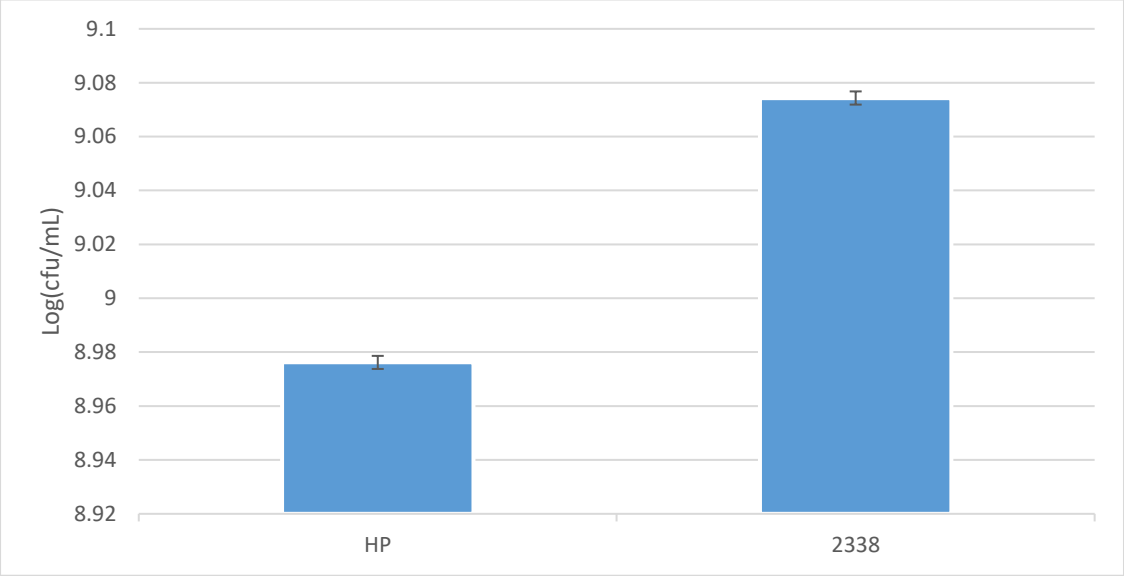

Figure 4-12. Colony forming unit (cfu) count of* Lc. Lactis *strain in each inoculum vial (cfu/mL). Error bars show standard deviation (n=3).

As clearly shown in the logarithmic graph, the number of bacteria in both *LC. lactis* sp. *cremoris* 2338 and *LC. lactis* sp. *cremoris* HP are close to each other. However, as we had the same incubation time for both spp., the lower activity of *LC. lactis* sp. *cremoris* HP (Figure 4-1), resulted in a lower number of final bacteria in each vial. Although the bacteria were injected in the similar method to the vials before incubation time, there is a possibility of difference in bacterial load to the vials even before the incubation time. The method which we used is a standard method and the level of consistency of the initial load is acceptable. The other evidence for this is the fact that the trials were carried out with repetitions in larger quantities (1800 mL in the fermenter) using 25 mL vials of each strains and we found that this strain had the lowest activity among all the strains studied.

Cell extract inoculums were enumerated to check the *lactococcal* count and compared with the cells in 10% RSM inoculum. It was shown that when the original method was adjusted, the number of lactococcal cells were similar to 10% RSM samples ($p<0.05$). This proved that there is

not a significant number of bacteria lost through the supernatant after the centrifugation step or any previously explained step afterwards. It also proved that there is almost a full recovery of the bacterial cells during the re-dispersion step.

4.11.2 Fermenter bacterial count

Bacterial count was carried out in order to have an estimation of the number of active bacteria inside the fermenter. In order to do this a sampling compartment for the fermenter was installed before sterilizing the fermenter. Once the fermenter was running with the fermenting medium, the sampling syringe was pulled to aseptically withdraw some of the fermenting medium to the attached air-tight glass bottle.

Figure 4-13. Fermenter sampling device.

The first few droplets were discarded as this initial amount coming out of the fermenter had been entrapped as a result of the atmospheric pressure inside the sampling tube when the formulated medium was added to the tank at the beginning of fermentation. Hence this was not a true sample of the whole. The bottle was sterilized again by flame and this procedure was done again. The sample was collected, diluted and incubated promptly for enumeration.

The results of our fermenter lactococcal count is presented in Figure 4.14.

A significant ($p<0.05$) increase in lactococcal counts were observed among all sampling times indicating that the fermenter system was suitable for the bacterial growth and development: i.e. the availability of nutrients and suitability of environment in terms of temperature, and redox conditions provided favourable conditions for the starter bacteria. It was decided to assess up to

18 hours from the onset of fermentation (the time inoculum is added to the fermenter at 32.5°C) because all of our fermentation trials lay within this timeframe.

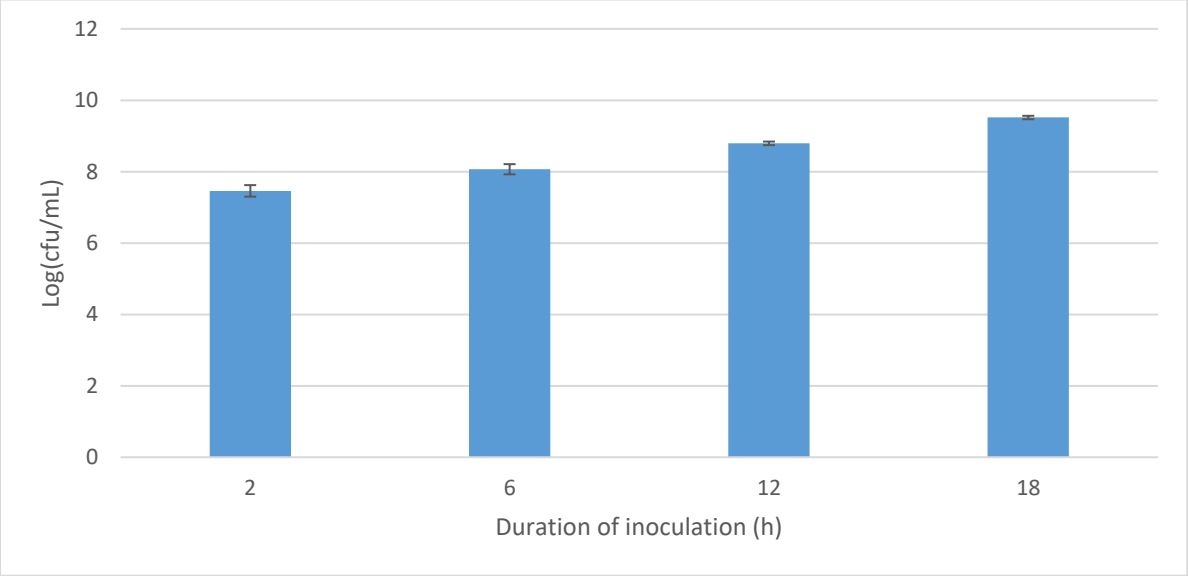

Figure 4-14. Mean Lactococcal count (cfu/mL) from fermenter after 2, 6, 12 and 18 hours of inoculation. Error bars show standard deviation (n=3).

4.11.3 Presence of bacillus

As mentioned before, the Bacillus bacteria can be a potential competitor with our target bacteria if the conditions are in their favour. It was also explained that the pH-stat conditions setting at 6, the rate of acidification of our 2338 spp. and proper sterilization in the beginning were the major steps to minimize the risk of presence of bacillus.

During our preliminary experiments, the fermented samples were assessed for the presence Bacillus (see method in section 3.3.5). The results were all negative for Bacillus strains. The high microbiological quality of the MPC85 powder (total aerobic plate count <10000 cfu/g, Product Bulletin, 071, version 2.1012, Fonterra, New Zealand) as well as sterility of the fermenter before each fermentation trial and aseptic lab practices ensured the minimized risk of Bacillus contamination. The dominance of the starter bacteria in the fermenter starting from inoculation (10^7-10^8 cfu/ml) was an important aspect of our designed PSLT fermentation system.

4.12 Chapter conclusion

In this chapter, the PSLT fermentation system principles were explained. The system was designed to facilitate the long-term fermentation trials. The trials were designed to remove any unwanted proteolytic activity from any source other than the lactic bacteria activity. Different *Lactococcal* sp. were evaluated and two most suitable ones were inoculated to the fermenter as a mixed strain starter culture. Their acidification and proteolytic activity were the basis of strain selection according to our preliminary experiments and the also the published literature. A novel method was developed to perform the fermentations at different durations. Unique methods were utilized to maximize the reproducibility of our results. These included LEP, pH=6 lactate and cell extract inoculum methods utilized. Our results showed evidences on the suitability of the developed method to perform the long fermentations with different durations under pH-stat conditions. Our results also demonstrated high cell density of the starter bacteria in the medium which was thought to be necessary for a detectable action on proteins. The impact of their activity on fermented-medium's proteins will be assessed in detail in the following chapters.

Chapter Five Evaluation of Nitrogenous compounds as an indicator of proteolysis in fermented milk and cheese

5.1 Introduction:

Cheese making is a process in which casein proteins of milk are converted into a curd by three main different methods i.e. acid treatment, enzyme treatment and heat treatment. As explained in Chapter Two, a majority of cheeses are produced by using a combination of both acid and enzyme treatments. The acid is normally produced by starter microorganisms, a group of lactic acid bacteria (LAB) which are considered in Chapter Three in detail. These bacteria are considered to be very important during ripening period of the cheese making process in ripened cheeses. Their main role in cheese making is to convert milk sugar (lactose) to lactic acid and therefore a reduction in cheese pH which increases the shelf life of the final product alongside several textural and aromatic impacts on final product. However, the LAB bacteria remain in the curd after processing and significantly impact the textural characteristics of the final product as a result of their proteolytic system activity. Since milk contains a limited quantity of free amino acids (FAA) and small size peptides, the *Lc. lactis* bacteria, which are auxotroph for several amino acids, rely on the casein protein hydrolysis (Mierau et al., 1996).

Proteolysis is a multi-parameter process with a range of participants and is regarded as one of the most important biochemical reactions (beside glycolysis of lactose and lipolysis of fat) happening during cheese ripening. In normal cheese making practices, the initial proteolysis by rennet is referred to as 'primary proteolysis' and this has been reported to be influential on cheese rheological properties (Creamer & Olson, 1982; Lawrence et al., 1987). However,

it is also well established that the bacteria are the most important player in development of characteristic textural and flavour attributes through proteolysis during cheese ripening (see Section 2.3).

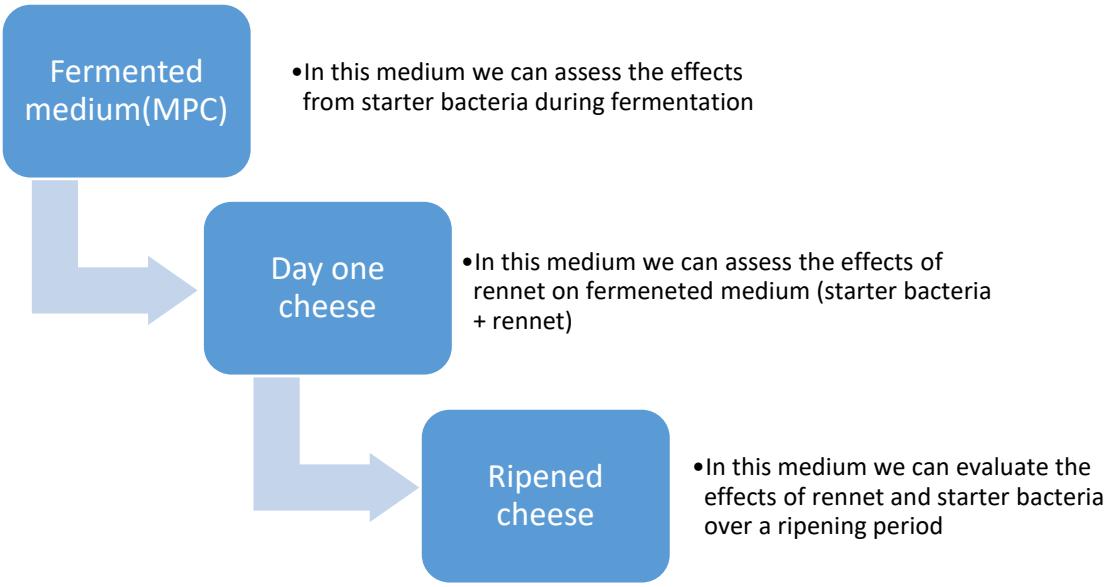

Figure 5-1. Assessment points to track down the proteolysis and the major contributors at each step. The focus in this study is before curd formation.

Cheese ripening is a rather long process (in some cheeses more than 2 years) and normally requires controlled temperature and atmospheric conditions. Ripening also affects the price of final product as it requires significant labour and investment to handle it.

If some of the proteolytic activity that normally occurs during ripening could be achieved in the cheese milk prior to curd formation, then some of the cost associated with ripening may be reduced. Under such circumstances the bacterial activity can be manipulated through optimising conditions to suit their requirements and their activity on the protein base of cheese milk especially the casein proteins.

As explained in Sections 2.2 and 2.6 and also Chapter 3, pH is an important parameter and its variations can affect all other aspects of cheese making parameters. In the present study, it was attempted to keep a predetermined fixed pH value for the experiments to maximize the interpretation of the possible.

5.2 Chemical analysis of fermented medium

The cheese milk base for this work was a milk protein concentrate powder with 85% protein (commercial product MPC4850 provided by Fonterra Research and Development Centre, Palmerston North, New Zealand) reconstituted to ~3.4 (w/w) protein. The basis of formulation adjustments of the medium is presented in Sections 3.1 and 3.2. The basis of concentration and pH selection are presented in Chapter Four, Fermentation. The treatments were selected to evaluate the effects of long-time fermentation at a fixed pH value. The rationale for selection of the pH-stat value and duration of each trial is described previously (3.1.2, 3.1.5 and 4.6). The focus of this study was to determine the effects of the length of pH-stat fermentations of the cheese milk on curd formation.

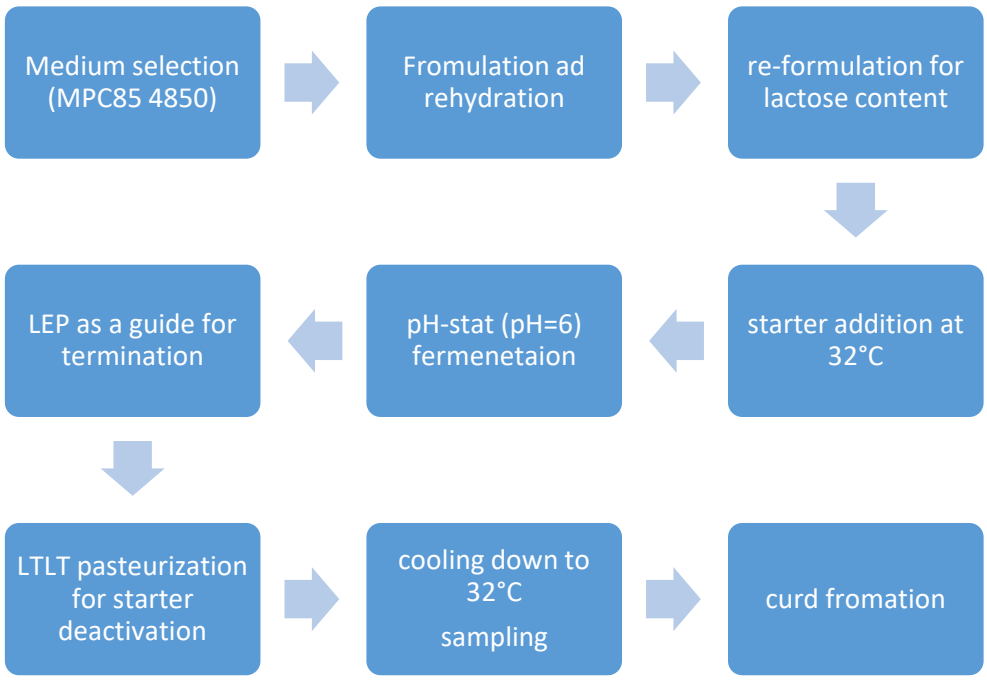

Figure 5-2. The process flow of pH-stat long-term formation before curd formation.

The protein content of the fermenting cheese-milk base as a function of fermentation time was measured using the Kjeldahl method (see Section 3.3.5) at sampling point (Figure 5.1). Rao, Pulusani and Rao (1982) reported a slight decrease in nitrogen content of a fermented medium compared to the unfermented media. They concluded this may be due to production and loss of

volatile nitrogenous compounds. However in this work the results showed no significant ($p<0.05$) change in protein content as fermentation progressed.

Table 5-1. Different parameters of fermented medium just before curd making. Mean± S.D. for protein and sodium contents. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Treatment	*Protein content (%w/w)	Added lactose (g)	Lactose concentration (%w/w)	**Expected** Fermentation duration (h)	[1]Conductivity (mS/cm)	**[2]Sodium ISE (mg/L)
NF	3.40±0.04	0	0.19	0	1.97±0.00[a]	280±15[a]
SF	3.44±0.05	3.4	0.4	~9	3.6±0.1[b]	780±11[b]
MF	3.44±0.04	6.8	0.59	~12	7.6±0.1[c]	1155±22[c]
LF	3.41±0.02	10	0.78	~15.5	9.8±0.1[d]	1479±25[d]

***n=4, **n=3*

Values followed by different superscript in one column are statistically (p<0.05) different.

1 Conductivity measurements recorded at the end of PSLT fermentation before lactate adjustments. For NF sample, conductivity measurement done at pH 6.7 before pH and lactate adjustments.

2 Sodium measurements carried out at the end of PSLT fermentation before lactate adjustments. For NF sample, sodium measurements carried out before lactate adjustments.

As the preparation method for all of the treatments were consistently similar, the protein content of all the fermented mediums before any further treatments expected to remain unchanged.

The pH values of all of the fermented mediums were recorded during the fermentation. A Schott Prolab 4000 measuring system was used to record the pH. The pH value started to drop once the starter bacterial cells initiate the fermentation. During this time, the lactose is metabolised to lactic acid and through this conversion the energy required for growth and development of bacteria is provided. The pH-stat system (see Section 3.1.7) continuously monitored the pH and kept it constant by controlling the pH to pH=6 through automated addition of alkali to neutralize the produced acid. If there was any minor difference with the target pH (pH=6) at the end of fermentation, then it was adjusted by using either 1N NaOH or 2M lactic acid.

The lactose content of each treatment was calculated based on the MPC85 lactose content according to manufacturer's ingredient specification (4%). The adjusted lactose content is the

sum of the initial lactose content plus the added lactose. The lactose addition rationale is reviewed in detail in Chapter 4.

The lactose content of the medium was calculated based on the intrinsic lactose content of the medium. For the non-fermented medium, the lactose was not consumed and remained unchanged. All of the fermented mediums have been fermented until the lactose elimination point (LEP). At this point there was no further lactose remaining in the fermenter.

One of the major reasons for lactate addition was to equalize the conductivity of the samples to keep consistency among the samples (with different length of fermentation and therefore different alkali added). However, it turned out to be impossible to make up the fermented medium from different treatments similar in terms of conductivity while maintaining the other parameter equal. The basis of calculations of lactate addition is presented in Chapter 3. The non-fermented sample necessitated the use of direct lactic acid addition to decrease the pH to 6 (no fermentation by bacteria). The amount of lactate added to this medium was equal to the amount of alkali which was added to the longest fermented medium during pH-stat fermentation.

5.3 Nitrogen fractionation as a guide to the extent of proteolysis

As reviewed in Chapter One, alongside calcium equilibrium, the proteolysis was reported to be the most influential parameter in defining texture and flavour characteristics of cheese (P F Fox et al., 2017). The solubility of nitrogenous compounds in different solvents is used as a measure of the degree of proteolysis and hence ripening of cheese (Bütikofer, Rüegg, & Ardö, 1993). The various methods of fractionation are explained in Chapter Two. The fractionation scheme was designed to discriminate the target components in order to extract them for further analysis. The tendency of nitrogenous compounds to dissolve in a solvent is the basis of such fractionations. The sample is treated by a predetermined solvent (under defined time, temperature, stirring, etc.) and this results in the dissolution of a portion of the original sample while the remaining protein based material remains undissolved. The soluble and insoluble fractions are then separated (using centrifugation and/or filtration, distillation, evaporation, etc.).

5.3.1 pH 4.6 fractionation

One of the most common methods to evaluate the extent of proteolysis in cheese is the index of soluble nitrogen as a percentage of total nitrogen (Bansal, Piraino, et al., 2009). A significant correlation between the %SN/TN and functional properties of processed cheese has been reported by Wang *et al.* (2011).

Different cheese making agents (coagulant, starter bacteria and non-starter LAB) contribute to the proteolysis. Figure 5-3 depicts the schematic effect of each of the above mentioned agents on proteolysis of cheddar cheese. The Figure shows that the presence of coagulant, and starter bacteria would result in the highest level of proteolysis in cheese.

Figure 5-3. Water Soluble Nitrogen (WSN) formation in cheddar cheese: (A) controlled microflora (absence of non-starter flora); (B) controlled microflora and starter-free (chemically acidified); (C) Controlled microflora with no rennet; (D) controlled microflora, no rennet and no starter (P F Fox et al., 2017).

During the treatments before renneting, samples have been fermented for different times by controlling lactose content. The lactose elimination method has been utilized in order to make sure there will not be any further fluctuation in pH during sample preparations. In order to keep

the consistency among the samples, sodium lactate was added to obtain desirable pH (as explained in Chapter 3).

The fermented mediums were also heat treated (low temperature long time) in order to deactivate the starter bacteria. The pH values were readjusted (using 2 M lactic acid) to pH 6.0 at the end of the cooling step (refer Figure 5.2). An aliquot was taken at the end of each fermentation trial and transferred to Kjeldahl tubes immediately for total nitrogen evaluation.

A further two aliquots were taken (about 40 mL each) for pH4.6 fractionation according to Sousa and McSweeney (Sousa & McSweeney, 2001).

The result of total nitrogen for the fermented medium is presented in Table 5-2.

Table 5-2. pH4.6 SN/TN of the treatments at the end of fermentation and the net increase in the water soluble nitrogen index. Non fermented, Short fermented, Medium fermented and Long fermented represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Fermentation	***%SN/TN**	**Water soluble nitrogen index**
Non fermented	13.46±0.16[a]	0
Short fermented	14.36±0.03[b]	1.23
Medium fermented	16.8±0.13[c]	3.08
Long fermented	17.90±0.07[d]	4.34

mean±S.D. (n=3),
Values followed by different superscripts are statistically (p<0.05) different.

The quantity of solubilized nitrogen as a percentage of total nitrogen is referred to as proteolysis index (Kuchroo & Fox, 1982). Although this term is commonly used when analysing curd and cheese, there is no report in the literature of using this term for the proteolysis before curd formation. The Proteolysis Index can show important aspects of bacterial activity before curd formation during long term fermentations. A significant ($p<0.05$) increase in pH4.6 SN/TN was observed among the samples as a result of longer PSLT fermentation. The results of the pH4.6

SN/TN are presented in Table 5-4. The results also show the net increase in pH4.6 SN/TN as a result of our treatments.

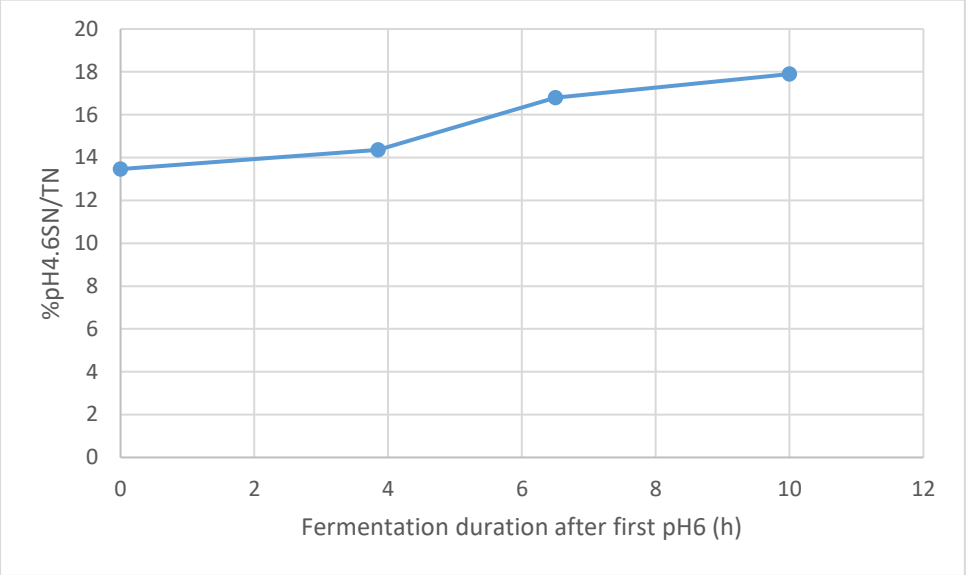

Figure 5-4. pH4.6-soluble nitrogen of samples as a percentage of total nitrogen.

As a result of our treatments (fermentations longer than common cheese making practices before curd formation), the bacterial cells start to use their comprehensive proteolytic system to hydrolyse the milk proteins as a source of nitrogen (detailed review is presented in Chapter 2). This would result in breakdown of the casein proteins into smaller size protein/peptide molecules which would be different from the original protein structure

In normal cheese making processes rennet is added within 1 hour of starter bacteria addition. In such a short period of time the bacteria are adapting themselves to the new medium and grow sharply. However, due to cheese making process parameters (*i.e.* temperature, pH at whey drainage, lack of fermentable carbohydrates, etc.) the bacterial growth will be significantly reduced (Upadhyay et al., 2004), and in some cases, such as mozzarella which includes high cook temperatures, terminated. In this study the environmental conditions were kept favourable for the growth and development of LAB bacteria (see Chapter 4).

On day one of cheese manufacture, the proteolytic effect is mostly arising as a result of rennet action. McCarthy and co-workers found a 5% value for %SN/TN in cheddar cheese(McCarthy,

Wilkinson, Kelly, & Guinee, 2016). They also have reported an increasing trend over ripening time reaching to almost 20% over 9 month ripening time. Sausa and McSweeney (2001) also reported a 5% increase in cheese at day one of Cooleeney cheese. Lane and Fox (1997) reported a similar value for proteolysis index for cheese at day one of production.

Fenelon *et al.* reported an increase in pH4.6SN/TN in low fat cheese during ripening (Fenelon, O'Connor, & Guinee, 2000). Sausa and McSweeney reported an increase of around 5% in pH4.6SN/TN over a period of 28 days in presence rennet and other proteolytic agents in Cooleeney cheese (Sousa & McSweeney, 2001). McCarthy and co-workers (2016) found a 5% increase in pH4.6SN/TN over around a month of ripening in cheddar cheeses. Hannon *et al.* reported the same net increase in cheese with modified enzymes to accelerate the flavour of cheese over a one moth ripening period(Hannon, Kilcawley, Wilkinson, Delahunty, & Beresford, 2006). Having reached a similar increase in the net proteolysis index (pH4.6SN/TN) in our longest fermentation (in absence of rennet as the most powerful hydrolytic agent) was promising.

5.3.2 70%Ethanol fractionation

The specifications of the EtOH fractionation method used in this research are presented in Chapter 3. This fraction is a sub-fraction of pH4.6 soluble fraction (refer Section 5.3.1) and the results showed in increasing trend in pH4.6SN/TN. According to the literature TCA and EtOH extract a similar range of nitrogenous compounds from cheese (Bansal, Piraino, et al., 2009). After considering the toxicity and environmental pollution of the solvents (Bansal, Piraino, et al., 2009; Bütikofer et al., 1993) in both methods and ease of removal of the solvents from the extracted material, ethanol was preferred to TCA. Peptides that are soluble in 12% TCA belong to a range of peptide sizes that vary from 2 to 22 residues of amino acids (600 Da < MM < 15 000 Da). The quantity of these peptides as a portion of total nitrogen is known as the ripening depth index (Roseiro, Garcia-Risco, Barbosa, Ames, & Wilbey, 2003).

Once these fractions are prepared, the insoluble fraction is ready to be freeze dried for further assessments. The soluble fraction dissolved in an abundant amount of solvent, undergoes a

further step of solvent removal to be ready for further processing. Rotary evaporation (see Chapter 3) was used to ensure the sample was solvent free and ready to be freeze dried.

These sample were analysed by HPLC for peptide profiling. The results of HPLC analysis of each fraction are presented in following Sections of this Chapter.

5.4 High performance Liquid chromatography

The method used for analysis is presented in 3.3.9.

5.4.1 Ethanol soluble fraction

Chromatograms of the reverse phase analysis of soluble fraction from NF, SF, MF and LF are presented in Figure 5.5. As shown in the graphs, once the duration of fermentation increased the number of emerged detectable compounds produced as a result of increased proteolysis. In the previous Section, an increase in the pH4.6 SN/TN was demonstrated, this fraction was then purified by removing all the non-soluble fraction. The net bacterial activity during treatments increased (as discussed in Chapter 4) and this is correlated with the observed increase in the number of peaks detected in the chromatography at 214nm with a reference array of 360nm. However, it is noteworthy that there is not a similar trend in the appearance/disappearance for all of the peaks. Further study is required to match the peaks with relevant compounds and analyse the trends individually.

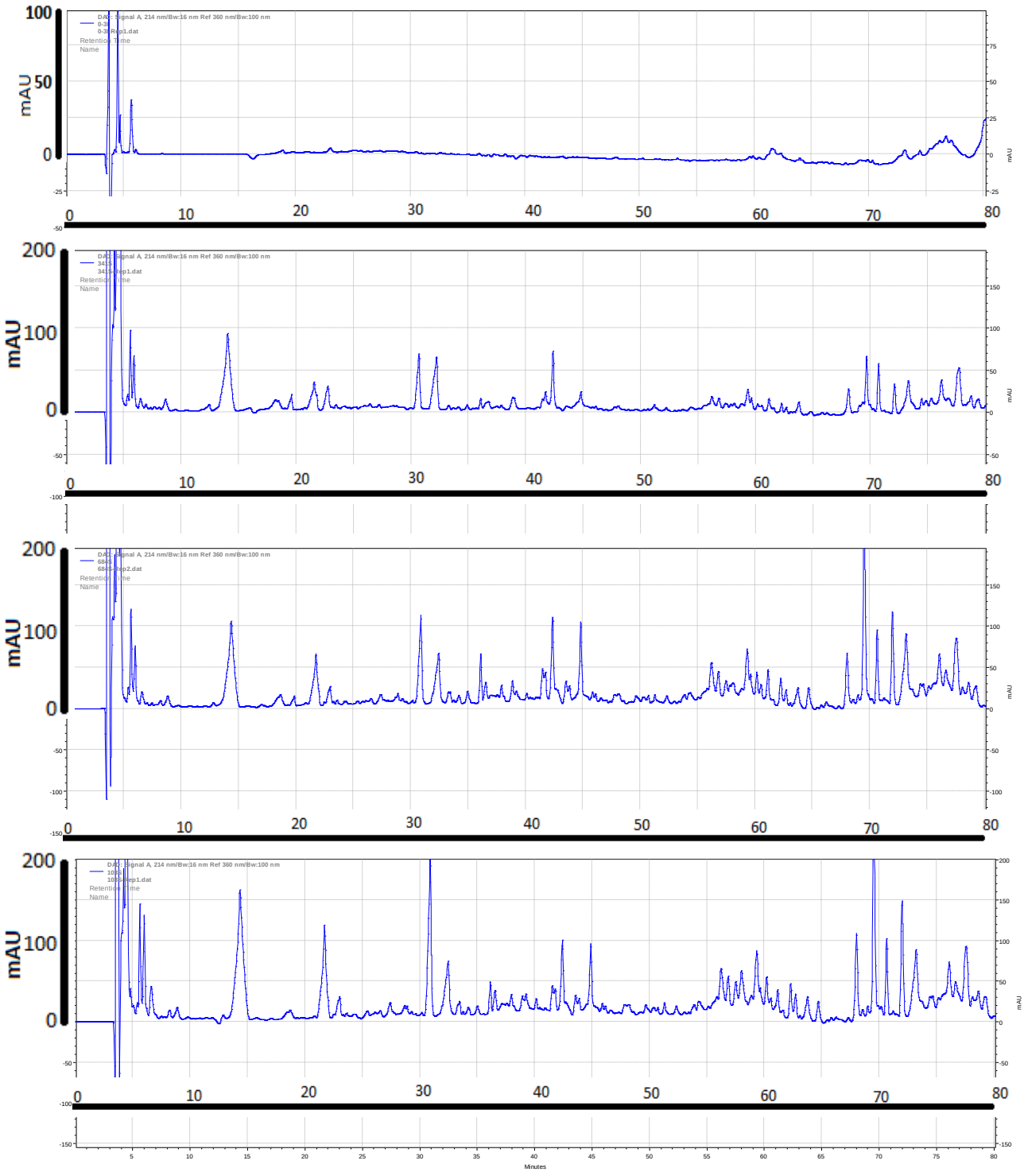

Figure 5-5. Chromatogram 70%EtOH soluble of the pH4.6S fractious of samples (from top to bottom: non-fermented, short fermented, medium fermented and long fermented respectively. X axis shows the retention time (min).

The results are in agreement to other researchers' work. de Llano Polo and Ramos reported an increase in the number of peaks detected during peptide profiling of water soluble fraction of blue cheese over ripening time (de Llano, Polo, & Ramos, 1991).

The evolution and increase of some of the major peaks by increasing fermentation times have been assessed. The peaks with retention times at 21.5, 31, 59 and 68 minutes were selected to evaluate the possibility of any correlation between the time of fermentation and the absorbance of the peaks (Figure 5.6). A linear regression (R^2 >0.96) was observed between some of the peaks absorbance and duration of fermentation at pH6. This may be related to the high specificity of LAB proteolysis system which was mentioned earlier in Chapter 2.

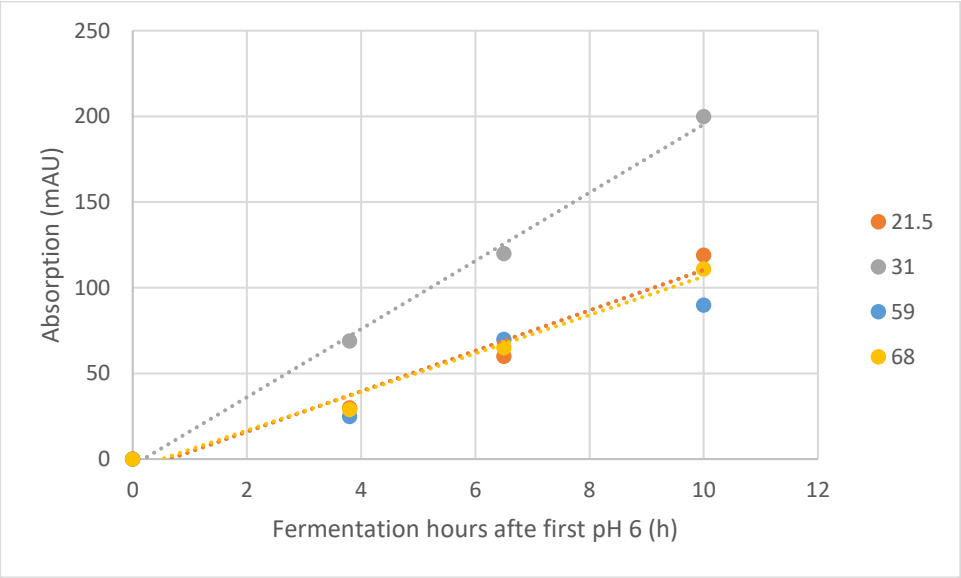

Figure 5-6. Changes in some of the major peaks with duration of fermentation after pH=6. The name of each series in the figure represents the retention time related to each individual peak.

It is noteworthy that not all the peaks are increasing. For instance, the peak evolved after 32 minutes has remained constant and the peak with retention time 19 in the short fermented chromatogram is disappearing once the fermentation duration is increased. Our results showed a lower number of peaks in EtOH-insoluble fraction compared to the pH 4.6 soluble fraction for individual samples. The height and area under chromatogram peaks were also lower in the same manner. The bacteria can only uptake the small seize peptides (up to 8 amino acids) and then

facilitate the metabolism of peptides by their comprehensive proteolytic system. On the other hand, smaller peptides have been discussed to be more soluble in EtOH, these may be the reason we had more peaks in the EtOH soluble fraction than the insoluble counterpart. These results were in accordance with the results from other researchers (Shakeel-Ur-Rehman, Pripp, McSweeney, & Fox, 1999; Sousa & McSweeney, 2001). There is possibly a relation between the PSLT fermentation using LAB and production of small sized peptides.

Quantitative evaluation of the chromatograms showed that there is a difference between the rate of increase in the peptide profile of samples with different extent of proteolysis. The group of peaks eluting between 30 to 45 minutes showed slower increase between the SF and LF whereas the peaks with retention time between 65-80 showed a greater increase in peak areas. This is in agreement with O'Mahony et al., who reported similar trends in cheeses made with different chymosin residual activities (O'Mahony, Lucey, & McSweeney, 2005). The suitability of these author's result to be compared to our results is arising from the fact that they limited the extent of pH4.6SN/TN formation and in some of their studied ripening periods, the proteolysis indices are close to our MF and LF fermented mediums. They related the greater increase in the late eluting peaks with longer ripening times to possibility of production of hydrophobic peptides which are eluting at later retention times during RP HPLC.

There were also a few of large peaks (compared to the rest) at the end of EtOH-insoluble fraction chromatogram. This was the time the acetonitrile solvent was maximum and dissolved all the hydrophobic compounds from the column (the peaks are not shown in the chromatograms).

Sausa and McSweeney reported similar result from evaluation of Cooleeney cheese. They analysed the pH4.6 soluble fraction by further fractionation using EtOH. They reported lower number of peaks in the insoluble fraction- with some large peaks at high hydrophobic area. They also reported higher numbers of peaks and higher peak areas in the EtOH-soluble fraction compared to EtOH insoluble. Furthermore, they demonstrated an increase in the number of peaks and the area under the chromatogram peaks as a result of longer ripening time (Sousa &

McSweeney, 2001). Gagnaire *et al.,* found the same pattern of peptide profile in Emmental cheese sampled at different stages of ripening (Gagnaire, Mollé, Herrouin, & Léonil, 2001).

The area under chromatogram peaks was calculated using equation below. This equation was used to calculate the total peak under chromatogram following by an integration on the chromatogram to include most of the visually detectable peaks.

$$Area = \int Abs * dt$$

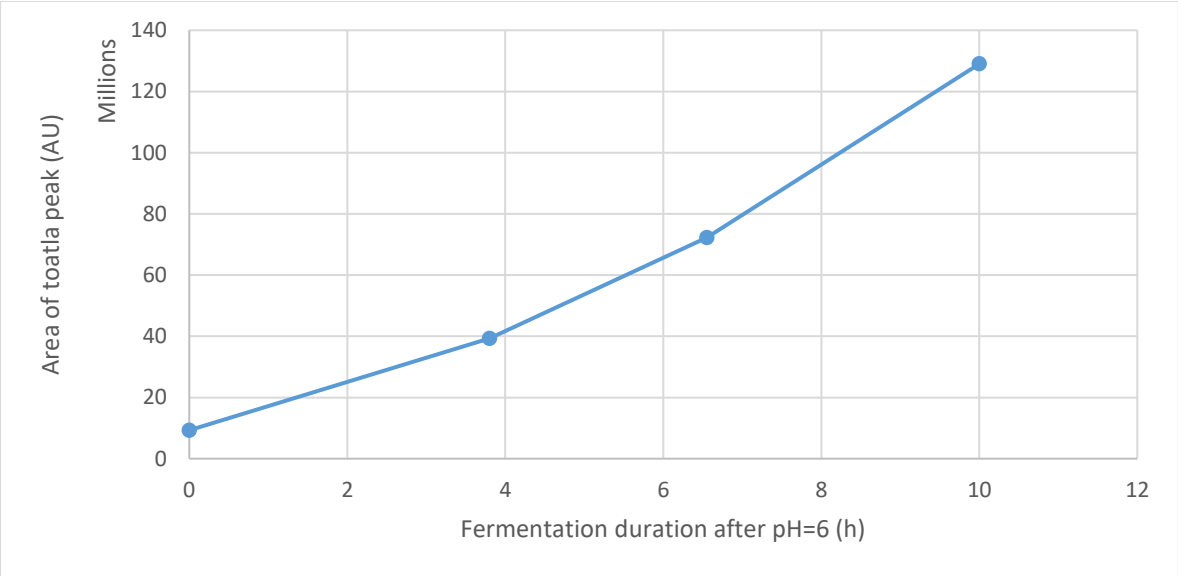

Figure 5-7.Increase in 'area of total peaks' of 70%EtOH soluble chromatograms for NF, SF, MF and LF treatments. Error bars show the standard deviation (n=4).

Our result also showed a significant ($p<0.05$) increase in the peak area under chromatograms of 70% soluble fraction when the duration of PSLT fermentation was increasing (Figure 5.7).

Fortula *et al.* reported an increase in the 'total peak area' of chromatograms of water soluble fractions when they treated the cheese samples to accelerate the cheese ripening (Furtula, Nakai, Amantea, & Laleye, 1994).

Polychroniadou *et al.* reported a correlation between total area of chromatograms and water soluble fraction of cheese (Polychroniadou et al., 1999). They connected the number of peaks in each chromatogram and total area under peak to the variability of peptides and molar absorption as a result of higher number of AA in each peptide. A further qualitative evaluation (extraction by

TCA or EtOH) was suggested by them to acquire more detailed information on the size and hydrophobicity of the peptides.

5.4.2 70%Ethanol Insoluble fraction

The results of reverse phase HPLC of the 70%EtOH insoluble fraction are presented in Figure 5-8. The graphs show an increase in the number of detectable components and also the height of peaks when the duration of the fermentation was increased.

De Llano, Polo and Ramos (1995)reported an increase in water soluble extracts of artisanal cheeses during ripening. They assessed samples of Afuega'l Pitu cheese and reported an increase in the absorbance when they monitored the chromatograms over a 60-day ripening time. They also reported a correlation between this parameter and measured water-soluble N. A positive correlation has been observed in our data, too. When the fermentation time increases, the value of pH4.6SN/TN increased and the overall absorbance of detectable compound in EtOH soluble fraction is also increased. Farkye, Madkor, & Atkins (1995) reported an increase in total area under chromatogram peaks of a model cheddar cheese system with various lactic acid bacteria blended into freshly made starter-free cheese. They reported at least 10% increase in the total number of peaks in samples treated with starter bacteria compared to the control (with no starter bacteria). Their finding showed an average of more than 8% total area of peaks for all samples (except for interestingly one sample lower than control).

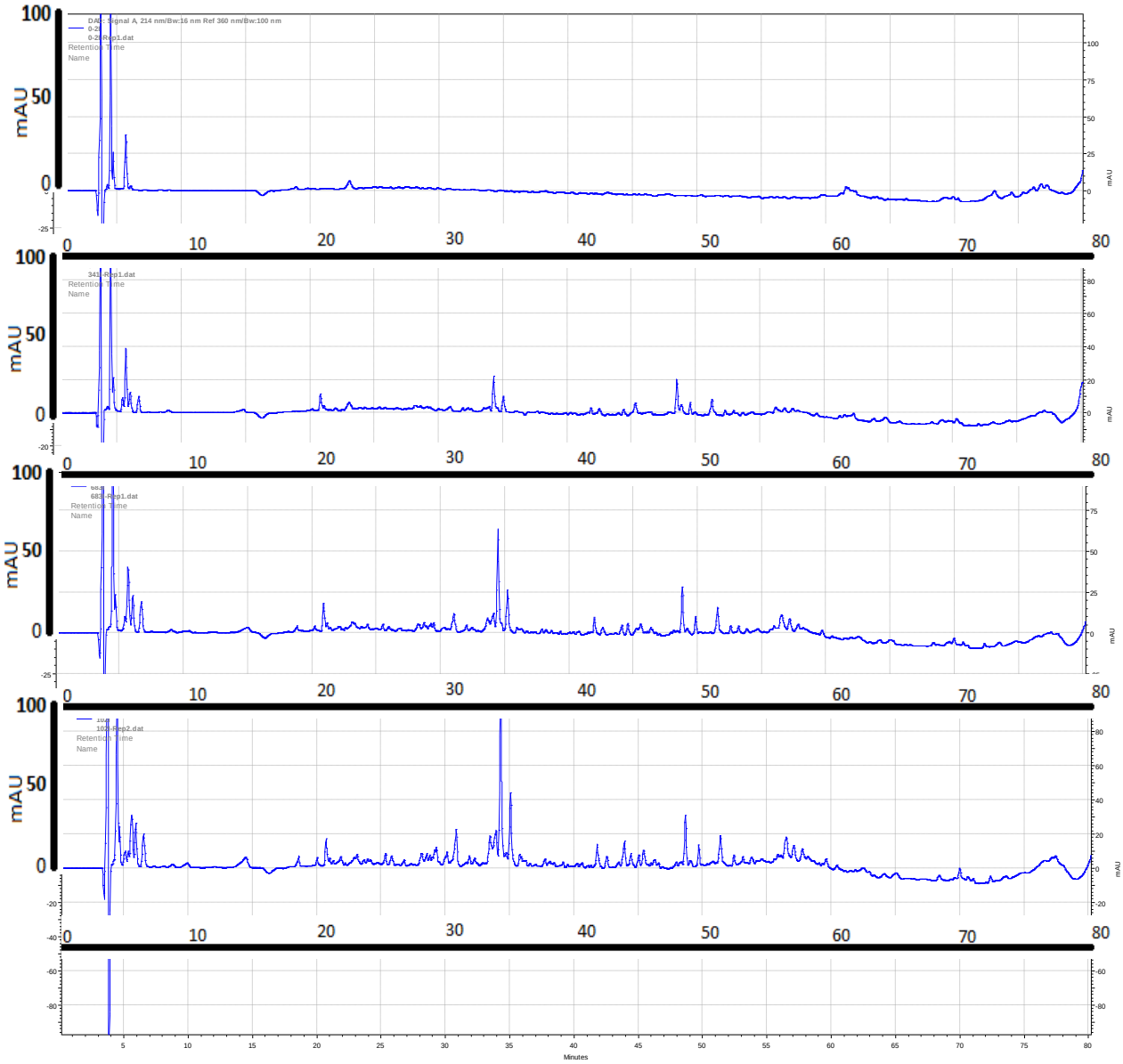

Figure 5-8. Chromatogram of the 70%EtOH insoluble fraction of the pH4.6S fraction for the samples (from top to bottom: non-fermented, short fermented, medium fermented and long fermented respectively. X axis shows the retention time (min).

The area under the chromatogram peaks was calculated and a significant ($p<0.05$) increase with fermentation time was observed. The results of this parameter are presented in Figure 5.9.

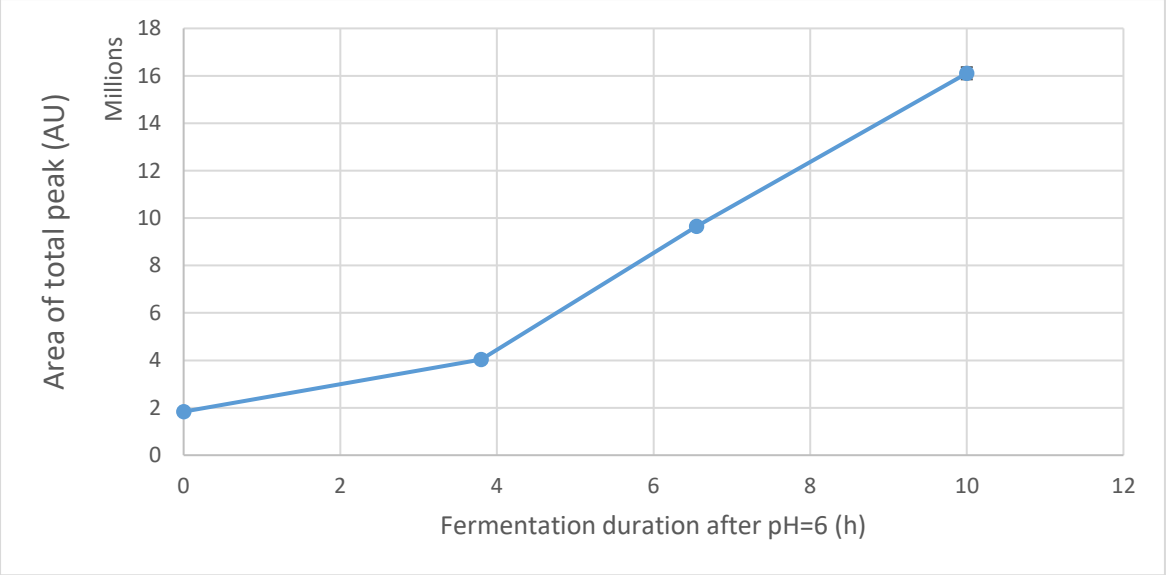

Figure 5-9. Increase in 'area of total peaks' of 70%EtOH insoluble chromatograms for NF, SF, MF and LF treatments. Error bars show the standard deviation (n=4).

5.5 Mass Spectrometry

Freeze dried samples of the 70%EtOH soluble fractions were analysed to evaluate the effect of PSLT fermentation on the evolution of small-sized peptides (m/z lower than 1200). This range was selected to assess the formation of small peptides which are mainly produced as a result of LAB starters in cheese. As the data form RP-HPLC are indicators of the tendency of peptide based on their hydrophobicity/hydrophilicity, it is not possible to make judgment on the size of the resultant peptides in the profile. Therefore RP-HPLC results can be complemented by the results from mass spectrometry. The value of mass spectrometry results in cheese proteolysis and ripening is arising from the fact that they can be an indicative of the small sized peptides produced during ripening. It is noteworthy that in cheese science and technology the production of small peptides are normally related to the activity of bacteria (including starter bacteria) and is reported as so-called 'depth of proteolysis' alongside with the free amino acid content. This is normally opposed to the 'extent of proteolysis' which accounts for the primary proteolysis as a result of rennet activity.

For mass analysis, the freeze dried samples were rehydrated using TFA 1/10 in MQ water. Aliquots of 200 µl were then ultrafiltered using Vivaspin 500 (GE Healthcare Bio-Science AB, Uppsala, Sweden) at 15000xg for 30 minutes. The method of mass spectrometry used was Quadrupole Time of Flight (Q-ToF). The samples were injected into an Agilent mass spectrometry machine (Agilent 6500 Series AccurateMass Q-TOF LC/MS) in replicates and an average of 90 scans were done per injection.

The chromatograms/spectrums were acquired using data acquiring software MassHunter Workstation (© Agilent Technologies, Inc. 2011) and processed using OpenChrom software (OpenChrom Community edition 1.1.0).

The mass spectra results are presented in Figure 5.10. The ratios of molecular mass to charge (*m/z*) of various compounds are shown in the figure. Relative intensities of samples with different levels of proteolysis are also shown in arbitrary units. Identification of peptides is normally achieved by doing tandem mass spectrometry, however this was not possible given resources limitations of this study.

Our results show an increase in the number of detectable peaks and the intensity of absorption in the chromatograms when the fermentation duration was increased. Our results are in agreement with published literature. Piriano *et al.* (2007) showed an increase in the number of peaks and intensity of absorption in the 70%EtOH soluble fraction of cheeses with higher levels of maturation.

In order to evaluate the level of proteolysis, the area under mass spectrometry peaks is an appropriate tool. It is especially efficient to evaluate the changes in mass spectrometry as evolution of new peptides would result in new mass and, therefore, a new peak. The results of peak areas of each chromatogram after integration are presented in Figure 5.10.

Our results demonstrate a significant ($p<0.05$) increase in the area under the peaks of mass spectrums. This is in agreement with other researchers' results. Fedele *et al.,* (1999) observed an increase in the abundance ratio of yoghurt samples when the duration of fermentation (and

therefore LAB activity) was increased from 1 to 6 hours. This is also in agreement with our other results for proteolysis assessment (pH4.6SN/TN).

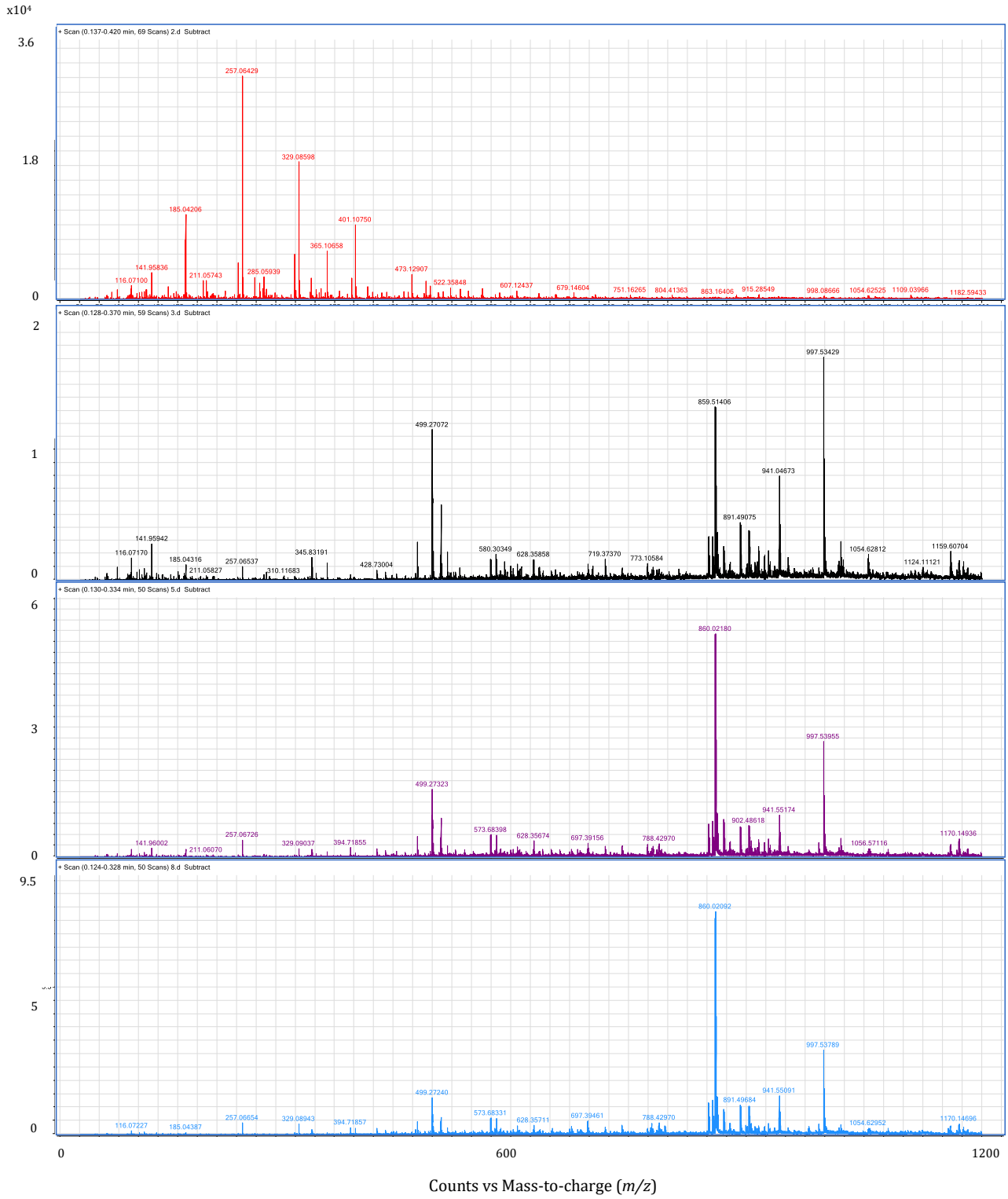

Figure 5-10. Chromatograms from LC/MS mass spectrometry of 70%EtOH soluble fraction for m/z values up to 1200. From top to bottom: NF, SF, MF and LF respectively. Y axis values are arbitrary units.

Figure 5-11. Q-ToF mass spectra of three 70%EtOH soluble fraction of three different cheeses with different extents of ripening. Top left: Cheddar cheese (day one); top right: Cheddar cheese (4 months) and bottom: Parmigiano-Reggiano fully ripened (printed from (Piraino et al., 2007).

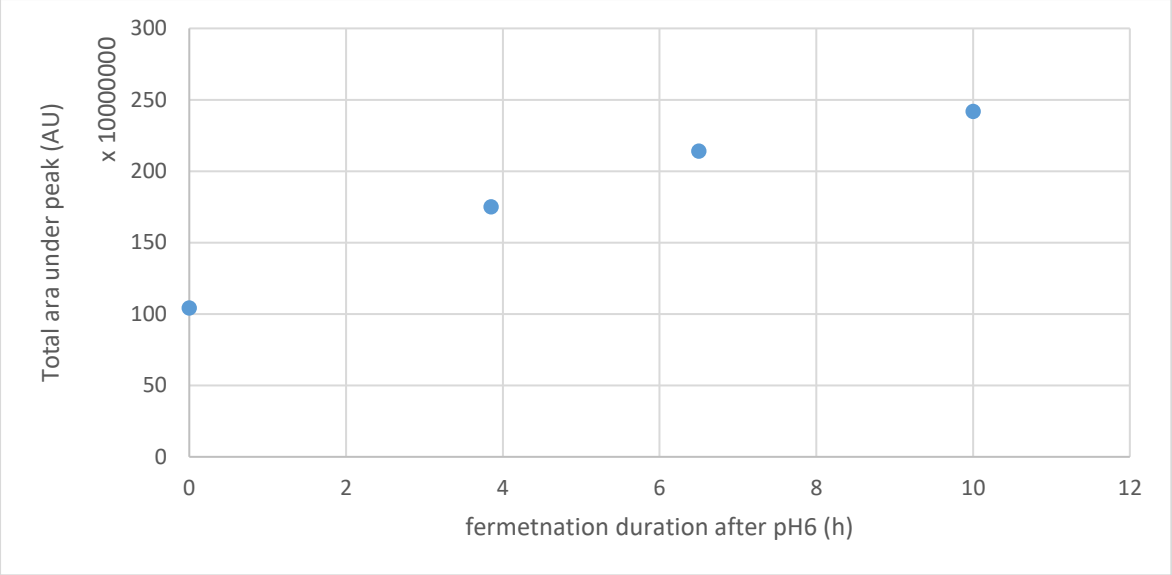

Figure 5-12.Total area under chromatogram acquired from Q-ToF Mass spectrums for NF, SF, MF and LF treatments. Error bars show the standard deviation (n=3).

5.6 Free amino acid evaluation

In order to evaluate the effect of PSLT fermentation on amino acid profile of ripened cheese, one randomly selected sample belonging to each fermentation duration was evaluated. The method utilized is presented in Chapter 3 with an exception. The cheeses made for this purpose were transferred to 4°C right after dewheying, pressing and brining steps (without leaving at 20°C for 48 hours). These cheeses were kept at this temperature for 12 months. Just before amino acid evaluation, they were drained carefully and the surface moisture were dried up using lint-free paper towel.

Our result showed an increasing trend in the formation of each individual amino acid when the duration of fermentation was increased (Figure 5-13). Leucine – a branched chain amino acid (BCAA) - showed the highest increase in the profile. A similar increase in Leucine was reported by Puchades *et al.* when the cheese was ripened at 6°C (Puchades, Lemieux, & Simard, 1989). Saldo, McSweeney, Sendra, Kelly and Guamis (2002) reported an increase in total amino acid content of cheese during ripening and have reported the results as grams of Leucine per 100g of cheese.

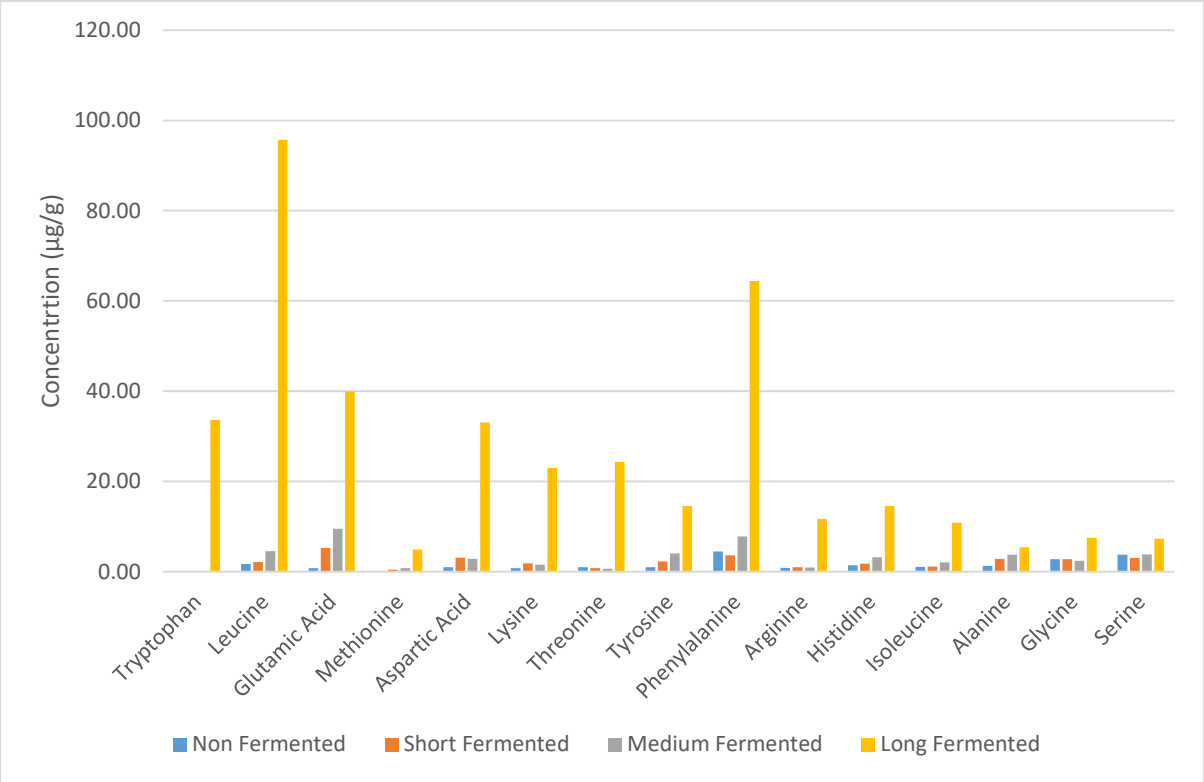

Figure 5-13. Accumulation of individual amino acid in ripened cheese made from PSLT fermented cheese milk with different fermentation durations.

Lane and Fox reported an increase in free amino acids over ripening in cheese made using controlled microbiological conditions. They reported a sharper increase in Total FAA in cheese sample made in presence of microorganisms when the compared with the result with cheeses made via direct acidification (GDL). They also evaluated the cheeses made by starter bacteria with no proteinase activity (the rest of proteolytic package was retained). Their results (Figure 5.14) showed a higher FAA over the ripening period compared to the cheeses with the absence of LAB (Lane & Fox, 1997).

Figure 5-14. Effect of starter bacteria on the liberation of free amino groups in cheddar cheese. open square: cheese made with normal LAB; filled square: starter with no proteinase; filled circle: GDL; open circle: GDL+ adjunct starter (lower quantity than normal) (Lane & Fox, 1997).

Total free amino acid is a common method for assessment of the extent of proteolysis in ripened cheese (see Chapter 2).

The release of free amino acids (the final product of proteolysis) is clearly related to the LAB intracellular aminopeptidase (P F Fox et al., 2017). The total FAA is higher in cheeses with high extent of proteolysis and defines the characteristic taste and flavour of them. The results of total free amino acid of our ripened cheeses after 12 months are presented in Figure 5.15.

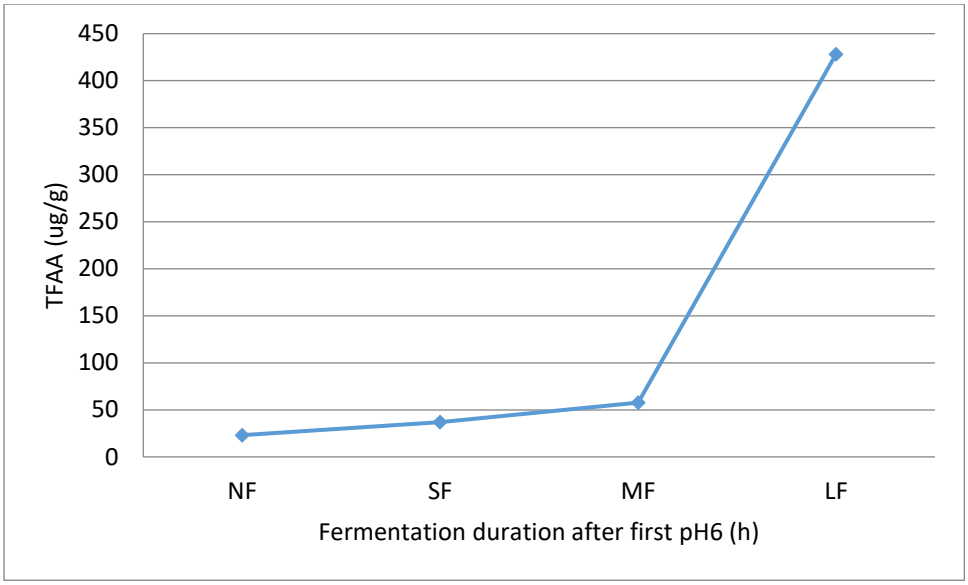

Figure 5-15. Effect of PSLT fermentation on total free amino acid (TFAA) of final cheese ripened at 4°C for 12 months. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Feeney *et.al.* reported a significantly lower proteolysis in mozzarella cheese when the ripening time was 15°C compared to 10°C for ripening times longer than three weeks (E P Feeney, Fox, & Guinee, 2001). This demonstrates the importance of the ripening temperature on activity of proteolytic enzyme in cheese. Masotti et al.,(Masotti, Hogenboom, Rosi, De Noni, & Pellegrino, 2010) reported a 19.2% increase in FAA content of Grana Padano cheese over a 12-15 months of ripening period with a maximum of 22.8% between 20 to 25 months. Our cheeses were ripened at 4°C (rather low temperature) and the sharp increase in FAA for LF treatment could be related to the proteolysis happened. Our results showed a significant ($p<0.05$) increase (longest fermentation as many as 20 time more than non-fermented) during the PSLT fermentation. Even for the SF sample, the net increase (>50%) in total FAA of our fermented samples was much higher than the results reported in the literature.

5.7 Chapter conclusion

An increase in the level of pH.4.6 soluble components and also free total free amino acids in final cheese has been assessed and the results indicate that the length of fermentation prior to curd formation has a significant role in the characteristics of manufactured cheese. The level of the net increase was identical to the extent of proteolysis reported to happen in cheese after more than at least one month of ripening (depending of type of cheese). When this value is compared to the cheese proteolysis, the presence of a very efficient proteolytic enzyme should be considered. The net increase in the concentration of the pH4.6SN/TN fraction in our treatments is the result of the proteolytic system of the LAB before rennet addition and curd formation.

O'Shea et al. reported an increase in the number of detectable peaks for cheddar cheese with higher levels of maturity. They also examined the total FAA in the studied cheese (mild, mature and extra-mature) and reported significant increases in that parameter. They concluded the FAA to be a more effective tool to discriminate the maturity of cheeses. However when it came to score the cheeses for flavour quality (defective or non-defective), the HPLC result was considered to be more advantageous (O'Shea, Uniacke-Lowe, & Fox, 1996). The HPLC peptide profile examines the cheese based on the hydrophilicity/hydrophobicity of peptide (as well as molecule size) and since the bitterness is related to the large hydrophobicity in the casein chain, HPLC can integrate better for that purpose.

A significant ($P<0.05$) increase in the level of pH.4.6 soluble components, 70%EtOH soluble and insoluble, very small peptides and also total FAA in final cheese has been assessed and this may be as a result of our pH-stat long fermentation treatments. The level of the net increase was identical to the extent of proteolysis reported to happen in cheese after more than at least one month of ripening (depending of type of cheese). When this value is compared to the cheese proteolysis, the presence of a very efficient proteolytic enzyme should be considered. The net increase in pH4.6SN/TN in our treatments has been designed to be as a result of proteolytic system of LAB before rennet addition and curd formation.

The resultant soluble fraction was further assessed by 70%EtOH by fractionation into two 70%EtOH soluble and 70%EtOH insoluble fractions. The 70%EtOH insoluble contains the large

peptides and 70%EtOH soluble smaller peptides and amino acids. The peptide profiles of these two fractions were separately analysed by reverse phase HPLC. For both fractions, the peptide profile showed an increase in the number of detectable compounds. A significant ($P<0.05$) increase was also observed in both total peak areas of the peptide profiles. As the 70%EtOH soluble fractions are considered as the depth of proteolysis, the results showed promising for possibility of having a cheese out of the fermented medium with manipulated levels of proteolysis before curd formation. The increase in these fractions is in coordination with the duration of fermentation and also the pH4.6SN/TN measured.

The extent of very small peptides ($M_r<1200$ D; around average 11 amino acid residuals) were also evaluated by Q-ToF mass spectrometry. The results showed a significant ($P<0.05$) increase in the total area under chromatogram peaks. These small peptides and amino acids have been reported to be the precursors of aroma and flavour of cheese. The increasing trend in these parameter is also in coordination with the duration of fermentation as well as the pH4.6SN/TN measured.

The total FAA values of the PSLT treated samples showed massive increase as the duration of fermentation increased. Compared to HPLC peptide profiles, total FAA is reported to be a more effective tool to discriminate the maturity of cheeses (O'Shea et al., 1996).

Chapter Six Effects of pH-Stat Long-Term Fermentation on Rheological Aspects of Curd formation and Cheese and Microstructure of Cheese

6.1 Introduction

The quality of food materials is defined by their characteristic physical, chemical and physicochemical characteristics. For cheese, so-called rheological attributes are as important as their nutritional facts. Rheology is the science of flow and deformation study of a material (Gunasekaran & Mehmet, 2002; Tunick, 2000). As described earlier in Chapter 2, rheological properties of food material is related to their micro and macrostructure, composition as well as its physicochemical specifications(O'Callaghan & Guinee, 2004).

As reviewed in Chapter 2, the rheological properties of cheese is influenced considerably by the characteristics of casein proteins (Peter Walstra et al., 2006). The intrinsically dynamic structure of casein micelles is influenced by the surrounding medium's conditions including pH, temperature, ionic strength, salt content, calcium ion association/dissociation, enzymatic activities, extent of proteolysis, acidity, bacterial and microorganisms' activities, rate of acidification, pH at whey drainage, etc.

Proteolysis -alongside CCP solubilisation- was reported to be the most important physicochemical pathway which can affect the textural quality of cheese (as well as cheese flavour and aroma) (Sousa et al., 2001; Upadhyay et al., 2004).The effect of the PSLT fermentation on the proteolysis of milk-based fermented medium was examined in Chapter 5. A significant ($P<0.05$) proteolysis identified by increasing the duration of PSLT fermentation duration. The effect of this unique proteolysis (proteolysis before coagulation) on the curd formation quality and textural attributes of cheese will be assessed in the present chapter. The first question to be answered

here is whether or not we can make a cheese curd and then what the textural characteristics of this cheese would look like.

The effects of PSLT formations on curd formation were assessed by non-destructive method of dynamin low amplitude oscillatory rheometry (DLAOR). The effect of the treatments on resultant cheese were also examined by high strain, destructive empirical methods.

In this chapter the term 'dynamic rheology' is used for small oscillation rheometry of the food material and 'static rheology' is used for textural evaluation of food cheese.

6.2 Curd formation and its rheological attributes

As mentioned before during cheese making procedure, the liquid state of cheesemilk gradually turns into a solid state. Curd formation is the first distinguished step of this quality of this physicochemical transformation could be evaluated utilizing Rheometer.

The sampling point for curd making assessment is presented in Figure 5.2. Once the adjustments finished, the pH6 sample was ready to be renneted and evaluated in terms of coagulation characteristics. A MCR-302 Rheometer (Anton Paar, Stütgart, Germany GmbH) was used to carry out the experiments. The specifications of the unit used, equipment setting, control parameters of trials, data acquiring setting and software and also the quantity of coagulant are presented in 3.2.5 and 3.2.12.

The effects of PSLT fermentation on gelation time (t_g) of the medium and gel strengthening quality are presented here. It is noteworthy that in order to have a better comparison in our dynamic rheological experiments, an extra sample was also assessed denoted in this chapter as SSF. This sample was taken at first pH drop of the fermented medium to 6 after about 320 minutes from inoculation. This was the time where the bacterial activity had passed the initial slow phase of activity during adaptation stage.

6.2.1 Gelation time

The basis of rennet coagulation is presented in Section 2.4. The gelation time (t_g) was considered as the time where G' exceeded G" (Karlsson et al., 2007). The results of the gelation times of PSLT fermented mediums are presented in Table 6.1. A decrease (non-significant at ($P<0.05$)) in the gelation time was observed among the samples as the duration of PSLT fermentation was increased.

Table 6-1. *Mean values ± S.D gelation time t_g of renneted MPC85 samples with different levels of fermentation. Adjusted time is the gelation time from the graph plus the time required for sampling of renneted medium before the first measuring interval of Rheometer. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Treatment	t_g from graph (min)	t_g Adjusted (min)
NF	5.33±0.29[a]	6.33±0.29
SF	5.16±0.29[a]	6.16±0.29
MF	4.83±0.29[a]	5.83±0.29
LF	4.33±0.57[a]	4.33±0.57

n≥3

Values followed by different superscript in one column are statistically (p<0.05) different.

NF: non-fermented, SF: short-fermented, MF: medium fermented and LF: long fermented.

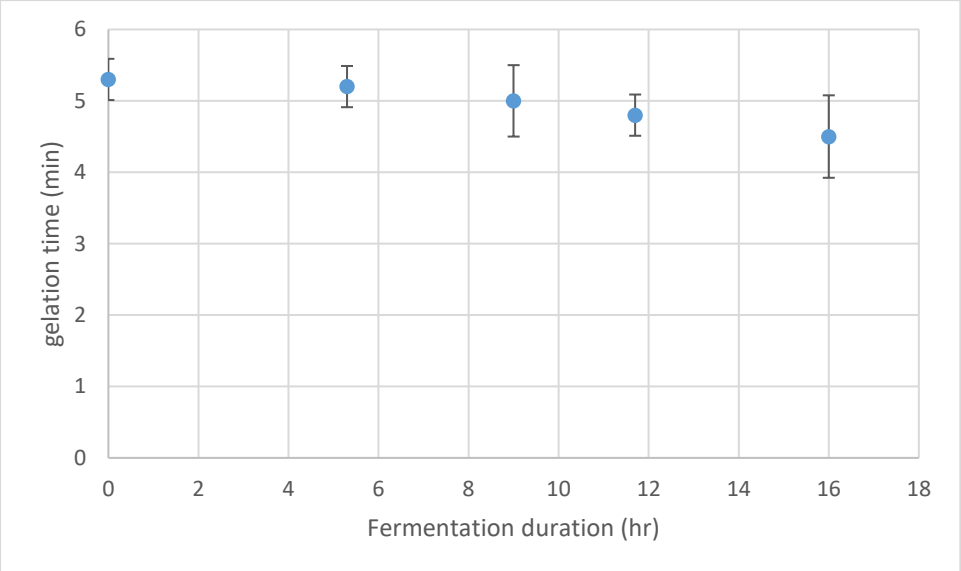

Figure 6-1. Effect of PSLT fermentation duration on gelation time of rennet induced gels. Error bars show standard deviation of measurements (n=3)

As discussed earlier in 2.4 the rennet coagulation is an enzymatic reaction that takes place in two different stages. The gelation time is the transformation from a sole to gel. This transformation lays at the end of the primary phase. This phase is pH dependent (Esteves et al., 2001; Waungana, Singh, & Bennett, 1998). The maximum velocity of enzymatic reaction is reported to be at pH=6. Furthermore, at pH values of around 6, a lesser extent of CMP removal from micelle was required to initiate the aggregation of caseins compared to normal milk pH. In the same manner, a higher aggregation rate and gel formation was reported to happen with decreasing pH. The fact that we preformed our experiments at pH=6 may be the reason gelation time happens quickly. This is in agreement with Zoon et al. (P Zoon et al., 1989). When they decreased the pH of rennet induced RSM from 6.65 down to 5.77, shorter gelation times were obtained. They reported a gelation time of between 410 seconds and 290 (very similar to our results) seconds for a pH range between 5.89-6.17.

As shown in Figure 6.1 gelation time showed no significant ($P<0.05$) difference between the samples with different duration of PSLT fermentation. The LAB lactocepines proteinases, which initiate the proteolysis to supply the organism with protein, has a lesser preference towards κ-caseins compared to other casein chains (See 2.3.1.4). This lower hydrolysis during our treatments may have resulted in a higher similarity in the stabilizing effect of the so-called hairy layer and can explain the similarity between the gelation times.

6.2.2 Evaluation of dynamic moduli of curd formation

The gelation profile of samples from different treatments are presented in Figure 6.2 and 6.3.

Mean values and standard deviations of coagulation time, G' and G" during gel firming were all calculated from three measurements. Representative coagulation date of the samples had been recorded and processed by Rheoplus software (RHEOPLUS/32 V3.41D091221, Anton Paar Germany GmbH) and the graphs have been made using Microsoft Excel (Microsoft ® Office, 2013).

The gel aging was continued at 1 Hz frequency and 0.5% strain for 100 minutes. The storage modulus of renneted-mediums is presented in Figure 6-2. A significant difference ($P<0.05$) in maximum dynamic moduli at the end of studied gel-stiffening time was observed between the samples with different extent of fermentation. A post ANOVA Tukey-test showed the difference lays between NF and SSF and other trials. The differences between SF, MF and LF samples were statistically non-significant ($P<0.05$).

The storage modulus is a measure of solid-like materials. The enzymatic reaction of rennet starts promptly as the temperature and pH of the liquid medium is optimum for its action and cleaves 105Phe-Met106 peptide bond in κ-casein. These optimum conditions for rennet coagulation facilitate the extensive hydrolysis of κ-caseins. This consequently resulted in destabilization of micelles partial aggregation and therefore sol/gel transition.

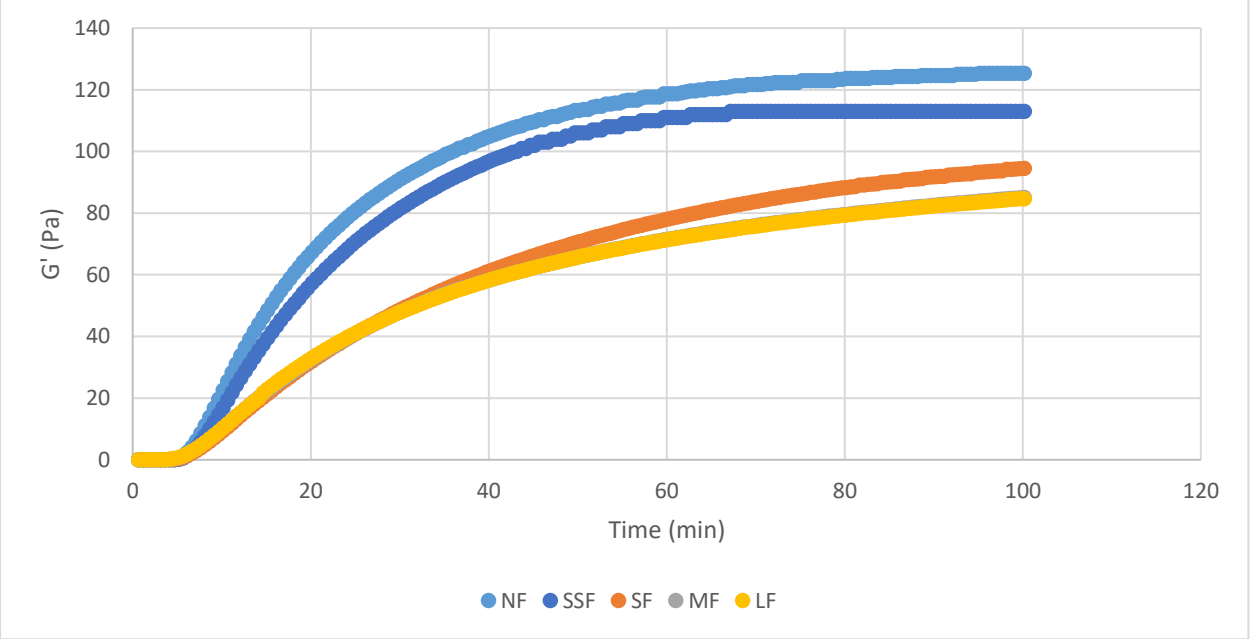

Figure 6-2. Development of storage modulus over time after renneting in rennet induced gels made using PSLT fermentations with different durations. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Due to the dynamic structure of rennet induced gels, they evolve over time as a result of rearrangements in the network structure (van Vliet, 2000). Sharp increase in the G' for NF and

SSF compared to slower rate for SF, MF and LF may be due to the differences in fusion of casein micelles and structural rearrangements of proteins. As a result of these ongoing changes, the contact area between the micelles would eventually result in aggregation of the paracasein particles with a higher number of interactions in the network.

As shown in Chapter 5, the PSLT fermentations with different durations had resulted in a proteolysis of proteins. This casein breakdown may have resulted in a decreased dynamic moduli values and maximum moduli reached at the end of the experimented gel stiffening period. Our result for maximum G' value of unfermented medium was in agreement with other researchers. The G' value of chymosin renneted skim milk (almost similar protein and fat content to our research) at pH=6 was reported to reach its maximum around 100 Pa and remained constant over the2 hours (Esteves, Lucey, Wang, & Pires, 2003). The difference in G' after 100 min from our study (120 Pa) compared to Esteves et al. (2003) result may be due to slightly higher protein content of our milk based medium. This higher protein content was reported by Waungana et al. (1998) to increase the G' values of rennet gels.

Our findings are also in agreement with the results from very few relevant published articles on proteolysis before curd formation. Srinivasan and Lucey found a decrease in stiffness of rennet-induced gels even at small amount of casein breakdown (Srinivasan & Lucey, 2002).

The intact casein proteins in non-fermented sample compared to the partially hydrolysed structure in longer fermented mediums may be the reason why the structure of fermented mediums are softer with lower maximum G', G" values at the end of gel stiffening period.

Proteolysis is reported to influence moduli by splitting the casein proteins which would result in the formation of loosely hanging strands (Zoon, 1988) which are smaller chains of casein with different interactive bonds to surrounding medium as well as neighbouring caseins . This consequently would result in a deceased number of structural bonds on the network which is being formed. Our results from PSLT fermented mediums showed an increase in the proteolysis arising from bacterial activity. The proteolysis may be the reason of lower dynamic moduli observed during gel formation in the fermented mediums.

The extent of the hydrolysis assessed in Chapter 5 was significantly different between all the samples. However, the NF, MF and LF (with 3.85, 6.5 and 10 hours of fermentation duration, respectively) samples did not show a significant difference in G' values after ~100 min of gel firming time. It is not possible to explain the exact reason for this based on our finding. The identifications and thorough evaluation of the peptide produced from mass spectrometry (amino acid sequence, the hydrophobicity of the peptides, etc.) may be a proper approach to answer this question. As gel structure is dynamic and the casein protein chains rearrange themselves according to the ongoing changes in the medium, the partial proteolysis of longest fermented mediums (MF and LF samples) may have aided in the improved rearrangement of the colloidal particles and therefore gel strength development (Srinivasan & Lucey, 2002; Dalgleish, 1983; Dalgleish, 2011).

The development of tan(δ) of over time, after rennet addition are presented in Figure 6-3. The values of tan(δ) remained unchanged once the initial fluctuations before gelation time (tan(δ)>0.4) has passed.

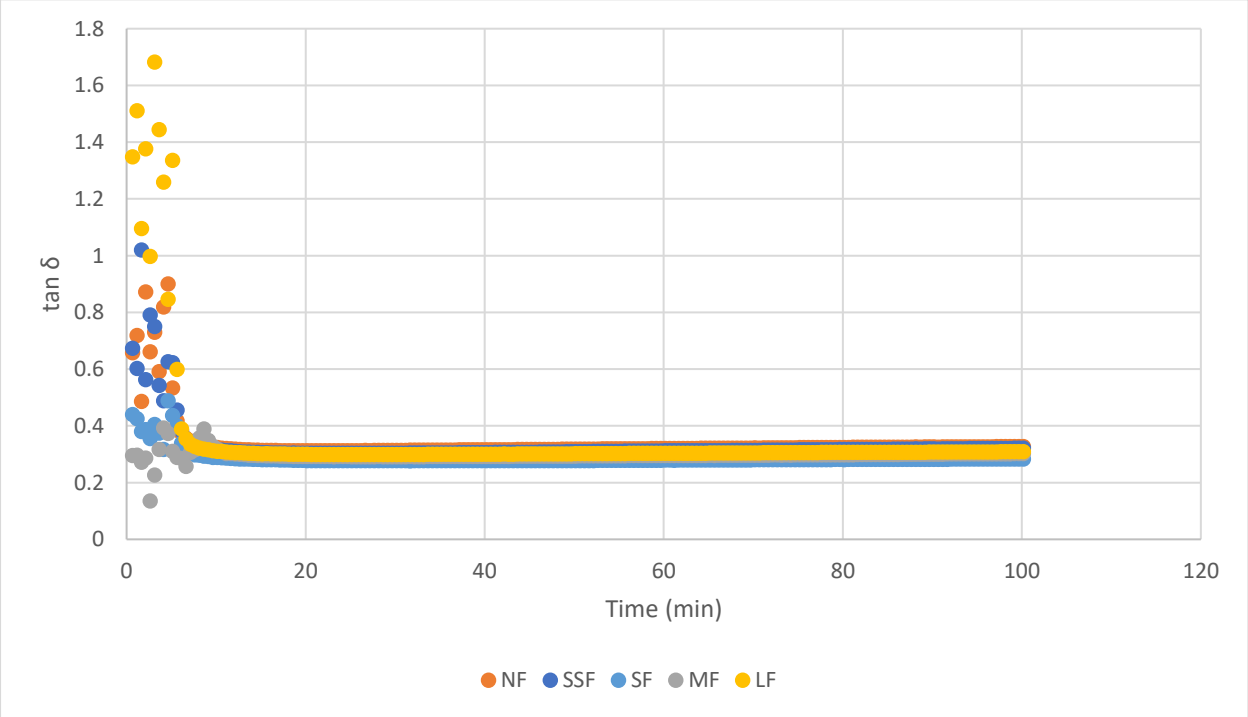

Figure 6-3. Development of damping factor of dynamic moduli over time after renneting in rennet induced gels made using PSLT fermentations with different durations. NF, SF, MF and

LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Esteves et al. (2003)reported a similar value (tan δ=0.34) for rennet gels made at 30°C using chymosin which remained unchanged over gel stiffening time once stability was reached. Zoon et.al also reported a similar value for skim milk gels made at pH values close to our pH (Zoon *et al.*, 1989).

The effect of timescale of applied strain (at a fix rate) on the rheological properties of coagulum was evaluated by doing a frequency sweep at the end of gel stiffening period. G' values of all coagula showed increasing trends when the timescale of deformation was shortened (figure 6-4). This is in agreement with Zoon et al. (1988).

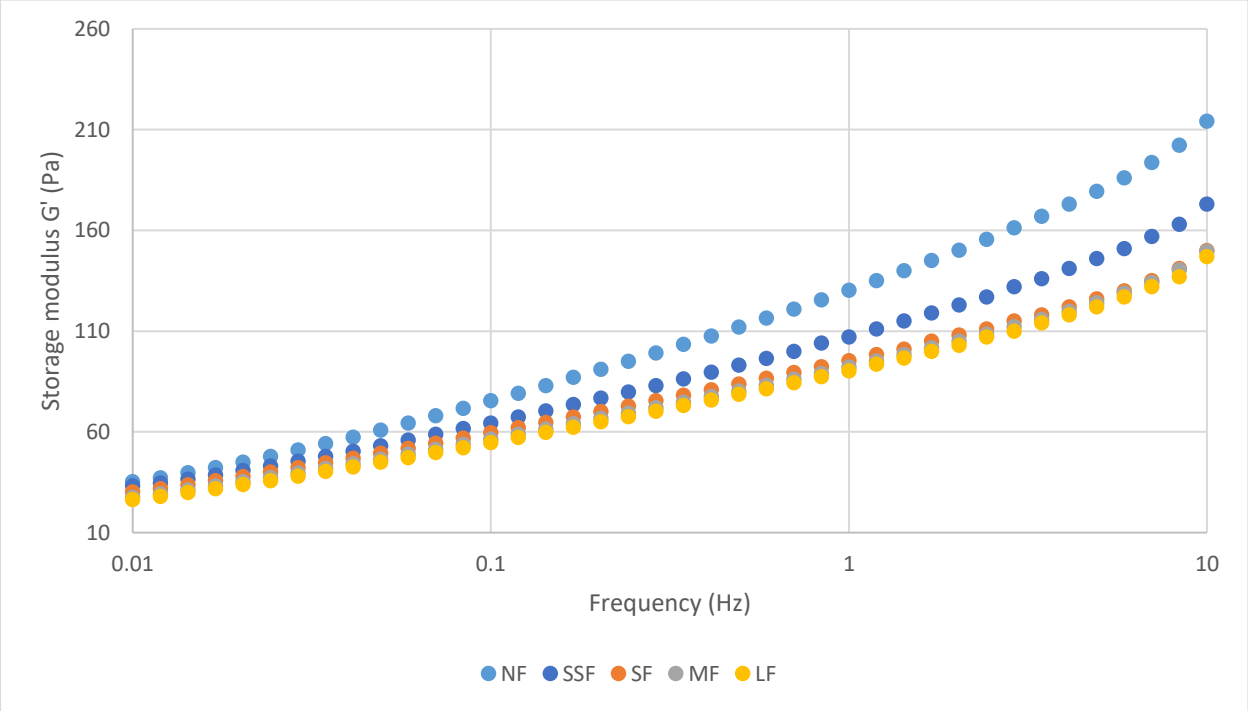

Figure 6-4. Effect of a frequency sweep on storage modulus of stiffened gel in rennet induced gels made using PSLT fermentations with different durations. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

A shorter relax time will be available for the bonds in the network, when the stress is applied at higher frequencies. For all samples, the G' values remained in the same order as gel stiffening

period. A significant difference (P<0.05) was observed between the G' values at higher frequencies. The samples with lower extent of fermentation, showed significantly higher G' values at higher frequencies. This may be related to the extent of undisturbed micelles before curd formation. According to van Vliet (2000), the gel formation of casein micelles follows a fractal structure and the cluster forming casein proteins of fractals of the gel network. Undisturbed caseins would aggregate as a result of GMP removal, and as a result of more similarity in the fractals, they will be distributed in a 3D network with higher uniformity (compared to the caseins which have been hydrolysed to some extent with various chain lengths). This uniformity may have resulted in firmer gel which can attain its shape more readily when an external strain is applied.

The effect of timescale of deformation on tan(δ) are presented in Figure 6-5. As shown in the Figure, the response of viscoelasticity of the samples are very similar. The behaviour of the viscoelastic gels were more similar in higher frequencies compared to lower ranges. Our findings are in agreement with Zoon et al. (1989) and Esteves, Lucy, & Pires (2002).

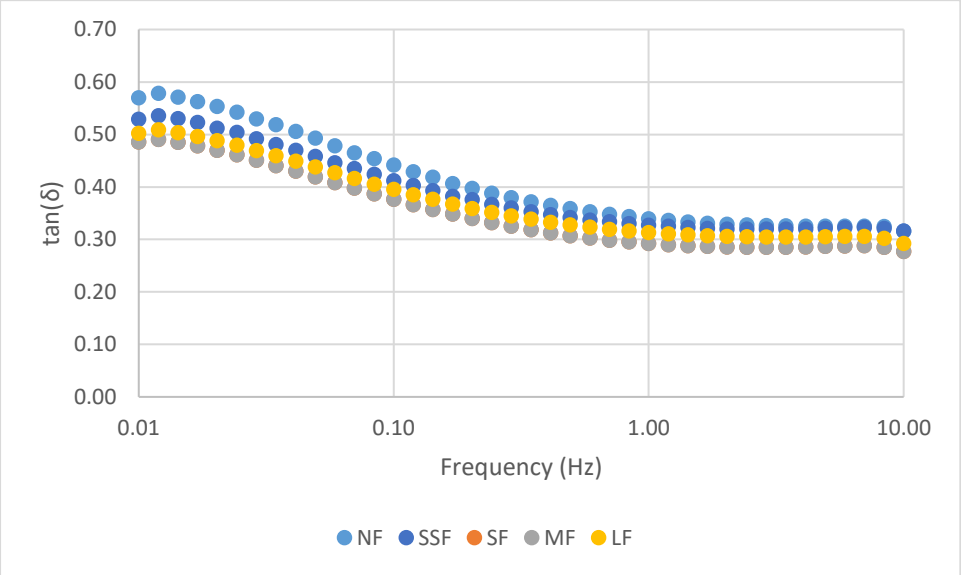

Figure 6-5. Loss tangent tan(δ) as a function of frequency for rennet induced gels made using PSLT fermentations with different durations. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

6.2.3 Chemical analysis of the formed coagulum

Chemical analysis of coagulum is presented in Table 6. The rationale and methods of required adjustments at the end of fermentation runs are presented in section 3.1. These analyses performed right after the formation of coagulum and before any further steps (cutting, syneresis, whey drainage, pressing, brining).

Table 6-2. Different parameters of cheese coagulum. Mean± S.D. for protein and conductivity measurements for in rennet induced gels made using PSLT fermentations with different durations. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Treatment	*Protein content (%w/w)	pH	Lactose remainder (%w/w)	Conductivity (mS/cm)	Sodium ISE (mg/L)
NF	3.39±0.03^{a}	6.03±0.04	0.19	10.3±0.2^{c}	1339±31^{b}
SF	3.41±0.04^{a}	6.04±0.05	0	9.5±0.0^{b}	1395±64^{ab}
MF	3.40±0.04^{a}	6.05±0.04	0	9.7±0.1^{a}	1423±45^{a}
LF	3.38±0.02^{a}	6.05±0.04	0	9.8±0.0^{a}	1460±31^{a}

n=3

Values followed by different superscript in one column are statistically (p<0.05) different.

NF: non-fermented, SF: short-fermented, MF: medium fermented and LF: long fermented.

The protein content and pH of the coagula at the end of gel aging showed no significant differences ($P<0.05$).

There was a minor but significant difference in the conductivity of the samples. This was expected as the NF samples had to be acidified by direct addition of 2M lactic acid. Payot and Flick found lactic acid to be the most influential component on conductivity measurements in a fermenting broth medium(Payot & Fick, 1997). On the other hand, the sugars do not contribute in conductivity measurements. This may have been the reason for the difference detected. It is noteworthy that adjustment of the conductivity to have identical values (while maintaining other

important parameters constant) sounds to be impossible as there would be an infinite steps of adjustments and readjustments to do.

The sodium content of coagulum showed a significant difference ($p<0.05$) among the sample. This may be as a result of differences in sodium content of Sodium Hydroxide 1N and Sodium Lactate where lactate contains lower sodium levels as some concentrated lactic acid is contributed in its formulation (and hence less Na at a fixed volume). There was no difference between the fermented samples with different durations.

The results showed strong evidences of the ability of developed system and selected approach to control the parameters and minimize their confounded effects.

By doing PSLT fermentation and deactivation of bacteria at the end of fermentations, and also doing lactose elimination approach, the pH was kept constant (and therefore the solubility of CCP remain unchanged). The conductivity and salt content of the medium were also adjusted to be as close as possible. It can be concluded that the net source of differences observed in the behaviour of the samples during coagulation may be related to the extent of LAB proteolysis during treatments.

6.3 Large strain rheometry

As reviewed in Chapter one, static rheometry is a versatile method to assess cheese textural attributes. One of the most utilized methods for cheese textural evaluation is uniaxial compression of cheese (Gunasekaran & Mehmet, 2002).

6.3.1 Chemical analysis of the final cheese

The protein content, pH values and moisture content of the final cheese are presented in Table 6.3. The protein content and moisture content of the cheeses showed no significant difference.

Table 6-3. [1]Composition and pH of final cheese made from samples with different extent of fermentation. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Treatment	*Protein content (%w/w)	pH	Moisture
NF	25.91±1.49[a]	6.03±0.07	66.42±0.87
SF	25.33±1.25[a]	6.06±0.09	66.09±0.86
MF	25.47±1.26[a]	6.09±0.06	67.07±0.92
LF	24.97±1.47[a]	6.07±0.1	67.20±1.06

Mean ± S.D. (n=3).

Values followed by different superscript in one column are statistically (p<0.05) different.

NF: No- fermented, SF: Short fermented, MF: Medium Fermented, LF: Long fermented

The pH values remained unchanged during cheese making. This was related to the absence of any residual lactose (after LEP) and active LAB in the medium. A slight increase (statistically ($p<0.05$) non-significant) in the cheese made from longest fermented medium was observed. This may be due to the higher extent of hydrolysis consequently resulting in the presence of more amino groups in the medium. This is in coordination with results from free amino acid evaluation.

6.3.2 TPA

Cheese samples were kept at 20°C in brine for 48 hours. After this time, the cheese blocks were taken out of the brine and the surface moisture of the cheese was dried by lint free hand towels (only 1-2 second of contact time for each side). The TPA samples were prepared and tested as explained in 3.3.13. The results of large deformation using TPA for hardness, adhesiveness, resilience, cohesion, springiness, gumminess, Chewiness, derived from the graphs, are presented in Table 6.4.

Table 6-4. [1,2]Mean± S.D. for hardness, adhesiveness, resilience, cohesion, springiness, gumminess and chewiness of cheese made from samples with different PSLT fermentation durations. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

treatment	Hardness (g)	Adhesiveness (g.s)	Resilience (%)	cohesion	Springiness (%)	gumminess	Chewiness
NF	3355.66±387^{a}	-55.54±11^{a}	13.31±0.93^{a}	0.29±0.03^{a}	65.75±3.21^{b}	957.32±118^{a}	632.30±104^{a}
SF	2664.67±264^{b}	-84.07±5^{b}	11.64±1.13^{a}	0.27±0.05^{a}	78.28±0.54^{a}	377.82±53^{b}	296.06±44^{b}
MF	2087.76±114^{c}	-123.51±4^{c}	9.29±0.66^{c}	0.21±0.05^{a}	66.97±6.58^{b}	355.73±64^{b}	240.63±59^{b}
LF	1315.96±176^{d}	-127.93±25^{c}	8.53±0.55^{c}	0.25±0.02^{a}	57.714.31^{c}	327.02±51^{b}	188.99±35^{b}

[1]Mean with different superscripts in the same column are significantly different (P<0.05),
[2]n=4

Mean values of all evaluated textural properties of cheeses decreased with increase in the duration of PSLT fermentations. A significant decrease was observed in the hardness of cheeses. This lower hardness may be related to the increase in the extent of proteolysis of casein proteins as a result of longer activity of LAB. In previous chapter we discussed the effects of PSLT with different duration on the proteolysis. Creamer and Olson reported the relevance of changes in cheese texture to casein proteolysis (Creamer & Olson, 1982) and reported a weakened cheese structure as a result of α_{s1}-casein hydrolysis. As presented in proteolysis results, there was a positive correlation between the duration of PSLT fermentation and formation of proteolysis products. We also tracked down some peptides formed during such fermentations. A peak at detected with m/z=1246. 86, showed the highest height in the mass spectrum which was assessed from 70% EtOH insoluble fraction (results are not presented). This peak identified by Piraino et al. to be as α_{s1}-casein (f14-23) (Piraino et al., 2007) and remained the peak with highest absorbance in all treatments. Another possible peptide derived from α_{s2}-casein was detected from mass spectrum. The peptide chain with m/z=719.42 was previously assessed by Rauh et al. to be α_{s2}-casein (f198-203). The cleaved peptide chains α_{s1}-casein(23-24) and α_{s2}-casein(197-198) were previously reported to be a substrate for lactocepines by different authors as reviewed by Upadhyay and McSweeney, Magboul, &Fox (2004). This cleavage alongside a significant amount of peptides removed from the caseins (Sections 5.3, 5.4 and 5.5) may have been the reason for a decreased hardness in cheese with longer fermentations.

Pearson's correlation showed a high correlation between the duration of fermentation, extent of proteolysis (as per pH4.6SN/TN) during fermentation and static high-deformation rheological attribute of final cheese (Table 6.5).

Table 6-5. Pearson's correlation coefficients between added lactose to the fermenter, fermentation duration, extent of proteolysis (pH4.6SN/TN) before curd formation, ethanol soluble and insoluble fractions peptide profiles, mass spectrometry peptide profile for small peptides, G'_{max} of gelation and hardness of cheese.

	lactose content (fermenter)	*fermentation duration*	*pH4.6SN/TN*	*70%EtOH S peak area*	*70%EtOH I peak area*	*Mass spectrometry peak area*	*Cheese hardness*	G'_{max}
lactose content (fermenter)	1							
fermentation duration	0.998**	1						
pH4.6SN/TN	0.984**	0.969**	1					
70%EtOHS HPLC peak area	0.985**	0.985**	0.978**	1				
70%EtOHI HPLC peak area	0.978**	0.970**	0.978**	0.994**	1			
Mass spec peak area	0.980**	0.983**	0.947*	0.936**	0.915164	1		
Cheese hardness	-0.998**	-0.999**	-0.975**	-0.992**	-0.981**	-0.972**	1	
G'_{max}	-0.883	-0.893	-0.832	-0.763	-0.764	-0.959*	0.867711	1

*****Correlation is significant at (P<0.001)***
****Correlation is significant at (p<0.05)***

Our results are in agreement with Wang and et al. (2011). They reported a high correlation (r= 0.96) between the pH4.6SN/TN and hardness of natural Cheddar cheese. Our results on different textural attributes were also in coordination with O'Mahony et al. (2005). They reported a significant decrease in hardness, cohesiveness, springiness and chewiness of all cheddar cheese made with increase in proteolysis levels. Similar to our findings, these authors reported a pH4.6SN/TN to have significant correlation with textural attributes of the cheese.

6.4 Evaluation of the microstructure of cheese by Confocal Laser Scanning Microscopy

Sample preparation, equipment, material and method utilized for evaluation of microstructure of cheese samples are presented in section 3.1.13.

6.4.1 Results

In the CLSM images provided, the green parts are dyed protein with fast green FCF and the darker spots are dispersed the surrounding water. A tendency towards an open structure was observed in images as the length of fermentation was extended (Figure 6-6). In the non-fermented, the protein structure looks very homogeneous and the uniformity of protein structure is discernible (Figure 6.6-A).

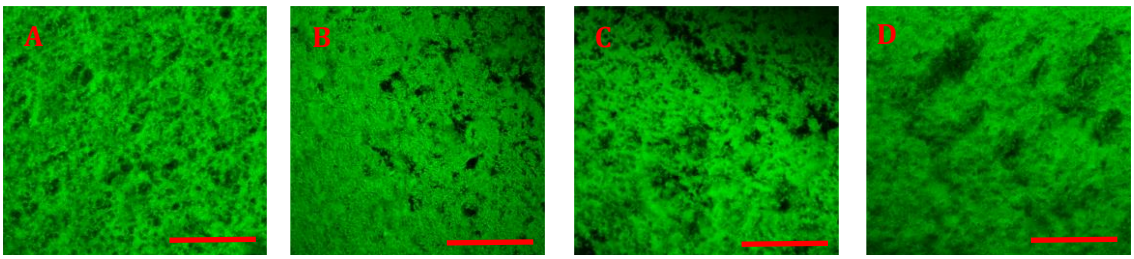

Figure 6-6.Confocal micrographs of day one cheese made from PSLT fermented sample with different durations. From left to right, NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively. Scale bars 40µm.

With increasing in the duration of PSLT fermentation, the size of the pores in the structure becomes larger. It is also clear in the CLSM images that the uniformity of the pores in the structure are changing towards the formation of fragmented pores which are more like a channel of moisture. This may be related to the differences in the extent of proteolysis which happened during the PSLT fermentations.

As the duration of fermentation increased, the higher extent of proteolysis (which was assessed in chapter 5) may have resulted in the cleavage of the casein proteins which form the backbone of the cheese structure. This hydrolysed protein does not contribute directly to the structure of the network and if not completely soluble in the surrounding liquid, they will be loosely connected to the structure forming proteins (Zoon, 1988) possibly via weaker interaction types (Lucey et al., 2003). This eventually may have resulted in a network in which the total number of protein bond in the evolving structure have reduced (Lucey et al., 2003). This is in coordination with our rheological results. As shown in the images, the more open structure observed in the samples with higher extent of PSLT fermentation may be connected to softer cheese structure in which the gel making strand had been limited to bond in all directions, as even as undisturbed chains.

Our results are also in agreement with other researchers. Srinivasan and Lucey reported an increase in the size of the pores in the gel network when the extent of hydrolysis before curd formation was increased (Srinivasan & Lucey, 2002).

6.5 Modelling

Mathematical modelling had been used to characterize the behaviour of rennet induced milk-based gels (Esteves et al., 2001; O'Callaghan & Guinee, 1996). One of the most commonly used methods developed by Carlson (Carlson, 1985)is based on the increased interconnectivity between the branching chains of caseins proteins during agglomeration. The advantage of this model is arising from the fact that it considers the rennet-induced milk coagulation as a two-phased process. This is agreement with the casein coagulation criteria (GMP cleavage and aggregation) reviewed in chapter 2. The ability of this model to agree with the experimental values of G' (O'Callaghan & Guinee, 1996) was the basis for choosing this model over others to evaluate the extent of fitting of our results with mathematical models. The model is based on evolution of storage modulus (G') over time after rennet addition. The model can be written as:

$$G' = G'_{max}\left[1 - ae^{-k_1(t-t_0)} + (a-1)e^{-k_2(t-t_0)}\right]$$

Where G'_{max} is the value of G' at infinite t time, t_0 is time elapsed after rennet addition and k_1 and k_2 as rate constants.

6.6 Technique used for curve fitting

A least squares optimization technique was utilized on experimental G'/t data. The model coefficients were adjusted by Microsoft Excel Solver Utility (Microsoft Office package, 2013), to minimize the sum of squared residuals of each (G',t) data set between the experimental and model prediction. The R^2 values were calculated as below:

$$R^2 = 1 - \sum_{t=0}^{t} \text{SS Res}$$

where (SS Res) is the sum of squared residuals and calculated as below:

$$\text{SS Res} = \sum_{t=0}^{t} (G'i - G')$$

In which G_i is the storage modulus measured experimentally and G is the storage modulus calculated from the model.

Absolute error and relative errors were also used to assess the accuracy of the model's predictions. Absolute error is regarded as the difference between G'_i and G' at any given data point and relative error is calculated as below:

$$\text{Relative error} = \frac{\text{Absolute error}}{\text{Experimental value}} \times 100$$

6.6.1 Assumptions

Carlson's model is incapable of to predict the values of G' before t_g and therefore assumes the renneting time (t_0) as the time of G' becoming any value higher than zero for the first time. The G' before that time is all assumed to be zero.

Assumption: if $t \leq t_g$ then G'=0;

6.6.2 Curve fitting results

Each experimental graph was fitted with Carlson's model separately. An example of curve fitting using experimental data for our medium fermented sample and predicted model values of G' as a function of time after gelation is presented in Figure 6-7.

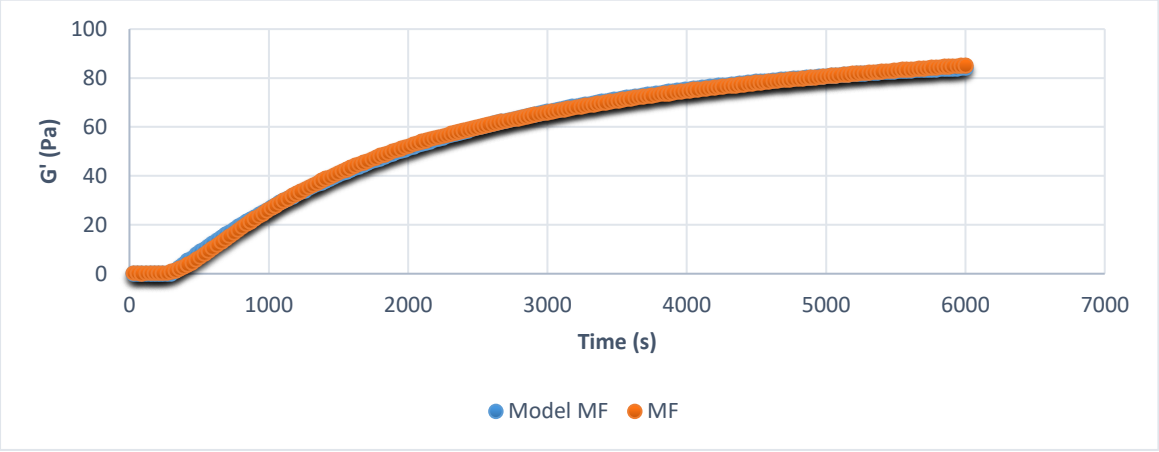

Figure 6-7 Storage modulus (G') development of rennet induced gel made with MF (as an example) experimentally (red markers) and Carlson's model (blue markers).

Table 6.6 shows the R^2 values of curve fittings. As shown in the table, the model was greatly able to predict the values for G' over time after t_g ($P<0.0001$).

Table 6-6-6. R^2 values and standard error obtained from the fitting of experimental data of G' deveolpemtn over time and Carlson's model predictive data for samples treated by PSLT feremtations with differnet duration. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Fermentation	NF	SSF	SF	MF	LF
R^2	0.999	0.999	0.999	0.999	0.999
Standard error	1.216475	0.70961	0.625432	0.677032	0.709671

(P<0.0001)

In an attempt to derive a model from our results, were considered to predict the G' values based on the duration of PSLT fermentation. Our results showed promising aspects of accurate prediction of the values for t_g and G'_{max} at a given fermentation duration. The Absolute and relative error in the t_g and G'_{max} between derived method and experimental data are presented in table 6.7.

As shown in the table, the results from derived model could significantly predict the parameters (t_g and G'_{max}) (r=0.97, P<0.05).

Table 6-6-7. Absolute and relative error in the t_g and G'_{max} between derived method and experimental data. NF, SF, MF and LF represent samples with 0, 3.85, 6 and 10 hours of fermentation after first pH=6, respectively.

Treatment	Absolute error G'_{max}	Relative error G'_{max}	Absolute error t_g	Relative error t_g
NF	0.82	0.63565891	0.100166667	1.821212121
SSF	-1.7	-1.51380232	0.026666667	0.516795866
SF	-8.399	-8.87468301	0.237166667	4.59625323
MF	-10.43	-12.2561692	0.0495	1.02484472
LF	-1.315	-1.55621302	-0.27933333	-6.451116243

6.7 Chapter conclusion

The fermented sample with PSLT system were all able to form a cheese curd. This showed the extent of the proteolysis was subtle which is a characteristic of bacterial proteolysis. In other words the structural changes due to the activity of studied lactic acid bacteria even at the longest fermentation trial were not destructive to the gel forming ability of the studied fermented medium. The gelation time was not affected ($p<0.05$) by the extent of fermentation over studied durations.

The G' values of gels made with non-fermented sample showed to be significantly higher than the fermented samples. This was expectable as the proteolysis opens up the structure of micelle by cleaving some backbones of the structure. Possible lower net number of effective bonds may also have resulted in a gel with lower strength as the extent of proteolysis increases. As a result of new conformation of the micelles in the colloidal system before gelation, new patterns of protein distribution in the serum phase were expected. This may have resulted in different rearrangement patterns of 3-D network forming strands with lower uniformity of distribution of protein in the serum.

Formation of such structure during gel formation may have resulted in differences in cheese structure. Higher extents of fermentation (and hence hydrolysis) may have resulted in cheese with different textural attributes.

Chapter Seven Concluding discussion and future work

The discussion that follows here is integrating the insights that gained in this research on the evaluation of the effect of long term starter bacterial activity before curd making on proteolysis of cheese milk and coagulation behaviour and final cheese texture. The experimental design and the analysis approach used in this study has been selected to provide new evidences in this field of study. The duration of fermentation in this study on cheese-milk (prior to curd formation) was selected to be much longer than the common practice in cheese making. The pH-stat long-term (PSLT) fermentation was designed to enable the study of starter bacteria in absence of other competitor contributors of normal cheese-making practices.

The peptide profiling analysis was designed to enable the highest possible accuracy in result's interpretation. By selecting the appropriate fractionation methods and analytical equipment's, the results provided novel evidences on the molecular size of the peptides produced during each particular fermentation, for this particular mixed strain inoculum.

7.1 pH-stat long-term (PSLT) fermentation system

A novel system was designed to conduct long term fermentations with different duration on a selected milk based material. Our results proved its ability to control the parameters well. Lactose elimination was proved to be efficient to control the duration of in preventing further fluctuations in the fermenter. Sodium lactate with desirable pH was proved to be efficient in controlling conductivity of the mediums with different duration of fermentation without causing any fluctuations in pH. MPC85 showed to be an ideal medium due to its low lactose and high casein content and appropriate gelation quality. The lactose adjustments in the formulation was perfectly controlling the length of fermentations with high reproducibility. Cell extract approach enabled us to have more control (compared to RSM based inocula) over uniformity of medium.

The microbiological results showed the selected LAB strains reach a high population in the fermenter and their activities overcome the fermenter conditions.

The novel PSLT fermentation system provided new insight on the rate of acidification of cheesemilk in five different *Lactococcus lactis* strains. Among five studied strains, *LC. lactis* sp. *cremoris* 2338 showed the highest rate of acidification of the studied mediums (both MPC85 and sodium caseinate) after 6, 12 and 18 hours of fermentation (under PSLT conditions). *Lc. lactis* sp. *cremoris* HP was on the other end of spectrum with lowest rate of acidification of the mediums. The bacterial cell count of the selected strains (*LC. lactis* sp. *cremoris* 2338 and *Lc. lactis* sp. *cremoris* HP) provided new information on the maximum number of the bacterial cell reach in a fermenter under defined conditions.

7.2 Proteolysis evaluation

The results from analysis of pH 4.6 soluble fraction (pH4.6SN) as a percentage of total nitrogen (proteolysis index) showed a significant ($P<0.05$) increase in proteolysis as the PSLT fermentation duration was increased. The results of this experiment provided new information related to the proteolytic activity of starter bacteria during PSLT fermentation. The increase in this parameter by the duration of fermentation was related to the activity of LAB proteolytic system for longer time in longer fermented mediums. The net increase in this parameter resulting from the longest PSLT fermentation of this novel study was comparable to cheeses made under normal conditions (in presence of rennet and bacteria) after one month of ripening, in some cases. The 70%Ethanol soluble (70%EtOHS) and insoluble (70%EtOHI) sub-fractions of pH4.6SN were acquired and assessed by peptide profiling. The results showed a significant increase ($p<0.05$) in the number of emerged detectable compounds produced as a result of proteolysis. As the experiments had been designed to evaluate the merely effects of starter bacteria on the cheesemilk prior to curd formation, the peptide profiles of 70%EtOHS and 70%EtOHI presented

in this study provided new information in this area. The introduction of rennet at the early steps of cheesemaking would result in different peptide profiles.

Quantitative analysis of some of increasing peak showed a high correlation between the evolution of the peak absorbance and the duration of fermentation. This was concluded to be related to LAB high specificity.

Quadrupole time-of-flight (Q-ToF) mass spectrometry was used to further assess the presence of small sized peptides (M_r<3500) which are the marker of activity of LAB in cheese. Our results showed significant (P<0.05) increase in the number of the peaks and total area under chromatogram as the duration of fermentation was increased. This was related to the activity of comprehensive proteolytic system of LAB.

Free amino acid (FAA) formation in ripened cheese made from PSLT fermented mediums with different duration showed a significant (P<0.05) increase in the FAA formation during ripening. The formation of small sized peptides during fermentations had facilitated this increase earlier during PSLT fermentations.

In normal cheese making practices where the rennet is added at early steps of cheese making, its proteolytic activity dominates the casein hydrolysis in cheesemilk. Rennet activity at early stages, alongside with comparatively minor activates from other proteolytic contributors, defines the 'extent of proteolysis'. On the other hand, the presence of small peptide and free amino acids in cheese is known as the 'depth of proteolysis'. The latter is mostly happening in the later stages of cheese maturation and governed by starter –and non-starter- bacteria. The depth of proteolysis influences the characteristic flavour and texture of cheese. Our results from both mass spectrometry of 70%EtOHS fraction and free amino acid evaluation provided new promising insight on the suitability of the starter bacteria activity to manipulate the flavour and texture of cheese, prior to curd formation.

7.3 Rheological aspects

This study aimed to investigate the effects of bacteria activity prior to curd formation on the textural attributes of gelation behaviour and final cheese textural attributes. The proteolytic activity of LAB on cheesemilk protein were expected to be the source of those effects. As we concluded so far the novel PSLT fermentation system enabled us to conduct long fermentation trials at pH=6.00. This facilitated the activity of bacteria (for a predetermined long time) without drastic pH changes which could have resulted in casein micelle destabilization and eventually acid gel formation. The proteolytic activity of the bacteria during the trials were also assessed through versatile proteolysis evaluation. The final step towards the goals of this research was to evaluate the effects of the LAB activities on gel formation behaviour of renneted coagulum and eventually textural attributes of final cheese.

7.3.1 Coagulation rheological attributes

Our results demonstrated all the samples with different durations of PSLT fermentations were able to form a gel when induced by rennet at pH6 at 32°C. The gelation time (t_g) was considered as the time where G' exceeded G" (Karlsson et al., 2007) of rennet induced gels of the samples was not affected ($p<0.05$) by differences in PSLT fermentation duration. In all samples, the gelation time (t_g) happened after a shorter time upon rennet addition compared to normal cheese making practices. It was concluded that this shorter gelation time to be related to the pH values of our samples from two different perspectives. Firstly, the rennet is more active at pH=6 compared to cheesemilk pH~6.6 in normal cheesemaking practices (Esteves et al., 2003; Waungana et al.,1998; Fox et al., 2017) and it optimum (maximum velocity of enzymatic reaction) at our gelation temperatures (*i.e.* ~32°C) is pH=6. At this pH, the aggregation of paracaseins trigger at lesser extent of CMP removal from micelles. Secondly, at a lower pH CCP solubilisation and consequently its bridge forming action between destabilized caseins facilitates the aggregation of paracaseins (Zoon et al., 1989). This will result in initiation of sol to gel transformation which could be detected as t_g (*i.e.* G'=G") by rheometer.

Study of dynamic moduli of gel formation showed a significant decrease ($P<0.05$) happening in the storage modulus G' of the samples with longer duration of fermentation. This was concluded to be the effect of proteolysis from bacteria that may have been affected the crosslinking protein chains in a 3D evolving network.

Previously, it was concluded that the proteolytic activity of the LAB during PSLT fermentation in our trials had resulted in significant ($p<0.05$) proteolysis of proteins in cheesemilk (showed by pH4.6SN/TN). In the same manner the peptide profiling provide evidences on significant ($p<0.05$) modification in peptide profiles (HPLC results) and formation of low molecular size peptide chains (LC MS/MS results). All these changes may have resulted in a decrease in net effective protein interaction in the coagulating medium. Srinivansan and Lucey (2002) reported a decrease in stiffness of rennet-induced gels even at small amount of casein breakdown. The formation of the 'hanging protein strands' which are not firmly attached to the micelles due to partial hydrolysis (Zoon, 1988) could explain the lower moduli in longer fermented samples.

The non-fermented and very short fermented samples showed higher G' values after ~100 minutes of gel firming. A value of around 0.33 was observed from the graphs of tan(δ) as a function of time of gel development. The slope of the graph remained unchanged over gel firming time after t_g. Similar values and trends were reported for gels made under conditions similar to this research (Zoon et al., 1989; Esteves et al., 2003).

The responses of G' at elevating frequency of strain was assessed by doing a frequency sweep test. A significant difference ($P<0.05$) was detected between the G' values at higher frequencies. The samples with shorter fermentation durations, showed significantly ($p<0.05$) higher G' values at higher frequencies. The non-fermented and very short fermented samples showed higher strength to resist high frequency fixed-strains. This was possibly due to uniformity of the subunits and the better development of crosslinks in the 3D network of gel prior and after rennet addition, respectively. According to van Vliet (2000) casein micelles gel formation follows a fractal structure in which the cluster forming casein proteins are the fractals in gel network. Therefore, the higher uniformity of the samples with no proteolysis or very low extent proteolysis would

result in higher uniformity of the fractals. This uniformity may have resulted in firmer gel formation in which the network can attain its shape more readily when an external strain is applied.

7.3.2 Final cheese texture and microstructure

Our results showed the bacterial proteolytic activity prior to curd formation have significant ($p<0.05$) impact on the he textural attributes of final cheese. The hardness, adhesiveness, resilience, springiness, gumminess and chewiness of the samples decreased significantly ($P<0.05$) with the increase in the PSLT fermentation duration. According to Creamer and Olson (1982) casein proteolysis could result in cheese textural changes. They reported a weakened cheese structure as a result of αs1-casein proteolysis. Our results from proteolysis in provided evidences on the proteolysis of our cheesemilk as a result of bacterial activities during PSLT fermentation trials. The proteolysis of caseins (pH4.6 SN/TN) and evolution of modified peptide profiles (HPLC results) with smaller sized peptide chains (LC MS/MS) as a result of proteolytic activity of starter bacteria during longer fermentation were assessed to be significant ($p<0.05$). Thus, that proteolysis may have been the reason for the differences in final cheese textural quality attributes.

A high correlation (negative) between the duration of fermentation, formation of pH4.6SN/TN as proteolysis index and cheese hardness was assessed. Other texture profile analysis (TPA) derived parameters were also impacted by the extent of hydrolysis during PSLT fermentations. Although the effect of protein hydrolysis were addressed by some researchers before, (e.g. O'Mahony et al. (2005)), the outcome of this study are the first evidences provided on the effects of starter lactic acid bacteria on textural attributes of cheese, prior to curd formation.

Microstructure of cheeses were imaged and it was observed (from image analysis) that there is a tendency towards formation of an open structure once the duration of PSLT fermentation of cheese milk was increased. When the extent of protein hydrolysis increased, the size of pores in gel network would increase consequently (Srinivasan and Lucey 2002). The formation of serum

channels rather than evenly distributed pores were visible. This was concluded to be in agreement with our proteolysis results and also cheese textural attributes.

7.4 Future work

Further research carried out by using the findings in this research can include:

- To assess the proteolytic activity of the other lactic acid bacteria used as a starter in the cheese industry using long term fermentation;
- Further assessment of the produced peptide profiles by the aid of peptide sequencing method to identify peptides produced and their pattern of appearance/disappearance during long term fermentation of LAB;
- Selection of the appropriate LAB starter type for the production of the desired cheese characteristics;
- To evaluate the effects of longer fermentation trials on the evolution of new peptide profiles and linking those to textural attributes of cheese, especially processed cheese with desirable textural attributes;
- To assess the effect of PSLT fermented cheese milk with different compositions (fat, moisture) on the textural attributes of resultant cheese;
- This research was mainly focused of cheese textural attributes; the results of this study suggest promising potential in flavour enhancement/manipulation of cheeses made from long-fermented cheese milks.

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