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Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork



Influence of milk pre-treatment, and changes in cheese structure and carbon dioxide solubility on the development of split defects in cheese

Thesis presented to the National University of Ireland for the degree of Doctor of Philosophy by

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October 2019

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Declaration

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

Signature: Date:

_____ _____

Prabin Lamichhane

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Abstract

The objective of this research was to understand the underlying issues leading to the development of undesirable split or crack defects within continental semi-hard cheeses made from a seasonally produced milk supply. Such defects result in poor aesthetic quality (a key retail requirement) and poor performance under high speed slicing for global food service markets, with consequential economic loss. Centrifugation (at centrifugal force of 9,000 × g) and incorporation of high heat-treated (HHT) centrifugate are common milk pre-treatment methods/practices prior to continental cheese manufacture. Centrifugation had little effect on the composition, texture, volatile profile and ripening characteristics of Maasdam cheese, except for significantly lower butyric acid levels. However, incorporating HHT centrifugate into the cheese milk significantly increased the levels of moisture in non-fat substance and decreased the hardness of the resultant cheeses. This may have the potential to influence subsequent eye formation characteristics, and possibly influence split or crack development. Primary proteolysis and levels of insoluble calcium content are considered to influence the fracture properties of cheese, including fracture stress and fracture strain. The present research found that (1) inhibition of rennet activity during ripening; (2) reduction of rennet activity during ripening; and (3) reduction of ripening temperature decreased the hydrolysis of α_{S1}-casein by ~95%, ~45%, or ~30%, respectively, after 90 d of ripening. During the same ripening period, ~35% of β-casein was hydrolysed for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of insoluble calcium as a percentage of total calcium decreased significantly from ~75% to ~60% between 1 and 90 d of ripening. Further results showed that although modulation of α_{S1}-casein hydrolysis is an effective means to maintain the strength of the cheese matrix during

ripening, maintaining higher levels of intact β-casein or insoluble calcium content (or both) within the cheese matrix results in reduced levels of shortness or brittleness of cheese texture. For the first time, dynamic microscopy was applied to understand the microstructural changes occurring in semi-hard eye-type cheeses during large-strain tensile deformation. It was observed that pre-existing micro-defects within cheese matrices led to the formation of undesirable slits or cracks. Gas behaviour, including solubility, is considered one of the critical factors for development of eyes, and also slits or cracks within cheese matrices. Therefore, CO_2 solubility behaviour was studied in casein matrices, representing the protein-water phase of cheese matrices. It was observed that the CO_2 solubility of casein matrices largely depends on the moisture-to-protein ratio, salt-in-moisture content, pH and temperature. Overall, this research provides a knowledge base to minimize or avoid development of splits or cracks defects and thus improves the quality and consistency of continental-type cheeses.

Publications

Peer-reviewed articles:

Lamichhane, P., Sharma, P., Kennedy, D., Kelly, A. L., & Sheehan, J. J. (2019). Microstructure and fracture properties of semi-hard cheese: Differentiating the effects of primary proteolysis and calcium solubilization. *Food Research International*, 125, 108525.

Lamichhane, P., Kelly, A. L., & Sheehan, J. J. (2018). Effect of milk centrifugation and incorporation of high-heat-treated centrifugate on the composition, texture, and ripening characteristics of Maasdam cheese. *Journal of Dairy Science*, 101, 5724-5737.

Lamichhane, P., Pietrzyk, A., Feehily, C., Cotter, P. D., Mannion, D. T., Kilcawley, K. N., Kelly, A. L., & Sheehan, J. J. (2018). Effect of milk centrifugation and incorporation of high heat-treated centrifugate on the microbial composition and levels of volatile organic compounds of Maasdam cheese. *Journal of Dairy Science*, 101, 5738-5750.

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Manuscript in preparation:

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Oral Presentation:

Prabin Lamichhane, Mark A. E. Auty, Alan L. Kelly and Jeremiah J. Sheehan. Dynamic *in situ* imaging of semi-hard cheese microstructure under large-strain tensile deformation: Understanding structure-fracture relationships. Walsh Fellow Regional Competition, November 22, 2019, Ireland. **(Prize won: 2nd Runner up)**

Prabin Lamichhane, Mark A. E. Auty, Alan L. Kelly and Jeremiah J. Sheehan. Structural breakdown behaviour of a model Maasdam-style cheese as studied using dynamic confocal scanning laser microscopy. ADSA Annual Meeting, June 23-26, 2019, Cincinnati, Ohio. Presented by Dr. J. J. Sheehan on my behalf.

Prabin Lamichhane, Prateek Sharma, Deirdre Kennedy, Alan L. Kelly and Jeremiah J. Sheehan. Differentiating between the effects of chymosin-mediated proteolysis, coagulant type, ripening temperature and calcium solubilization on fracture behavior of Maasdam-style cheese. ADSA Annual Meeting, June 23-26, 2019, Cincinnati, Ohio. Presented by Dr. J. J. Sheehan on my behalf.

Prabin Lamichhane, Mark A. E. Auty, Alan L. Kelly and Jeremiah J. Sheehan. Structural breakdown behaviour of a model Maasdam-style cheese as studied using dynamic confocal scanning laser microscopy. The 8th International Symposium on Food Rheology and Structure - ISFRS, June 17- 20, 2019, Zurich/Switzerland.

Prabin Lamichhane, Mark A. E. Auty, Alan L. Kelly and Jeremiah J. Sheehan. Structural breakdown behaviour of a model Maasdam-style cheese as studied using dynamic confocal scanning laser microscopy. IFSTI 47th Annual Food Science and Technology Conference, Dec 6th and 7th 2018, University College Cork.

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Poster presentations:

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Chapter 1: Structure-function relationships in cheese: A review

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1.1 Abstract

The quality and commercial value of cheese are primarily determined by its physico-chemical properties (e.g., melt, stretch, flow, and color), specific sensory attributes (e.g., flavor, texture, and mouthfeel), usage characteristics (e.g., convenience), and nutritional properties (e.g., nutrient profile, bioavailability, and digestibility). Many of these functionalities are determined by cheese structure, requiring an appropriate understanding of the relationships between structure and functionality to design bespoke functionalities. This review provides an overview of a broad range of functional properties of cheese and how they are influenced by the structural organization of cheese components and their interactions, as well as how they are influenced by environmental factors (e.g., pH and temperature).

1.2 Introduction

Overall, the global consumption of cheese has been increasing continuously and is projected to increase by ~13.5% between 2016 and 2025 (OECD/FAO, 2016). Simultaneously, consumers/end-users have increasingly been demanding enhanced physico-chemical properties, sensory and nutritional quality, and optimal usage characteristics of cheese, all at a reasonable cost. This is primarily driven by factors such as growing consumer awareness of the role of diet in health and well-being, the potential to use structure to influence flavor release and sensory experience, and the extensive use of cheese as an ingredient in food retail applications. Such expanding consumer demands have triggered the focus of food researchers and cheese producers toward the improvement in the quality of existing products or the design of new innovative products.

It is now well recognized that many of the desirable properties of cheese are largely determined by its structure. For example, structure plays an important role in determining the mechanical, rheological and cooking properties of heated and unheated cheese (Lucey, Johnson, & Horne, 2003; Guinee, 2016), eye-formation in several types of hard (e.g., Swiss-type or Emmental) and semi-hard (e.g., Maasdam type) cheese (Daly, McSweeney, & Sheehan, 2010), and texture perception (Rogers et al., 2009). More recently, it has also been reported that food structure plays a key role in flavor release (Taylor, 2002) and in the digestion and the absorption of nutrients (Parada & Aguilera, 2007; Singh, Ye, & Ferrua, 2015). Apart from containing basic nutrients, the nutritional value of food can also be enhanced by introducing health-promoting and bioactive compounds, such as polyphenols and peptides. In this context, the cheese matrix can potentially be used as a delivery vehicle for bioactives and probiotics (Sharp, McMahon, & Broadbent, 2008;

Rashidinejad, Birch, & Everett, 2016). Thus, a better understanding of the complex interrelationship between structure and functionality, i.e., the so-called structure-function relationship, is necessary to design of cheese types with specific functionalities. However, the full extent of the relationships between structure and functionality of cheese is not fully understood. The aim of this review is to provide an appropriate knowledge of how cheese structure may be manipulated to control and predict the functional properties of cheese.

1.3 Cheese components and structure

Caseins, the main structural component of cheese, are present in the form of a network in the cheese matrix in which fat globules, water, minerals, bacteria, and dissolved solutes such as lactose, lactic acid, soluble salts, and peptides are all interspersed. The spatial arrangements of these components and their interactions determines the structure of cheese, which is influenced by relative volume fractions of each component and their properties (e.g., residual charge on the casein, composition of membrane materials of fat globules, and state of minerals, water, and fat), cheese manufacturing procedures, maturation conditions, and environmental conditions (e.g., pH, temperature, and solvent quality/ionic strength), among other factors.

Like other food types, cheese encompasses a hierarchical structure, with scales that span from the molecular to the macroscale (Figure 1.1). At a macroscopic level, cheese is the assembly of curd particles (resulting from cutting of the gel in the case of brine-salted cheeses), or curd chips or pieces (resulting from milling of curds and dry salting, such as in Cheddar and Stilton cheese manufacture) (Guinee, 2016). Eyes, slits/cracks, visible crystals and mechanical openness are also macro-structural

features of cheese. At microscopic level, cheese is composed of microstructural components, such as the casein network, fat globules, and water droplets. At further higher levels of magnification (nano or molecular scale), microstructural components of cheese are formed from molecules and atoms. Structures at the macro, micro, nano and molecular levels of organization all have an important role in various properties of cheese. Various techniques to study cheese structure, such as microscopy, rheology, magnetic resonance, dynamic light scattering, have been reviewed extensively (e.g., Everett & Auty, 2008; El-Bakry & Sheehan, 2014).

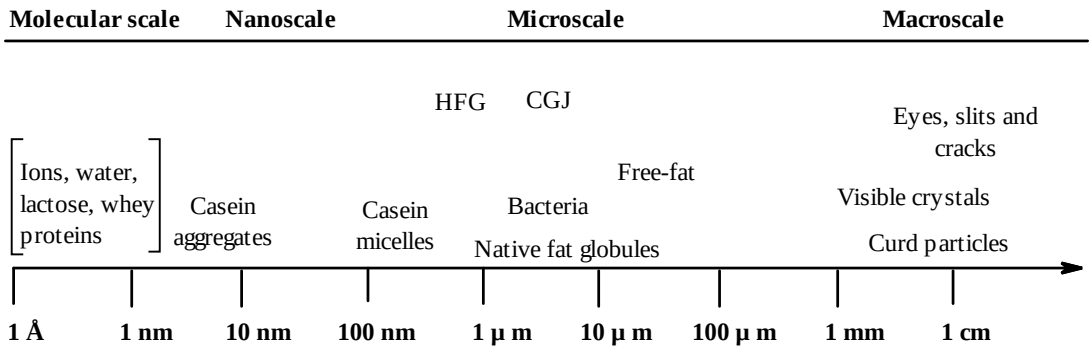


Figure 1.1. Characteristic length scales in cheese. HFG = homogenized fat globules, CGJ = curd granule junction.

From a materials science perspective, cheese can be viewed as a 2-phase composite material (also called "filled gels" or "gelled emulsions") containing fat globules as a filler in a protein gel matrix (Barden, Osborne, McMahon, & Foegeding, 2015). Several researchers used this approach to study the role of milk fat and protein network on the mechanical and rheological properties of cheese (Rogers, McMahon, Daubert, Berry, & Foegeding, 2010; Barden et al., 2015; Thionnet, Havea, Gillies, Lad, & Golding, 2017).

1.4 Molecular interactions within the cheese matrix

Various molecular forces and interactions that act between the cheese components are considered important as they can influence the functionality of cheese. For example, it is suggested that the localized balance of the attractive and repulsive forces between caseins controls the melting of heated cheese (Lucey et al., 2003). Moreover, the nature and extent of interactions of flavor compounds and nutrients with the food matrix can influence their release patterns in the mouth during mastication and in the gut during digestion, and this can in turn affect the sensorial and nutritional properties of food (Parada et al., 2007; Gierczynski, Guichard, & Laboure, 2011). For such reasons, knowledge of molecular interactions and forces that act between cheese components is vital.

Some studies have characterized the interactive forces in milk gels and cheese curd using different dissociating agents such as urea, sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA) (Lefebvre-Cases et al., 1998; Gagnaire, Trotel, Graët, & Léonil, 2002; Zamora, Trujillo, Armaforte, Waldron, & Kelly, 2012). These dissociating agents are known to disrupt specific types of bond or interaction; for example, hydrophobic interactions and hydrogen bonds can be disrupted by SDS or urea, respectively, whereas ionic bonds involving calcium salts are broken by the chelating effects of EDTA (Zamora et al., 2012). Lefebvre-Cases et al. (1998) characterized the interactive forces in rennet- and acid-induced milk gels using different dissociating agents, and the results of their study suggested that hydrophobic interactions and calcium bonds were the most important forces for the stabilization of the structure of rennet milk gels. The contribution of hydrogen bonds seemed comparatively less important for the stability of rennet gel structure than the

aforementioned forces. In acid-induced milk gels, hydrophobic and electrostatic interactions and hydrogen bonds have been shown to be important forces, whereas the contribution of calcium bonds have been found to be less important, most probably due to solubilization of colloidal calcium at low pH (Lefebvre-Cases et al., 1998). Calcium bonding, electrostatic interactions, and hydrogen bonds (to a lesser degree) contribute to the formation and stability of the *para*-casein matrix (after pressing) in Emmental cheese (Gagnaire et al., 2002). The major interaction forces responsible for the structural organization of cheese components are defined as outlined below.

1.4.1 Electrostatic interactions

Electrostatic interactions are important for food components that have a permanent electrical charge, such as dipoles or ions (McClements, Decker, Park, & Weiss, 2009). Cheese is a complex system, and many components present in the cheese matrix are known to have electrical charge. For example, casein contains several amino acid residues with ionizable groups along their polypeptide chains, including phosphoseryl residues (Horne, 1998).

Electrostatic interactions between charged species are sensitive to the surrounding environment, particularly pH and ionic strength. The electrical charge of ionizable groups of food components depends on their pKa values relative to the pH of the surrounding aqueous solution (McClements et al., 2009). The aqueous phase of the cheese matrix contains several monovalent (e.g., Na^{+}) and multivalent ions (e.g., Ca^{2+}), and their levels determines the ionic strength of the aqueous phase of the cheese matrix. The magnitude and range of the electrostatic repulsion between the caseins in the cheese matrix may decrease with increasing ionic strength of the

surrounding aqueous solution due to electrostatic screening effects (McClements et al., 2009).

Calcium-sensitive caseins can cross-link with free calcium ions *via* calcium bridging, which is a type of electrostatic interaction (Dalgleish, 1983). Such interactions are considered important for the aggregation of renneted casein micelles during coagulation of milk (Dalgleish & Corredig, 2012). The binding of calcium by casein is suggested to decrease with increasing ionic strength and with decreasing temperature (< 40°C) (Horne & Lucey, 2014).

1.4.2 Hydrophobic interactions

Hydrophobic interactions are strong attractive forces between hydrophobic side groups of molecules in aqueous solution. The molecular origin of hydrophobic interactions is the fact that water molecules can form relatively strong hydrogen bonds with other water molecules, but not with non-polar groups (McClements et al., 2009). Caseins have a significant fraction of non-polar regions along their polypeptide chain (Horne, 1998). Thus, it is expected that the hydrophobic interactions may play an important role in determining the casein interactions in the cheese matrix (Lucey et al., 2003). The strength of hydrophobic interaction tends to increase with increasing temperature (McClements et al., 2009). Thus, it is believed that these interactions may make a significant contribution to the functionality of heated cheese (Lucey et al., 2003).

1.4.3 Hydrogen bonding

Hydrogen bonds are simply an interaction between an electronegative atom (e.g., O, N, F and Cl) and a hydrogen atom covalently bound to similar

electronegative atoms. This is the one type of electrostatic force which tends to decrease in strength as the temperature increases (McClements et al., 2009). Hydrogen bonding is known to play a major role in hydration of proteins (Petukhov, Rychkov, Firsov, & Serrano, 2004). Proteins, including caseins, contain several groups, such as carbonyl, amine, amide, and hydroxyl, which are able to interact with water through hydrogen bonding. Hydration of casein is considered important for the development of desirable texture and cooking properties of some cheese-types, such as Mozzarella (Guo, Gilmore, & Kindstedt, 1997). Moreover, several studies have also suggested that the texture and cooking properties of low-fat cheeses can partially be improved by increasing the water-binding capacity of the protein matrix through approaches, such as modulation of pH, and varying the level of colloidal calcium phosphate (**CCP**) and sodium chloride (NaCl) of the cheese matrix (Paulson, McMahon, & Oberg, 1998; Sheehan & Guinee, 2004; McMahon, Paulson, & Oberg, 2005; Johnson, Kapoor, McMahon, McCoy, & Narasimmon, 2009).

1.4.4 Disulfide bonding

Disulfide bonds are a covalent bond formed between two thiol groups. This bond is considered important for cheese made from high heat-treated milk (e.g., Queso Blanco). High heat treatment of cheese milk unfolds heat sensitive whey proteins, exposing thiol groups which can form disulfide links with other reactive thiol groups of whey protein and casein through classical thiol-disulfide exchange reactions (Kethireddipalli & Hill, 2015). This type of reaction is influenced by redox potential (Poole, 2015) although there is little published on this with regard to cheese matrices.

1.5 Functional properties of cheese

1.5.1 Key properties for use as an ingredient

Cheese is extensively used as an ingredient in many foods. Two main functional requirements of cheese when used as an ingredient are: (1) machinability (the ability of cheese to be shredded/diced/cut/sliced); and (2) specific cooking and melting properties (Lucey, 2008). Functional requirements of cheese largely depend on end-use application. For example, when cheese is used a topping on pizzas and lasagna, it needs to melt and stretch in a specific manner, whereas melting is undesirable when visual identity and shape of cheese on cooking is required, such as for Queso-Blanco and Paneer, although a certain degree of softening is desirable (Guinee, 2016). The various functional properties required when cheese is used as ingredients have been extensively discussed (Lucey, 2008; Guinee, 2016).

1.5.2 Texture perception

Texture is an important factor determining the quality and identity of food, including cheese (Lawrence, Creamer, & Gilles, 1987; Foegeding & Drake, 2007). For cheese, the main evaluation of texture occurs in the hand (touch/feel/bend) and mouth (during mastication). However, in some eye-forming cheese types, such as Emmental and Maasdam, visual properties are also important. Proper eye development, such as number, size, shape, luster and distribution, is an important textural property in those cheese types (Lucey et al., 2003; Daly et al., 2010).

During consumption, food is subjected to a complex series of oral manipulations, including ingestion, size reduction, and mixing with saliva, to form a bolus for safe swallowing, collectively termed as oral processing (Foegeding, Çakır,

& Koç, 2010). Behavior of food during oral processing, such as breakdown patterns and extent of interaction (coating) with the oral surfaces, is thought to play an important role in the texture perception, and the structure and chemical composition of food can influence their behavior during oral manipulation (van Vliet, van Aken, de Jongh, & Hamer, 2009; Foegeding et al., 2010). For example, the desirable texture of full-fat Cheddar cheeses compared to low-fat cheeses is considered to be partly due to role of fat in the desirable breakdown patterns of cheese during oral manipulation (Rogers et al., 2009; Foegeding et al., 2010). Similarly, the desirable texture of aged cheese is attributed to the age-related structural changes in the protein matrix, resulting in specific breakdown pattern during chewing (Rogers et al., 2009). Moreover, several studies of emulsion gels have reported that the properties of emulsion droplets, extent of droplet-matrix interactions, distribution of emulsion droplets, and characteristics of the gel matrix can all influence texture perception (Sala, van de Velde, Cohen Stuart, & van Aken, 2007; Liu, Stieger, van der Linden, & van de Velde, 2015; Oliver, Berndsen, van Aken, & Scholten, 2015). Thus, a fundamental knowledge of how the structure of cheese influences a specific textural response could be useful for designing cheese with desired texture profiles.

1.5.3 Flavor release and perception

Like texture, flavor (comprising taste and aroma) is also an important attribute of cheese. The heterogeneous mixture of several hundred volatile and nonvolatile flavor compounds in cheese are the result of complex biochemical reactions during maturation, such as proteolysis, lipolysis and glycolysis (McSweeney, 2004). During consumption, flavor compounds are released from the food matrix and diluted with saliva, which need to be transported to the flavor receptors in the mouth and nose for flavor perception to occur (Taylor, 2002). The

correct balance and concentration of a wide range of flavor compounds, their release profile during oral processing, and the concentration and the rate at which those flavor compounds reach the receptors can all influence overall flavor perception (Taylor, 2002).

Food structure appears to play a key role on release of flavor compounds. Several studies on pure gels (e.g., protein gels, carrageenan gels), mixed gels (e.g., whey protein-polysaccharides), emulsion-filled gels (where emulsion droplets are embedded within a gel matrix) or solid lipoproteic colloid foods have shown that the gel structure affects volatiles and tastant release profile (Stieger & van de Velde, 2013; Kuo & Lee, 2014); in general, weaker gel textures and more porous structures had higher flavor compound release during mastication. For example, Kuo et al. (2014) reported that the rate of sodium release increased with increasing porosity and pore size of solid lipoproteic colloid foods. Proteolysis weakens the structure of protein network due to breakdown of the protein network; thus, it may be assumed that proteolysis facilitates the release of sapid compounds during mastication (Sousa, Ardö, & McSweeney, 2001).

Other factors, such as properties of the flavor compounds (e.g., volatility, solubility and affinity towards the food matrix), and the subject's oral processing behavior (e.g., chewing, saliva flow rate, and air flow rate through the mouth and nose), can also influence the release of flavor compounds from the food matrix during oral manipulation (Taylor, 2002; Gierczynski et al., 2011). In addition, it is now well accepted that overall flavor perception can also be influenced by perceptual interactions between various sensory modalities (e.g., aroma, taste and texture). For example, Visschers et al. (2006) reported that the intensity of aroma perceived by

subjects decreased with increasing firmness of the food. In another study, the odor of Comté cheese enhanced the perception of saltiness in model cheeses (Lawrence et al., 2011).

Based on the above considerations, it is clear that the flavor profile of food/cheese can potentially be enhanced by modifying food structure. This approach could be manipulated to facilitate reduction of sodium and saturated fat without significantly altering flavor attributes or to ameliorate flavor defects in reduced-fat and reduced-salt cheeses. Such approaches merit further research.

1.5.4 Nutritional properties

Cheese, a nutrient-dense dairy product, is a good source of proteins, vitamins, and minerals, particularly calcium, and phosphorous. However, the traditional method for evaluation of the nutritional quality of food, i.e., based on its composition, has recently been criticized since the method often neglects the effect of food matrix (structure) on nutrient release and absorption (Parada et al., 2007; Singh et al., 2015). For example, in an *in vivo* study using six mini-pigs, Barbé et al. (2013) reported that a renneted-gel matrix slowed down the rate of digestion of protein and absorption of amino acids as compared to liquid milk, probably due to lesser accessibility for digestive enzymes. In another study, Lamothe, Corbeil, Turgeon, and Britten (2012) studied the digestion pattern of Cheddar and Mozzarella cheeses using an *in vitro* stomach model. The results of their study suggested that the degradation of protein and the kinetics of fatty acid release are closely associated with the physical characteristics of the cheese matrix; cheeses that exhibited greater cohesiveness and elasticity were more slowly degraded during digestion and gave slower rates of fatty acid release. More recently, some studies have reported that

calcium in cheese can influence the free fatty acid bioaccessibility by producing insoluble calcium soaps with long-chain fatty acids at intestinal pH conditions (Ayala-Bribiesca, Turgeon, & Britten, 2017). A better understanding of the role of cheese structure on digestion and absorption of nutrients within the gastrointestinal environment is a key to design cheese with enhanced nutritional quality. This area has been reviewed in detail recently by Singh et al. (2015).

1.5.5 Delivery of bioactives and probiotics

Several studies have reported the potential for using the cheese matrix as a delivery vehicle for bioactives, such as, vitamins (Madziva, Kailasapathy, & Phillips, 2006), minerals, and polyphenols (Rashidinejad et al., 2016). For example, Rashidinejad et al. (2016) successfully used full-fat hard cheese as a delivery vehicle for liposomal nanoencapsulated green tea catechins. Moreover, the cheese matrix can also serve as a vehicle for probiotic delivery (Sharp et al., 2008). High buffering capacity and the dense protein network of cheese are thought to protect probiotic bacteria against the harsh acid environment in the stomach (Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009), making cheese a potentially suitable carrier for probiotics.

However, it should be noted that many bioactives have an undesirable taste and odor, such as, metallic taste of mineral salts, bitter taste of peptides, and fishy taste and odor of marine oils rich in omega-3 fatty acids (Augustin & Sanguansri, 2008), which can alter the sensory properties of cheese. Moreover, the metabolites from high numbers of viable and metabolically active bacterial cells can also alter the sensory attributes of cheese. Therefore, these details need consideration when using cheese as a delivery vehicle for bioactive compounds and probiotics.

1.6 Role of structural elements and their interactions on functional properties of cheese

The composition and the structural organization of cheese determine its functionality. In this section, we therefore focus on how the properties and the structural organization of the different phases of cheese, and the interactions between them influence cheese functionality. For the sake of simplicity, we have divided cheese structure into four phases: (1) protein phase, (2) fat phase, (3) aqueous phase, and (4) gas phase, particularly carbon dioxide (CO_2).

1.6.1 Protein phase

1.6.1.1 Formation and rearrangement of protein network

Formation of a protein network is a crucial step in cheese manufacture. The destabilization of casein micelles is one of the first steps in the manufacture of cheese. The mechanisms of destabilization of casein micelles by different means have been discussed extensively elsewhere (Dalgleish et al., 2012). The destabilized casein micelles aggregate into chain and clusters, leading to formation of a three-dimensional gel.

Several studies have reported that the factors, such as, concentration of casein (Karlsson, Ipsen, & Ardö, 2007), properties of casein micelle (e.g., casein micelle size) (Logan et al., 2014), and coagulation conditions (e.g., pH, temperature, and concentration of rennet) (Wium, Pedersen, & Qvist, 2003; Ong, Dagastine, Auty, Kentish, & Gras, 2011a; Ong, Dagastine, Kentish, & Gras, 2012), can all influence the coagulation process. This may influence the arrangement of casein into protein matrix and also the microstructure and the quality of the final cheese. For example,

milk renneted at lower pH (pH 6.1) gave gels with more compact protein network than in gels renneted at higher pH (pH > 6.3) (Ong et al., 2012). Moreover, the texture of resulted Cheddar cheese was different, i.e., cheese made using milk renneted at lower pH (pH 6.1) had lower chewiness, gumminess, cohesiveness and springiness than cheese made using milk renneted at higher pH (pH 6.7 or 6.5). Increased solubilization of CCP, accelerated rennet activity and reduced charge repulsion between micelles at a lower milk pH are most likely to alter the rate and extent of aggregation, possibly leading to different microstructures of gels and cheese curds (Ong et al., 2012). In other studies, the coarseness of the protein network of the gel or cheese increased with increasing coagulation temperature (Wium et al., 2003; Ong et al., 2011a). This is probably due to enhancement of the rearrangement of protein network and increasing strength of hydrophobic interactions at higher coagulation temperatures. Moreover, the calcium-binding by *para*-casein is suggested to increase with increasing temperature within the normal milk-coagulation temperature regime, which may influence the aggregation kinetics of fully renneted casein micelles (Dalgleish, 1983; Horne & Lucey, 2014). This suggests that the functionality of the final cheese can be modified by optimization or modulation of initial cheese-making conditions. Thus, the influence of initial cheese-making conditions on the final properties of cheese should not be underestimated.

Rennet-induced gels are inherently unstable and likely to undergo intraparticle, interparticle and interstrand rearrangements (Mellema, Walstra, Van Opheusden, & Van Vliet, 2002). An ultimate result of such rearrangements is syneresis (expulsion of whey) (Mellema et al., 2002). The rate and extent of syneresis is promoted by various cheese-making processes, such as, cutting, stirring, scalding and pressing (Dejmek & Walstra, 2004). Syneresis is considered as an

essential step during cheese manufacture since it affects the composition and texture of final cheese, as reviewed extensively by Dejmek et al. (2004). However, in some cheese-types, such as Quark (also called Quarg) and cottage cheese, syneresis can also occur in the finished product during storage, termed wheying-off, which is generally considered as undesirable (Guinee, 2016).

To date, there is significant knowledge on how milk composition and renneting conditions affect the gel structure. However, the link between gel structure and macroscopic behavior of the gel, such as, syneresis and water-holding, is not yet fully understood. Structural parameters, such as, dimensions of protein strands, network pore size, and volume fraction of the pores and protein network can be characterized at different structural levels by using different microscopic techniques (Langton & Hermansson, 1996; Ong, Dagastine, Kentish, & Gras, 2011b). Such structural information is relevant in understanding the effects of milk composition and gelation conditions on the macroscopic behavior of gels, such as, syneresis and water-holding properties of gel.

1.6.1.2 Casein-mineral interactions

In cheese, significant levels of minerals are associated with the protein network (Lucey & Fox, 1993). Calcium (Ca) and phosphate (PO_4) are the two most important minerals found in cheese, and are present in both soluble and in colloidal form. However, it is well recognized that the calcium and phosphate associated with the casein are an important structural unit in cheese. The level of calcium associated with casein (micellar calcium) varies widely between cheese types, ranging from less than 5 mg/g protein in Feta and Cottage cheese to ~ 24 mg/g protein in Gouda and Emmental cheese (Remillard & Britten, 2011).

Modulation of levels of colloidal calcium in the cheese matrix can alter the texture and cooking properties of cheese. For example, decreased levels of colloidal calcium is associated with the softening of cheese texture (at least in Cheddar; O'Mahony, Lucey, and McSweeney, 2005) and increased melt and flow properties (O'Mahony, McSweeney, & Lucey, 2006; Choi, Horne, Johnson, & Lucey, 2008), attributed to the reduction in calcium-induced casein-casein interactions (Lucey et al., 2003). This mechanism is supported by the studies of Pastorino, Hansen, and McMahon (2003a) and McMahon et al. (2005), who observed a more homogenous microstructure in cheeses with low levels of calcium than those with high levels of calcium, when observed using scanning electron microscopy (SEM); this indicated the proteins in the former were less aggregated than in the latter cheeses.

Micellar calcium levels in cheese are considered important in conferring an elastic texture to cheese (Lucey et al., 1993), which is important in the case of eye-forming cheese types, such as, Emmental and Gouda, to accommodate gas produced during warm-room ripening for smooth eye formation (Daly et al., 2010). Moreover, an elastic texture is also important for sliceability of cheese without fracturing or crumbling or sticking to cutting devices (Guinee, 2016).

It has also been reported that increased hardness due to increase in micellar calcium levels slowed down the disintegration during *in vitro* digestion, which in turn can affect nutrient bioaccessibility (Ayala-Bribiesca, Lussier, Chabot, Turgeon, & Britten, 2016).

1.6.1.3 Age-related changes in the protein matrix

During maturation, the structure of the protein network changes due to complex physical and biochemical changes in the cheese matrix, such as, proteolysis by various proteolytic agents, demineralization of casein, and hydration of the casein networks (at least in Mozzarella) as reviewed by Guinee (2016).

Recently, fermentation-produced camel chymosin has received attention because of its much higher ratio of milk clotting to general proteolytic activity than bovine chymosin (Kappeler et al., 2006). Cheddar cheeses made using recombinant camel chymosin were generally found to be harder with less bitter and brothy flavors, and with lower levels of proteolysis than cheeses made from bovine chymosin or microbial rennet (Hannilase) (Bansal et al., 2009; Soodam, Ong, Powell, Kentish, & Gras, 2015). Moynihan et al. (2014) suggested the use of recombinant camel chymosin to extend the techno-functional shelf-life performance of low-moisture part skim Mozzarella since its baking properties, such as, blister quantity, strand thickness, hardness, and chewiness, on baked pizzas were maintained for a longer time during storage than in cheeses made with bovine calf chymosin. Apart from residual coagulants, indigenous milk enzymes and enzymes produced by starter and non-starter bacteria also contribute to the proteolysis of cheese, particularly in high-cooked cheese varieties in which the residual chymosin activity is very low, most probably due to heat-denaturation of chymosin (Sousa et al., 2001; Sheehan, Oliveira, Kelly, & McSweeney, 2007). Plasmin is considered the most important indigenous milk proteolytic enzyme, and its activity in high cook cheese varieties (e.g., Emmental and grana-type cheeses) is comparatively higher than those in low-cook cheese varieties, (e.g., Cheddar), most probably due to thermal inactivation of inhibitors of both plasminogen activators and plasmin

(Sheehan, 2013). Plasmin has an optimum pH of ~ 7.5 and thus has a major contribution in the ripening of cheese types with high pH (~7), such as, mold-ripened (e.g., Camembert) and smear-ripened (e.g., Tilsit) cheese varieties (McSweeney, 2004; Sheehan, 2013). The role of plasmin and other indigenous milk enzymes in casein hydrolysis and their contribution to the quality of cheese has been extensively reviewed (Sousa et al., 2001; Kelly & McSweeney, 2003; McSweeney, 2004; Kelly, O'Flaherty, & Fox, 2006).

Varying degree of hydrolysis of casein in different cheese types have been reported (Table 1.1). The rate and extent of casein hydrolysis is influenced by factors, such as, cheese type, ripening temperature, level and types of coagulant and cheese compositions (e.g., moisture in non-fat substance, MNFS) (Table 1.1). α_{s1}-Casein has been considered to be the principal structural element in several cheese varieties, such as, Cheddar and Emmental (Lawrence et al., 1987; Gagnaire et al., 2002), with the hydrolysis of α_{s1}-casein thus being associated with a weakening of the protein network (Creamer & Olson, 1982). However, more recent studies have shown that softening of cheese in the early stages of ripening is primarily due to solubilization of CCP (O'Mahony et al., 2005).

Table 1.1. Extent of hydrolysis of casein in different cheese varieties

Cheese type	Level of casein hydrolysis	Age	Coagulant type	% MNFS[4]	% S/M[4]	Methods	References
Cheddar	48.8%, α_{s1}-CN[1] 16.8%, β-CN[1]	45 d at 7°C	single strength calf rennet extract (0.15 mL/kg milk)	55	3.75	urea-PAGE	Bogenrief et al. (1995)
Cheddar	44.7%, α_{s1}-CN[1] 59.8%, β-CN[1]	45 d at 7°C	*Cryphonectria parasitica* (0.051 mL/kg milk)	55.7	3.69	urea-PAGE	Bogenrief et al. (1995)
Cheddar	~80-85%, α_{s1}-CN[1] ~10%, β-CN[1]	90 d at 8°C	chymosin (0.3 mL/L milk)	57	2.85	urea-PAGE	O'Mahony et al. (2005)
Cheddar	~78%, α_{s1}-CN[1]	180 d at 8°C	fermentation-produced calf chymosin (0.3 mL/L milk)	56	3.3	urea-PAGE	Bansal et al. (2009)
Cheddar	~50%, α_{s1}-CN[1]	180 d at 8°C	fermentation-produced camel chymosin (0.035 mL/L milk)	56	3.3	urea-PAGE	Bansal et al. (2009)
Part-skim Mozzarella	~50-80%, α-CN[1]	28 d at 4°C	single strength calf rennet extract (0.42 mL/L milk)	64-66	ND[4]	SDS-PAGE[4]	Fife, McMahon, and Oberg (1996)
Mozzarella (direct acidification)	~55%, α_{s1}-CN[2] ~15%, β-CN[3]	15 d at 5°C	double-strength rennet, chymosin (0.025 mL/kg milk)	66	2.5	capillary electrophoresis	Dave, McMahon, Oberg, and Broadbent (2003)
Mozzarella (direct acidification)	~90%, α_{s1}-CN[2] ~50%, β-CN[3]	15 d at 5°C	double-strength rennet, chymosin (0.1 mL/kg milk)	66	2.5	capillary electrophoresis	Dave et al. (2003)
Swiss cheese	~75-80%, α_{s1}-CN ~75-80%, β-CN	60 d (7d at 4°C, 21 d at 21°C, and 32 d at 4°C)	*Cryphonectria parasitica* (0.05 mL/kg milk)	55	ND[4]	capillary electrophoresis	White, Broadbent, Oberg, and McMahon (2003)
Emmental	~50%, α_{s1}-CN ~40%, β-CN	40 d (20d at 12°C, and 20 d at 24°C)	calf rennet (0.3 mL/kg milk)	54	ND[4]	urea-PAGE	Sadat-Mekmene et al. (2013)

[1]Expressed as % levels at 1d of ripening
[2]Initial (100%) intact α_{s1}-CN level is based on the peak area of day-1 α_{s1}-CN plus the peak area of day-1 α_{s1}-CN (f24-199) times 1.1314 (to account for the loss of 23 of the 198 peptide bonds in α_{S1}-CN)
[3]Initial (100%) intact β-CN level is based on the peak area of β-CN at day 1
[4]ND = not determined; [4]MNFS = Moisture in non-fat substance; [4]S/M = salt-to-moisture ratio; [4]SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Some studies indicated that the specific hydrolysis patterns of casein and the resulting peptide profiles can influence the melting and stretching properties of cheese. For example, Bogenrief and Olson (1995) observe that a degree of melt of Cheddar cheese was more closely related to the extent of β-casein hydrolysis than the hydrolysis of α_{s1}-casein. In another study, Emmental cheeses made with *Lactobacillus helveticus* as a starter culture exhibited greater stretchability (2.5 times higher) than those with *Lactobacillus delbrueckii* (Richoux, Aubert, Roset, & Kerjean, 2009). Moreover, the stretchability of cheese was strongly correlated with the proportion of hydrophobic peptides in the pH 4.6-soluble nitrogen fraction. This finding is further supported by the study of Sadat-Mekmene et al. (2013), who also observed high stretchability in Swiss-type cheese made with two different strains of *Lactobacillus helveticus*, i.e., ITGLH77 and ITGLH1. Moreover, the stretchability was correlated with hydrophobic peptides, regardless of casein origin (i.e., whether α_{s1}-CN, α_{s2}-CN or β-CN), and with a lower degree of proteolysis. These hydrophobic peptides may interact with the protein matrix or with other large peptides via hydrophobic forces, possibly forming fibers in the cheese matrix (Richoux et al., 2009).

1.6.2 Fat phase

During cheese manufacture, milk fat globules are entrapped within the protein gel network, and processes, such as, scalding, cheddaring, hot water stretching and pressing, can cause aggregation, coalescence and disruption of the fat globules. In the cheese matrix, fat globules can exist as intact (spherical fat globules covered with native membrane materials), aggregated (clumps of circular fat globules), coalesced (spherical but larger than typical milk fat globules), elongated

(especially in pasta-filata cheese-types), or even non-globular forms (Michalski et al., 2007; Rogers et al., 2010; Ong et al., 2011b) (Figure 1.2). The microstructure of fat globules can influence the physical properties of cheese. For example, although Everett and Olson (2003) did not find a correlation between fat-globule-circularity and free-oil formation in Cheddar cheese, fat globule size (Feret's diameter) in Mozzarella cheese has been positively correlated with meltability and free-oil in a study by Ma, James, Zhang, and Emanuelsson-Patterson (2013).

Several factors, such as, the fatty acid compositions, native milk fat globule (NMFG) size, the level of fat, and the properties of fat globule membrane materials can all influence various properties of cheese, such as, texture, opacity, and rheological and cooking properties of cheese.

1.6.2.1 Fatty acid composition

The fatty acid composition of milk fat (which is influenced by factors, such as, stage of lactation, breed of cow, genetics, and diet composition; Månsson, 2008) can alter the rheological and textural properties of cheese. Palmitic acid (C16:0) and oleic acid (C18:1) are the major saturated and unsaturated fatty acids in milk that have high and low melting points, respectively (Coppa et al., 2011); a higher ratio of C18:1 to C16:0 is known to produce more creamy and less firm cheese (Coppa et al., 2011; Bocquel et al., 2016). Bocquel et al. (2016) observed ~ 30% decreases in hardness of Raclette cheese when the ratio of C18:1 to C16:0 in cheese milk increased from 0.8 to 1.0. In the case of Raclette cheese, the increased hardness increases the risk of cracks forming, which significantly affects the quality of cheese (Bocquel et al., 2016). The impact of fatty acid composition on the "mouthfeel" of cheese is not yet fully understood. However, it may be assumed that the complex

crystallization behavior due to fatty acid composition can alter the in-mouth coalescence of fat globules during oral processing, which can in turn affect the fat-related sensory perception. For example, high solid fat content in emulsion droplets enhances the coalescence of emulsion droplets and reduces friction during oral processing of emulsion-filled gels (Liu et al., 2015).

1.6.2.2 Native milk fat globule size

The size of NMFG ranges from < 0.2 to > 15 µm, with an average diameter of ~ 4 µm (Huppertz & Kelly, 2006). Studies have shown that the cheese manufactured from milk with different fat globule size differ compositionally and texturally. For example, Camembert and Emmental cheese produced from milk with small fat globules (SFG, ~ 3 µm), separated using microfiltration, had higher moisture content, softer texture, and underwent greater proteolysis during ripening than cheese made from milk with large fat globules (LFG, ~ 6 µm) (Michalski et al., 2003; Michalski et al., 2004). More recently, Logan et al. (2017) reported that the Cheddar cheese made from milk with SFG (~ 2.7 µm) was less firm at the early stages of ripening, and was less cohesive, less chewy and less springy throughout maturation than cheese made from milk with LFG (~ 5 µm). However, the exact effect of NMFG size on cheese properties was not determined in these studies since the effect of NMFG size is confounded with the cheese moisture level.

In another study, Michalski et al. (2007) made Emmental cheeses from milk with SFG and control milk, and adapted the process to obtain similar moisture content. The authors found that Emmental cheeses with SFG exhibited higher stretchability and elasticity, and improved sensory characteristics compared with control cheeses, despite the moisture content being similar for both cheeses. This

may be attributed to the impact of NMFG size on microstructure of cheese. Emmental (Michalski et al., 2007) and Cheddar (Logan et al., 2017) cheeses made from SFG appeared less aggregated and less coalesced than control cheeses or cheeses made from LFG, when observed using confocal laser scanning microscopy (CLSM). Moreover, more intact fat globules covered with phospholipids were observed in Cheddar cheeses from SFG than those from LFG when observed using CLSM (Logan et al., 2017). A recent study of an emulsion-filled gel system has reported that the mechanical properties of emulsion gels were influenced by the magnitude of droplet clustering or aggregation. Droplet clustering or aggregation enhanced the stiffness of emulsion-filled gels (Oliver et al., 2015).

Moreover, the fat globule size can alter the casein strand formation during rennet-induced coagulation of milk; this could be another reason for observed differences in the properties between the cheeses made from milk with small and large fat globules. Depending on the size of NMFG and the pore size of the protein network, the NMFG can act as an "inert-filler", "structure-breaker" (Michalski, Cariou, Michel, & Garnier, 2002) or has even been suggested as being held weakly within the protein matrix (Everett & Olson, 2000; Logan et al., 2015).

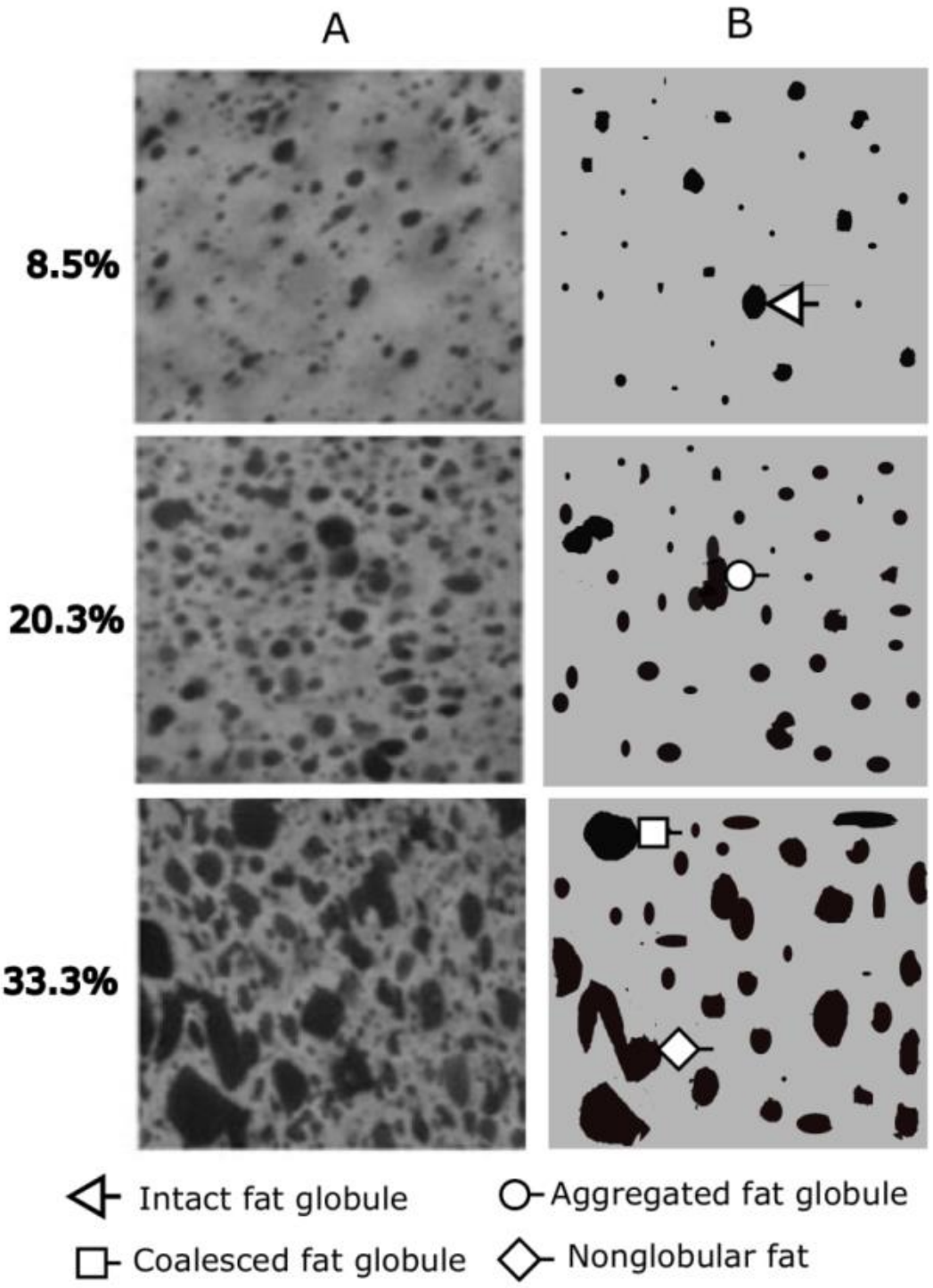


Figure 1.2. Column A shows confocal laser scanning microscopy images (100 μm × 100 μm) of Cheddar cheese (age: 12 wk) at 3 different fat levels (8.5%, 20.3% and 33.3%). Column B shows a schematic representation of Cheddar cheese microstructure, with intact fat globules, aggregated fat globules, coalesced fat globules, and nonglobular fats. The dark and gray areas represent fat globules and protein network within the cheese matrix, respectively. Adapted from Rogers et al. (2010) with permission.

1.6.2.3 Level of fat

A reduction in levels of fat without a proportionate increase in the levels of moisture will increase the concentration of casein in the protein matrix, leading to a compact protein matrix and a lower degree of fat coalescence (at least in Cheddar) (Guinee, Auty, & Fenelon, 2000; Rogers et al., 2010) (Figure 1.2). Such changes on the cheese structure may have consequences for texture, opacity, and rheological and cooking properties of cheese (Guinee et al., 2000; Johnson et al., 2009; Rogers et al., 2009; Rogers et al., 2010). The impact of fat reduction on texture, flavor, cooking properties and color of cheese has been reviewed by Johnson et al. (2009).

To better understand the role of milk fat content on the mechanical and rheological properties of cheese, and to simplify the complex cheese system, model filler particles, such as, Sephadex beads (Barden et al., 2015) or glass beads (Thionnet et al., 2017) have been used instead of milk fat in some studies. These studies suggested that the mechanical properties of cheese depend on the rheological properties of both the gel matrix and filler particle and on the volume within the cheese occupied by the filler particles (Barden et al., 2015; Thionnet et al., 2017). This knowledge may be useful in developing the replacement of milk fat in low-fat cheeses with other fat-like components, such as, hydrocolloids (Thionnet et al., 2017).

1.6.2.4 Interactions between fat globules and protein matrix

The interactions between milk fat globules and the protein matrix in cheese largely depend on the composition of fat globule membrane materials. Although a subject of debate (Everett et al., 2008), it is generally accepted that the native milk fat globule membrane, which is composed mainly of specific proteins and

phospholipids, does not chemically interact with the surrounding protein matrix (Michalski et al., 2002).

The nature and extent of interactions between fat globules and protein network can be controlled by modifying the surface properties of fat globules. Such modulation of fat-protein interactions can alter the cheese structure, which in turn can affect the mechanical, rheological, and sensorial properties of cheese. For example, Everett et al. (2003) compared the microstructure and rheological properties of Cheddar cheese manufactured from recombined milk containing fat globules coated with casein or whey proteins. The microstructure of fat globules appeared elongated and clustered in cheeses made from milk with fat globules coated with α_{s2}-casein (a relatively poor emulsifier) compared to other experimental cheeses in which fat globules were coated with other proteins. Moreover, the former cheeses fractured at a lower strain and with a lower stress than the other experimental cheeses.

The impact of interactions between fat globules and protein matrix on texture perception of cheese is not yet fully understood. However, several studies on emulsion-filled gel systems reported a significant impact of filler-matrix interactions on the texture perception. Emulsion-filled gels are prepared by embedding emulsion droplets into a gel matrix, and are a representative model food system for a broad variety of food products, including cheese (Sala et al., 2007). Depending on the properties of the emulsifiers on the surface of emulsion droplets, emulsion droplets can either be bound to the gel-matrix (called bound filler) or not (called unbound filler) (Sala et al., 2007). Unbound fat has been found to be related to the enhancement of fat-related sensory perception rather than bound fat droplets in

emulsion-filled gels (Sala et al., 2007; Liu et al., 2015). The unbound droplets underwent more coalescence than bound droplets during oral manipulation, leading to lower friction and enhancement of fat-related sensory perceptions (Liu et al., 2015).

1.6.3 Aqueous/serum phase

1.6.3.1 Water

Water in cheese can be broadly classified as bound or bulk water. Bound water is strongly associated with protein and other components of the cheese matrix, and this water is not available as a solvent, whereas bulk water is loosely associated within the protein matrix, and retains a large solvent capacity and is freezable at −40°C (McMahon, Fife, & Oberg, 1999). Bulk water may be either present within the channels surrounding the fat (free water) or entrapped within the protein matrix (entrapped water). The distribution and state of water in cheese depends on factors, such as, cheese-type and age. In most cheese varieties, most of the water is present within the protein matrix. However, in young Mozzarella cheese, a significant amount of water is present in the fat-serum channel. During aging, this water is gradually absorbed into the protein matrix, which has been confirmed by studies undertaken using nuclear magnetic resonance technique (Kuo, Gunasekaran, Johnson, & Chen, 2001; Smith, Vogt, Seymour, Carr, & Codd, 2017). Moreover, during maturation, hydrolysis of each peptide bond releases two new charged groups (NH_3^+/COO^-) which can bind the available free water and thus can alter the state of water in cheese (Creamer et al., 1982). This might be a possible reason for a slight decrease in water activity (a_w) in Cheddar cheese during ripening, from a mean of ~ 0.965 at 1 d to ~ 0.956 at 270 d (Hickey, Guinee, Hou, & Wilkinson, 2013).

Cheese generally becomes softer as the levels of moisture increase. Two main reasons have been reported for the texture softening effect of moisture, i.e., (1) water in the cheese matrix plays the role of a plasticizer (low-viscosity lubricant) (Marshall, 1990), and (2) increasing the levels of moisture results in a corresponding decrease in the levels of casein, which is the principal structuring component (McMahon et al., 2005). However, the impact of water on melt properties of cheese is rather complex. Increasing total moisture content of cheese does not necessarily increase the meltability of cheese (Pastorino, Ricks, Hansen, & McMahon, 2003c; McMahon et al., 2005). Instead, melt properties of cheese are reported to be more related to casein-water interactions (which are largely influenced by pH, ionic strength and the levels of CCP) than total moisture content (McMahon et al., 1999).

1.6.3.2 Components of the aqueous/serum phase

The components present in the aqueous phase, such as nitrogen fractions (whey proteins, enzymes, peptides or free amino acids), minerals, carbohydrates (lactose, galactose and glucose) and organic acids, and their levels influence the environment, mainly pH, a_w and ionic-strength, of the cheese matrix (Salaün, Mietton, & Gaucheron, 2005; Hickey et al., 2013). This can in turn affect the structure of the protein phase in cheese and thus on the texture, rheological and cooking properties. Moreover, the correct balance and concentration of components of the serum phase can influence the flavor profile of cheese. Components, such as hydrophobic peptides, lactose, lactate, and free amino acids, have been found to be positively associated with bitter, sweet, sour and umami flavor intensities, respectively (Møller, Rattray, Bredie, Høier, & Ardö, 2013).

The level of ions and their valance determines the ionic strength of serum phase of the cheese matrix, which can alter protein interactions. Salt (NaCl) has a major contribution to the ionic strength of the cheese matrix, since a varying amount of salt, ranging from ~ 0.5% (w/w) to 6% (w/w), is added in cheese, mainly for flavor and preservation (Guinee, 2004). Addition of salt up to certain concentrations can promote protein-water interactions, probably due to a "salting-in" effect, leading to hydration and swelling of the casein matrix (Guinee, 2004). Several studies have elucidated the role of salt in hydration and solubilization of casein. Guo et al. (1997) observed higher levels of intact casein in the serum phase obtained from centrifugation (~12500 × g for 75 min at 25°C) of brine-salted Mozzarella cheese than in unsalted cheese. Pastorino, Hansen, and McMahon (2003b) observed a more homogeneous protein matrix with less serum pockets in Munster cheese injected with salt than cheese without salt injection, as observed by SEM. Everett, Guinee, and Johnson (2014) reported an increase in NaCl-soluble proteins with increasing concentration of salt solution up to 6% (w/w) when unsalted Cheddar curd was immersed in varying brine concentrations (0 - 25%, w/w). However, very high salt concentrations can promote protein-protein interactions, probably due to a "salting-out" effect, leading to protein aggregation and contraction of the cheese matrix.

Salt in the cheese matrix not only contributes to the saltiness of cheese, but can also enhance the flavor intensity of sapid compounds; moreover, salt can suppress the unwanted flavor, e.g., bitterness (Møller et al., 2013). Thus, reduction in salt content is sometimes associated with flavor defects. For example, the flavor profile of Cheddar cheese deteriorated when the level of salt was reduced by 50% (Møller et al., 2013). In another study, consumer liking for low-salt cheeses was low

and they were able distinguish even a 30% salt reduction (Ganesan, Brown, Irish, Brothersen, & McMahon, 2014).

The impact of salt in structure, texture, rheological and cooking properties of cheese has been discussed extensively (Guinee, 2004; Everett et al., 2014). A general overview of the roles of salt in cheese is depicted in Figure 1.3.

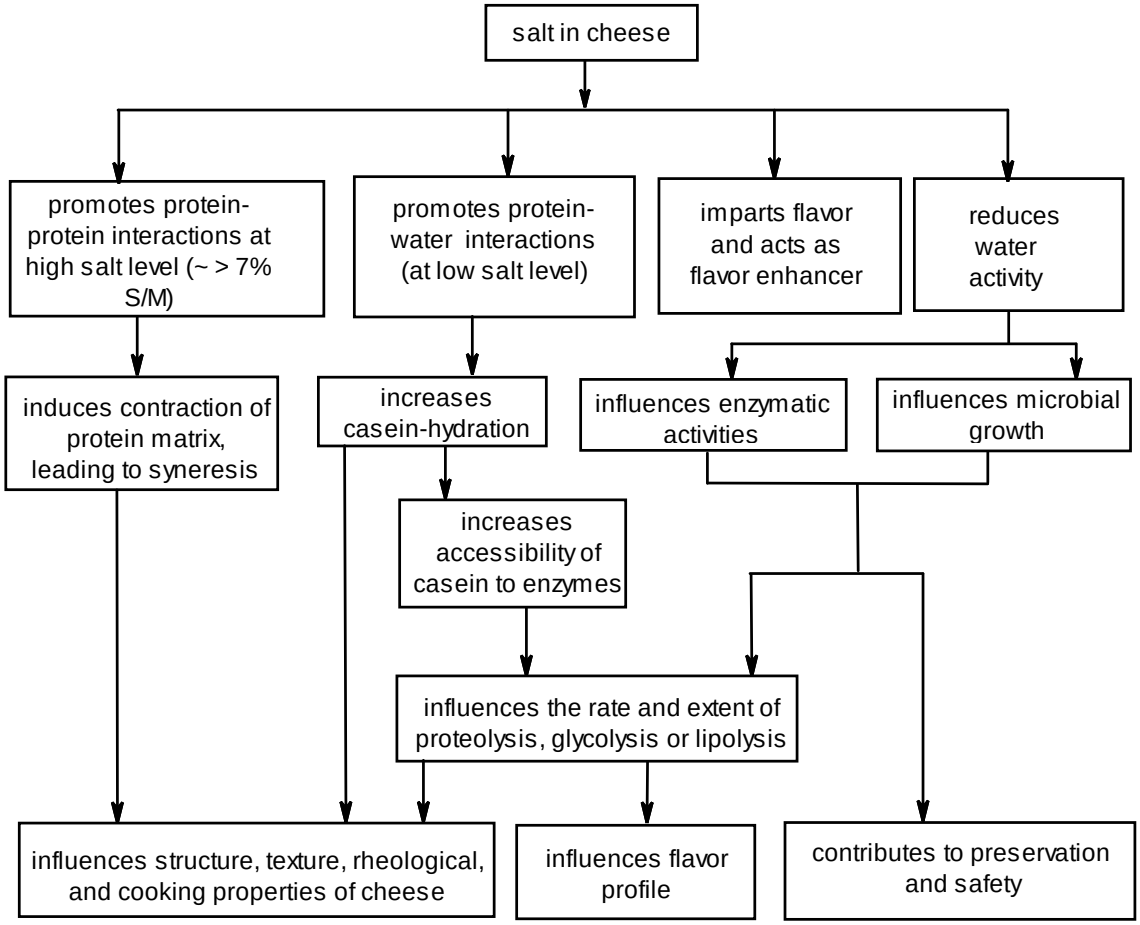


Figure 1.3. Role of salt in cheese. S/M: salt-to-moisture ratio.

1.6.4 Gas phase (particularly carbon dioxide)

Formation of smooth eyes in eye-forming cheese types, such as, Emmental and Maasdam, is considered an important quality parameter. Cheese matrix structure, the rate and extent of gas production and its behavior in the cheese matrix (e.g., solubility and diffusivity), and the presence of nuclei are known to play an important role in desirable eye formation (Daly et al., 2010). In this section of review, we focus on the role of CO_2 in the eye formation.

An appropriate understanding of CO_2 production, and its solubility and diffusivity in the cheese matrix, is necessary to obtain desirable quality of eyes without splits and cracks. Carbon dioxide in the cheese matrix is mainly produced due to lactate fermentation by propionic acid bacteria (PAB) during the warm room ripening. The rate and extent of CO_2 production is influenced by factors, such as, strains of PAB, ripening temperature, and cheese composition (Daly et al., 2010). Acerbi, Guillard, Aliani, Guillaume, and Gontard (2016a) determined the rate of production of CO_2 in semi-hard cheese to be ~ 10 - 15 mmol kg/day at constant temperature (25°C) and salt-to-moisture ratio (S/M, 2%, w/w). Carbon dioxide solubilizes in the fat and aqueous phases of cheese. However, its solubility is largely temperature-dependent, i.e., the solubility of CO_2 in the fat phase is lower at low temperature (e.g., 4°C) than at high temperature (at least up to 20°C); the opposite holds true for the solubility of CO_2 in water (Jakobsen, Jensen, & Risbo, 2009). The solubility of CO_2 in semi-hard cheese was determined as ~ 37 mmol/kg at 2°C and ~ 30 mmol/kg at 25°C (Acerbi, Guillard, Guillaume, & Gontard, 2016b). Thus, any changes in the ripening conditions or cheese composition or both can alter the solubility of CO_2 within the cheese matrix, which in turn can affect the internal

pressure of cheese. It is necessary to control the internal pressure of cheese, since over-pressure can lead to slits or cracks, which are unappealing to consumers. Moreover, these cheeses can produce a lot of fines or broken portions during size reduction operations, such as slicing and dicing, resulting in lost revenue to manufacturers (Martley & Crow, 1996). On the other hand, small or no eyes ("blind" cheese) will be formed if the gas pressure is inadequate.

Carbon dioxide produced in the cheese matrix diffuses within the cheese matrix or escapes from the cheese. The diffusion of CO_2 within the cheese is thought to obey the second law of Fick [Equation 1.1; where D is the effective diffusivity coefficient (m^2/s)], which describes the change in concentration (c) with time (t) at any place (x) as a function of the local concentration gradient for a mono-directional diffusion (Acerbi, Guillard, Guillaume, Saubanere, & Gontard, 2016c).

$$\frac{dc}{dt} = D\left(\frac{d^2c}{dx^2}\right) \qquad [1.1]$$

Diffusion of CO_2 to nuclei generates pressure at nuclei which are the primary site for eye formation. Diffusivity of CO_2 within the cheese matrix is one of the most important factors impacting eye growth in eye-forming cheese types, and it is influenced by cheese composition and structure, and ripening conditions. For example, a higher level of diffusivity of CO_2 has been observed in more aged semi-hard cheeses compared to young cheeses, and this has been attributed to age-related changes in the cheese matrix, such as, proteolysis and demineralization of casein (Acerbi et al., 2016c).

1.7 Impact of environmental factors on structural and functional properties of cheese

1.7.1 pH

It is widely recognized that pH has a strong influence on the texture, rheological and cooking properties of cheese, mainly via altering the casein-casein, mineral-casein and casein-water interactions, through its effect on casein charge and calcium solubility. At higher pH values (~5.4), the proportion of calcium associated with casein (micellar calcium) is relatively higher than at low pH (unless colloidal calcium is solubilized by other means, such as addition of calcium chelators; Choi et al., 2008; McAuliffe, Kilcawley, Sheehan, and McSweeney, 2016). Higher levels of micellar calcium promote casein-casein interactions within the cheese matrix. Such strong casein-casein interactions are known to increase the structural rigidity of the cheese matrix; as a consequence, cheese tends to be more firm, elastic and less meltable (McMahon et al., 2005). At intermediate pH (~ 5.1), casein-casein interactions decrease as the negatively charged regions of the casein (e.g., phosphoserine residues) are exposed due to partial solubilization of micellar calcium, and the resultant cheese tends to be softer and more meltable (Lucey et al., 2003). However, lowering the pH toward 4.7 increases the strong casein-casein interactions as the casein approach their isoelectric point, and adversely affects the melt, flow and stretch properties of cheese (Lucey et al., 2003; Pastorino et al., 2003a).

It seems that that pH may have an indirect effect related to its influence on the distribution of calcium (soluble or casein-associated) at pH above 5 (at least in Cheddar and direct-acidified non-fat Mozzarella cheese) (Pastorino et al., 2003a; McMahon et al., 2005). Below 5, pH seems to have a direct effect, i.e., charge

neutralization. If the cheese curd has very low levels of calcium, then increasing pH may simply increase protein-water interactions, probably due to increases in electrostatic repulsion forces between the charged groups of protein, which is illustrated by the work of Monteiro, Tavares, Kindstedt, and Gigante (2009). Those authors investigated the effect of pH on the microstructure and functionality of hot-pack cream cheese, in which calcium level is very low, by using exposure to acetic acid or ammonia vapor to modulate pH post-manufacture. The microstructure of cheese appeared more continuous or swollen with increasing pH (Figure 1.4), indicating that the casein are increasingly hydrated with increasing pH; moreover, cheese firmness decreased, whereas cheese meltability increased, with increasing pH.

The pH of cheese can alter the size of the protein aggregates and their arrangement in the protein matrix (Hall & Creamer, 1972; Lawrence et al., 1987; Pastorino et al., 2003a). Pastorino et al. (2003a) developed a model for the protein matrix of cheese at different pH, and reported that the diameter of protein aggregates were relatively higher at pH 5.3 (10 to12 nm) than at pH 4.7 (2 to 4 nm) in their model. Moreover, the protein aggregates at pH 5.3 have relatively more well-defined structure than those at pH 4.7. The proposed model is in agreement with the study of Hall et al. (1972), who observed bigger protein aggregates (10 to 15 nm) in Gouda cheese (pH, ~ 5.3) than in Cheshire cheese (3 to 4 nm), with relatively low pH (~ 4.6), when examined using SEM; moreover, the protein in latter cheese is less well-organized.

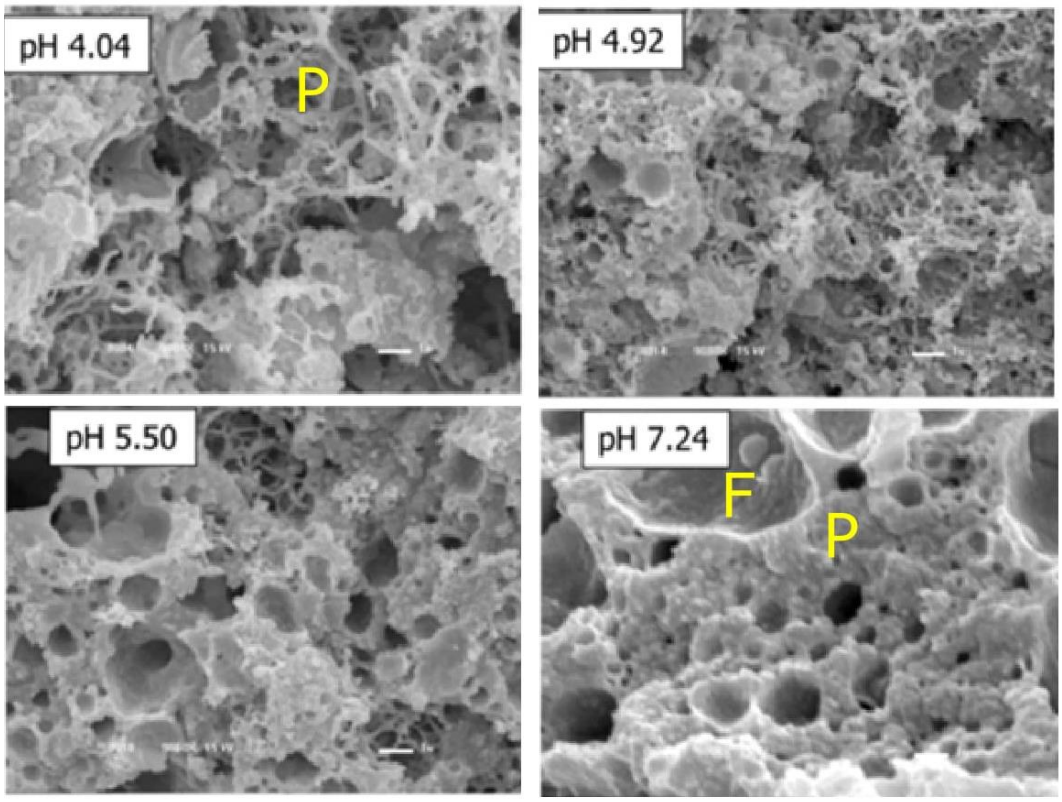


Figure 1.4. Scanning electron micrographs (9,000 ×) of hot pack cream cheese with different pH values. Cheese sample were exposed to ammonia vapor to increase the pH or acetic acid vapour to decrease the pH. The pH of the untreated control cheese was 4.92. P = protein matrix, F = spherical imprints in the protein matrix left by fat globules that were extracted during sample preparation. Scale bar represents 1 µm. Adapted from Monteiro et al. (2009) with permission. Copyright (2009) Institute of Food Technologists.

Taneya, Izutsu, Kimura, and Shioya (1992) also reported a less well-defined protein network structure of curd at pH 5.0 than at pH 5.4 when observed with transmission electron microscopy, and the authors concluded that curd having pH 5.4 was suitable for stretching during manufacture of string cheese. Pastorino et al. (2003a) speculated that the size of the protein aggregates and their arrangement in the cheese matrix may alter the texture, rheological and cooking properties of cheese, since the strength of material is known to be influenced by factors such as the extent of cross-linking, and the orientation or the structural regularity of the constituents of the material (Pastorino et al., 2003a).

It is well known that mold-ripened cheeses (e.g., Camembert and Brie), have a macroscopic pH-gradient between the surface and interior of cheese (McSweeney, 2004). However, it has recently been found that cheese can have a microscopic pH gradient. Burdikova et al. (2015) observed pH micro-heterogeneity in natural cheese matrices using fluorescence lifetime imaging microscopy (FLIM) (Figure 1.5). The local variation of pH in cheese matrix may influence the molecular interactions at the local level, which may lead to local heterogeneity in the microstructure of cheese. Nevertheless, more research is needed to gain greater understanding in this area.

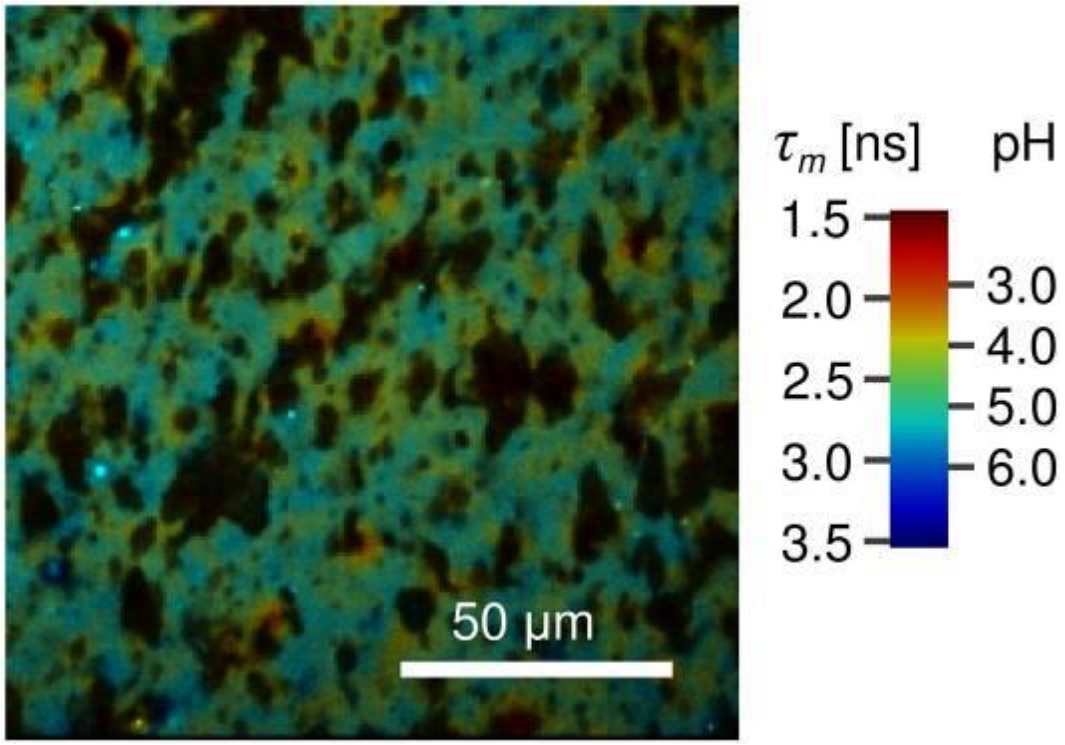


Figure 1.5. Fluorescence lifetime imaging microscopy (FLIM) image of natural cheese sample stained with Oregon Green 488. Shown are apparent local variations of fluorescence lifetime and thus pH. Localized spots with pH as low as 4.0 are observed. The dark areas most likely represent fat within the cheese matrix. The pseudocolor scale of the FLIM images is calibrated both in lifetime (τ_m) and pH values. Reprinted with permission from Burdikova et al. (2015). Copyright (2015) Lausanne: Frontiers Research Foundation.

1.7.2 Temperature

Temperature influences the structure of cheese through its effect on the components of cheese and their interactions, including changes in the physical state of fat and the molecular interactions between the casein. These changes in the structure of cheese are important to the textural, rheological and cooking properties of heated or unheated cheese. At low temperatures (below 20°C), a significant proportion of milk fat is in a solid state (Lopez, Briard-Bion, Camier, & Gassi, 2006). Lopez et al. (2006) observed more than half (~54% of total fat content) of the milk fat present in Emmental cheese is in crystallized form at 4°C. Solid milk fat in cheese is known to act as reinforcing fillers, contributing to elastic properties of unheated cheese (Rogers et al., 2010; Shima & Tanimoto, 2016). Moreover, it has been suggested that the contact area between the casein increases with decreasing temperature as they expand at low temperature, probably due to weakening of hydrophobic interactions (Lucey et al., 2003). Thus, the firmer texture of cheese at low temperature (< 20°C) is considered due to the combined effect of higher proportion of solid fat and increased contact area between casein. The firm texture of cheese at low temperature is generally suitable for size reduction operation since it is easier to cut cheese cleanly than at higher or ambient temperature (Lucey, 2008).

During heating of cheese, the proportion of liquid fat increase dramatically; at ~ 40°C, almost all fat in cheese is in liquid state (Lopez et al., 2006). Liquid fat acts as a plasticizer between casein strands, making cheese softer and flexible (Shima et al., 2016). Although fat has an important role in the initial softening of cheese during heating, it is now well accepted that the casein interactions have the major role on the melt properties of cheese. Lucey et al. (2003) proposed a mechanism for melting of cheese during heating based on the dual-binding model

proposed by Horne (1998). The authors speculated that the localized balance of the attractive and repulsive forces between casein controls the cheese melting and the behavior of cheese at elevated temperature. During heating, casein networks in cheese are thought to contract, probably due to strengthening of hydrophobic interactions. Magnetic resonance studies have also indicated that the contraction of the protein network in cheese with increasing temperature, since levels of free water in cheese increased as it was heated from 20 to 60°C (Vogt et al., 2015; Smith et al., 2017). Contraction of the casein network is suggested to reduce the size of the contact area between casein, leading to weakening of the cheese matrix (Lucey et al., 2003). Weakening of the cheese matrix during heating is indicated by the changes in the rheological parameters, i.e., decreases in dynamic moduli (G' and G″) and an increase of loss tangent with increasing temperature. More recently, mid-infrared and synchronous fluorescence spectroscopies, coupled with chemometrics, have been suggested as valuable tools for understanding the role of temperature on the melt behavior of cheese (Boubellouta & Dufour, 2012).

Kim, Lim, and Gunasekaran (2011) investigated the impact of baking temperature (180°C for 25 min) on the properties of reduced-fat and full-fat Cheddar cheeses by reacting heated cheeses with different dissociating agents, such as, SDS, EDTA, and urea; the results indicated that the skin formation in reduced-fat cheeses was the result of a high degree of protein-protein interactions, which involved disulfide bonds and hydrophobic interactions and, to some extent ionic bonds with calcium.

1.8 Conclusions

This review summarizes the current understanding on structure-function relationships in cheese. A fundamental knowledge of how the structure of cheese influences various functionalities is necessary to design cheeses with enhanced physico-chemical properties, and of optimal sensory and nutritional quality. Such knowledge is particularly important for the improvement of the quality of cheeses, such as, those with low fat content, since fat reduction is often associated with undesirable changes in texture, flavor and cooking properties. Similarly, reduction in sodium has also been linked particularly with flavor defects. Cheese structures have been shown to play an important role in texture perception and in release of flavor compounds during mastication. Structuring of the cheese matrix for controlled release of nutrients, and delivery of bioactives and probiotics is an area of key importance in the development of functional cheese with beneficial properties beyond basic nutrition. A detailed knowledge of molecular interactions and forces that act between cheese components is vital as they can influence the functionalities of cheese, such as physico-chemical properties of heated and unheated cheese, and also the release patterns of flavor compounds or nutrients in mouth during mastication or in the gut during digestion. This also creates a need to further develop analytical methods for determination of molecular forces or interactions within the cheese matrix. Similarly, advanced microscopy techniques allied with image analysis, mathematical modeling, and computer simulations will also help to establish a greater knowledge of the link between structure and functionality of cheese. In addition, the growing number of studies using model food systems, such as, emulsion-filled gels, offers potential and such approaches need to be applied to research and innovation in natural cheeses. Overall, an appropriate knowledge of

structure-function relationship is key to the design of future cheese types with specific functionalities.

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Chapter 2: Effect of milk centrifugation and incorporation of high heat-treated centrifugate on the composition, texture and ripening characteristics of Maasdam cheese

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2.1 Abstract

This study investigated the impact of centrifugation (9,000 × g), as well as the incorporation of high heat-treated (HHT) centrifugate into cheese milk on the composition, texture and ripening characteristics of Maasdam cheese. Neither centrifugation nor incorporation of HHT centrifugate into cheese milk had a pronounced impact on the compositional parameters of any experimental cheeses, except for moisture and moisture in non-fat substance (MNFS) levels. Incorporation of HHT centrifugate at a rate of 6 to 10% of the total milk weight back into centrifuged milk increased the level of denatured whey protein in the cheese milk and also increased the level of MNFS in the resultant cheese compared to cheeses made from centrifuged milk and control cheeses; moreover, the former cheese had ~3% higher moisture content on average than the latter cheeses. Centrifugation of cheese-milk reduced the somatic cell count by ~95% relative to the somatic cell count in raw milk. Neither centrifugation nor incorporation of HHT centrifugate into cheese milk had a significant impact on age-related changes in pH, lactate content and levels of primary and secondary proteolysis. However, the value for hardness was significantly lower for cheeses made from milk containing HHT centrifugate than for other experimental cheese types. Overall, centrifugation appeared to have little impact on composition, texture and ripening characteristics of Maasdam cheese. However, care should be taken when incorporating HHT centrifugate into cheese milk, because such practices can influence the level of moisture, MNFS and texture (particularly hardness) of resultant cheeses. Such differences may have the potential to influence subsequent eye development characteristic, although no definitive trends were observed in the present study and further research on this is recommended.

2.2 Introduction

Various milk pre-treatment methods have been applied prior to cheese-making to enhance quality, consistency and functionality of different cheese varieties (Kelly, Huppertz, & Sheehan, 2008; Johnson, 2017). Centrifugation of milk using a special centrifuge (also called Bactofuge^{TM}) at a centrifugal force of ~9,000 × g is a pre-treatment methods widely used by the cheese industry for removal of *Clostridium* spores prior to cheese-making. After centrifugation, milk is divided into two streams, i.e., (i) centrifuged milk containing low bacterial cells and spores count, which account for ~97% of the feed volume and (ii) centrifugate containing high bacterial cells and spores count, which account for ~3% of the feed volume (Kosikowski & Mistry, 1990).

Some cheese producers apply high heat treatment to the centrifugate to inactivate bacterial cells and spores and recycle the stream back into centrifuged milk prior to cheese-making, to minimize protein losses as it contains ~7% protein (Kosikowski et al., 1990). High heat treatment of milk results in denaturation of whey proteins (Rynne, Beresford, Kelly, & Guinee, 2004), which can form complexes with whey proteins (in the serum phase) and casein micelles (Donato & Guyomarc'h, 2009). Such complexes are believed to hinder the aggregation of destabilized casein micelles during rennet-induced coagulation of milk (Vasbinder, Rollema, & de Kruif, 2003) and thus reduce the ability of the gels to synerese, leading to cheese curd with higher levels of moisture and moisture in non-fat substance (MNFS). Moisture in the cheese matrix acts as a plasticizer between the protein strands and softens the cheese texture (Lamichhane, Kelly, & Sheehan, 2017). Moreover, the higher moisture and MNFS content within the cheese matrix can enhance the microbial and enzymatic activities (Beresford, Fitzsimons, Brennan,

& Cogan, 2001), which can alter the ripening characteristics of cheese (Rynne et al., 2004; Rynne, Beresford, Kelly, & Guinee, 2007).

Some *Clostridium* spp. have been reported to be associated with late blowing defect (LBD) of cheese, which is manifest as production of gas (e.g., CO_2 and H_2) and formation of high levels of butyric acid, resulting in down-graded cheeses (Klijn, Nieuwenhof, Hoolwerf, van der Waals, & Weerkamp, 1995; Le Bourhis et al., 2007; Garde, Arias, Gaya, & Nuñez, 2011). Although the impact of centrifugation on efficacy of removal of *Clostridium* spores from milk and LBD of cheese have been a research focus for several studies (Langeveld, 1971; Su & Ingham, 2000), its impact on composition, texture and ripening characteristics of cheese has to date received little attention. As well as removal of *Clostridium* spores from milk, centrifugation also removes milk bacterial cells and somatic cells from milk, by ~87% and 75-95% of the total count, respectively (Te Giffel & Van Der Horst, 2004; Wieking, 2004).

Maasdam is a brine-salted, large-eye forming semi-hard cheese combining the traits of both Swiss and Dutch-type cheeses. Both lactic and citric acid fermentation occur during the first 24 h of manufacture and propionic acid fermentation occurs during warm-room ripening. Very little research has been published on the physicochemical properties and ripening characteristics of Maasdam and similar cheese-types, such as Jarlsberg.

The aim of this study was to evaluate the effect of (i) centrifugation and (ii) the incorporation of the high heat-treated (HHT) centrifugate into cheese-milk on the composition, pH, primary and secondary proteolysis, lactic acids levels and texture of Maasdam cheese during ripening. In this study, centrifugation refers to the

separation of bacteria and spores at a centrifugal force of ~9,000 × g whereas centrifugal separation refers to separation of milk into cream and skim milk.

2.3 Materials and methods

2.3.1 Milk supply and treatments

Raw whole milk was obtained from a local dairy company. From raw milk, three different cheese milk streams were prepared (Figure 2.1). Part of the raw milk was separated at 55°C (centrifuge disc separator, Westfalia) to give skim milk and cream. Control cheese milk (CT) was prepared by adding a portion of the resultant cream to skim to achieve a protein: fat ratio of 1.13: 1. The remaining whole milk was centrifuged (Bactofuge Disc Separator, Alfa Laval, type: D3187M) at a centrifugal force of ~9,000 × g to provide centrifuged whole milk and 'centrifugate' (also called 'sludge' or 'bactofugate'), which accounts for approximately 3-6% of the total milk feed. Centrifuged whole milk was then separated to give skim and cream. Centrifuged cheese milk (CF) was prepared by adding portions of the cream into the skim milk to achieve a protein: fat ratio of 1.13: 1. High heat treatment (120°C for 26 s, APV plate heat exchanger) was applied to centrifugate to inactivate spores and bacteria, and this centrifugate was combined with a portion of centrifuged cream and skim milk to produce the third cheese milk, i.e., centrifuged milk containing HHT centrifugate (CFHHT). As the protein content of centrifugate after high heat treatment varied between 3.76 and 6.36 (%, wt/wt) between trials, HHT centrifugate was added to centrifuged milk on a protein basis rather than weight basis, i.e., approximately 12% (wt/wt) of the total protein was from HHT centrifugate in CFHHT milk (on weight basis, HHT centrifugate was added at a level of 6.6 to 10.3%, wt/wt, depending on the protein content of HHT centrifugate). All

cheese milk types (CT, CF and CFHHT) were standardized to a protein to fat ratio of ~1.13: 1, and pasteurized (72°C for 15 s) before cheese manufacture.

2.3.2 Cheese manufacture

Three experimental Maasdam cheese types, i.e., cheese made from control milk (CT cheese), centrifuged milk (CF cheese) and centrifuged milk containing HHT centrifugate (CFHHT cheese), were each manufactured on three different occasions in replicate cheese-making trials over a 3 month period. Standardized and pasteurized cheese milks were pumped into cylindrical, jacketed cheese vats. Each vat contained automated variable speed cutting and stirring equipment (APV Schweig AG, Worb, Switzerland). All cheese milks (380 kg/vat) were inoculated at 31°C with frozen direct vat inoculate cultures (Chr. Hansen Ltd., Cork, Ireland): (1) mixed strains of mesophilic bacteria (C950, 18 mg/kg milk), consisting of *Lactococcus lactis* ssp. *cremoris, Lactococcus lactis* ssp. *lactis,* and *Leuconostoc*; (2) *Lactobacillus helveticus* (LH-B01, 4.8 mg/kg milk); and (3) propionic acid bacteria (PAB) (PS-60, 7 mg/kg milk). Calcium chloride (34%, wt/vol) was added at a level of 0.3 mL/kg milk to each vat. Rennet (Chy-Max Plus, ~200 IMCU/mL; Chr. Hansen Ltd., Cork, Ireland), diluted ~1:10 with deionized water, was added at a level of 0.2 mL/kg milk after a 40-min ripening period at 31°C.

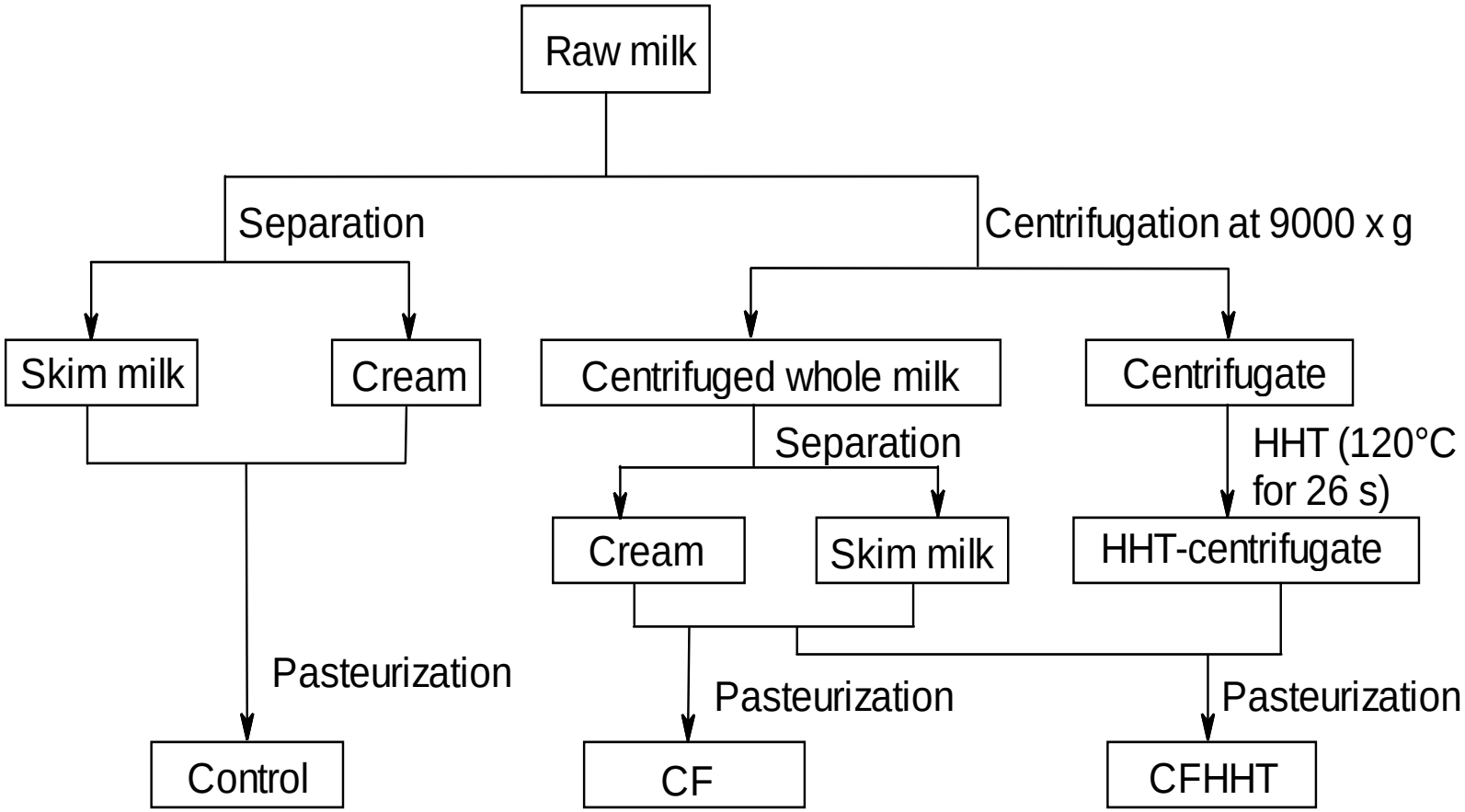


Figure 2.1. Flow charts of the preparation of cheese milks, i.e., control, centrifuged (CF) and centrifuged milk containing high heat-treated centrifugate (CFHHT). Abbreviations: HHT, high heat treatment, HHT-centrifugate, high heat-treated centrifugate

All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments, New Castle, DE, USA) and the resultant curd particle/whey mixture was allowed to heal for 7 min before being stirred continuously for another 7 min. Stirring was then stopped and a portion of whey (34 kg/100 kg cheese milk) was removed. After whey removal, reverse osmosis (RO) water at ~50°C (23 kg/100 kg cheese milk) was used to cook the curd to 37°C at a rate of 0.2°C/min with continuous stirring. After the curd washing and cooking steps, whey and curd were drained into a pre-press vat and curds were vertically pre-pressed under warm whey for 25 min, with increasing pressure from 3 to 5 kPa. Whey was then drained from the pre-press vat and subsequently the pre-pressed curd was cut into 10 kg wheels (3 wheels from each vat), placed into 10-kg molds and pressed vertically under increasing pressure from 3.3 to 14 kPa for ~3.5 hours. When the pH of the cheese curds reached around 5.49 to 5.51, cheese wheels were transferred to a saturated brine solution (23% , wt/wt, NaCl, 0.56% $CaCl_2$, pH 5.2 and 18°C) for 24 hours. After brining, cheese wheels were vacuum-packed in CO_2 permeable bags, and transferred to the ripening room. The cheeses were ripened at 8°C for 10 d (pre-ripening), at 23°C for 30 d (warm-room ripening), and finally stored at 4°C for 140 d.

2.3.3 Rennet coagulation properties

In all three replicate cheese-making trials, 2 min after rennet addition and stirring, a representative sample of milk was removed from the vat and placed into a cell of a small amplitude oscillatory rheometer (AR 2000ex, TA Instruments). A concentric-cylinder measuring geometry, consisting of a cylindrical bob and cup, was used. The dynamic changes in rheology during the coagulation process were

monitored using a dynamic time sweep analysis with an angular frequency of 1.0 Hz, and a strain of 0.01 at 31°C, within the linear viscoelastic region (strain < 0.03) reported for rennet milk gels (Mateo et al., 2010). Total time to reach a G′ value of gels of 35 Pa (at which cutting of the gel in the cheese vat was initiated) after rennet addition was calculated.

2.3.4 Milk and cheese composition analysis

The composition of raw milk, centrifugate and pasteurized cheese milks were analyzed using a Fourier Transform Infrared spectrophotometer (MilkoScan FT 120, Foss Electric, Hillerød, Denmark). Raw milk and centrifuged milk samples were analyzed for somatic cell count (SCC, cells/mL) with a fluoro-opto-electronic counter (Fossomatic FC, Foss, Hillerød, Denmark). Casein number, non-protein nitrogen, and levels of whey protein denaturation as percentage of total whey protein of HHT centrifugate and pasteurized cheese milk samples of one representative trial were determined as described by Rynne et al. (2004). Grated cheese samples were analyzed at 11 d at least in duplicate for moisture, fat, protein, salt, calcium and pH, as described by Sheehan, Fenelon, Wilkinson, and McSweeney (2007a).

For determination of lactose and galactose content, cheese samples were extracted as described by Zeppa, Conterno, and Gerbi (2001). Grated cheese samples (10 g) were mixed with 50 mL 0.013 *N* H_2SO_4 and stomached for 10 min using a stomacher (Iul Instruments, Barcelona, Spain) before centrifugation at 7,000 × g for 5 min. The supernatant was then filtered using a 0.2-µm nylon filter. The extracted samples were then analyzed by HPLC (Waters Alliance 2695 separation module, Milford, MA) with an Aminex HPX-87C Carbohydrate column 300 × 7.8 mm (Biorad, Hertfordshire, UK) under the following working conditions: injection

volume, 50 µL; mobile phase, 0.009 *N* H_2SO_4; flow rate, 0.5 mL/min; column temperature, 60°C; refractive index detection (Waters 2414 RI detector, Milford, MA). Quantification of lactose and galactose was based on the external standard method as described by Hou, McSweeney, Beresford, and Guinee (2014).

2.3.5 SDS-PAGE analysis

The individual proteins in the raw milk, centrifugate before and after HHT, and cheese milks were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All milk samples were diluted, using Milli-Q water, to a protein concentration of ~6 µg/µL. A portion of the diluted samples were further diluted with SDS sample buffer [NuPAGE LDS Sample Buffer (4X), comprised of lithium salt, glycerine, sulphuric acid and monododecyl ester]. For reducing SDS-PAGE, samples were treated with dithiothreitol [NuPAGE Sample Reducing Agent (10X), Carlsbad, CA; concentration, 500 mmol/L] at a level of 10% (vol/vol) of the total sample volume mixture. All samples were heated at 70°C for 10 min, cooled, and loaded on SDS-PAGE gels (NuPAGE 12% Bis-Tris mini gels, Carlsbad, CA) at a rate of 10 µg per well before running in SDS running buffer [NuPAGE MOPS SDS Running Buffer (1X), Carlsbad, CA] at constant voltage of 200 V for 50 min using Mini Gel Tank (XCell SureLock Mini, Thermo Scientific, Dublin, Ireland). After electrophoresis, the gels were stained as described by McCarthy et al. (2012). SDS-PAGE gels were scanned using an Epson V700 film scanner (Epson, Suwa, Nagano, Japan). The identities of principal protein bands in the milk samples were determined using prestained protein molecular weight marker (PageRuler Prestained Protein Ladder, 10 to 180 kDa, Thermo Scientific, Dublin, Ireland).

2.3.6 pH and, L- and D-lactate analysis

The pH of cheese samples were measured on cheese slurry prepared by mixing 20 g of grated cheese and 12 g of de-ionised water at different time points throughout ripening (Sheehan et al., 2007a).

The sample extraction method as outlined for lactose and galactose content was used for D- and L-lactic acid content determination. The extracted samples were analyzed for D- and L-lactic acid content using HPLC (Waters Alliance 2695 separation module, Milford, MA) equipped with chiral column [Chirex 3126 (D)-penicillamine, column 150 x 4.6 mm, Phenomenex, Cheshire, UK] as described by Hou et al. (2014).

2.3.7 pH 4.6 soluble nitrogen (% of total nitrogen) and free amino acids

The levels of pH 4.6 soluble-nitrogen and free amino acids (**FAA**) of the cheeses were measured after 1, 11, 41, 65, 97, 140 and 180 d as described by Fenelon and Guinee (2000) and Sheehan et al. (2007a), respectively.

2.3.8 Texture profile analysis of cheese

Texture properties were analyzed by TAHDi texture profile analyzer (TPA; Stable Micro Systems, Goldalming, Surrey, England), equipped with a 70 mm (diameter) compression plate and a 100 kg load cell. Cheese was cut into six cube-shaped samples (25 mm^3), using a Cheese Blocker (Bos Kaasgreedschap, Bodengraven, the Netherlands), wrapped in tin foil and stored overnight at 4°C. Cheese samples (~4°C) were compressed to 40% of its original height in two consecutive bites at a rate of 60 mm/min (Henneberry, Wilkinson, Kilcawley, Kelly,

& Guinee, 2015). TPA parameters were calculated as previously described by Chevanan, Muthukumarappan, Upreti, and Metzger (2006).

2.3.9 Statistical analysis

Three experimental cheese types were each manufactured on three different occasions in replicate cheese-making trials. Analysis of variance, using IBM SPSS software version 24 (IBM Corp., 2016), was applied to determine the effect of treatment on milk and cheese composition. A split-plot design was used to determine the effects of treatments (centrifugation or addition of HHT centrifugate into cheese milk), ripening time and their interactions on pH, lactic acid to protein ratio, lactic acid, proteolysis, and texture. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple-comparison test was used for paired comparison of treatment means at a 5% level of significance. IBM SPSS software version 24 (IBM Corp., 2016) was used to perform Pearson correlation between lactate to protein ratio and pH.

2.4 Results and discussion

2.4.1 Raw milk, centrifugate and cheese-milk composition

The average fat, protein, and lactose contents of raw milk used for the three replicate cheese making trials were 4.01(%, wt/wt), 3.41(%, wt/wt), and 4.73 (%, wt/wt), respectively. Somatic cell counts (SCC) of the raw milk between trials ranged between 1.2×10^5 and 2.4×10^5 cells/mL. The SCC of milk depends on factors such as breed and parity, stage of lactation, udder health, and also individual and environmental factors, as well as management practices (Li et al., 2017; Panthi,

Jordan, Kelly, & Sheehan, 2017). In general, the SCC of milk from healthy cows is less than 2×10^5 (Li, Richoux, Boutinaud, Martin, & Gagnaire, 2014). The efficacy of centrifugation process (9,000 × g) for removal of SCC was ~95% relative to the SCC in raw milk, in close agreement with the study of Wieking (2004), who reported the efficacy of centrifugation as ~95%. It is generally accepted that the SCC of cheese milk negatively influences the cheese making and final cheese quality (Panthi et al., 2017). However, in contrast, a recent study (Li et al., 2017) suggested that the somatic cells *per se* have minimal impact on the cheese making and final cheese quality. This suggests that more research is needed to better understand the role of somatic cells in cheese quality.

The composition of centrifugate before and after HHT and cheese milks is shown in Table 2.1. The level of whey protein denaturation (WPD, as percentage of total whey protein) in centrifugate after HHT was 68.30%. It is widely recognized that HHT denatures whey proteins; for example, Rynne et al. (2004) observed that 34% of total whey protein was denatured when milk was heated at 87°C for 26 s. The protein and lactose content of centrifugate decreased, although not significantly, after HHT. This was probably due to slight dilution with process flush water when utilizing a low volume of centrifugate (~40-45 kg) in pilot-scale processing.

Although fat, protein, lactose contents of cheese milks were not statistically different, the level of WPD was ~2.5-3 fold higher in CFHHT milk (14.2%) than in CT (5.8%) and CF (4.8%) milks. The low level of WPD in CT and CF milk was attributed to pasteurization (72°C for 15 s) of milk, whereas the level of WPD in CFHHT milk was attributed to both pasteurization and incorporation of HHT

centrifugate. The level of WPD in pasteurized milk was in close agreement with that reported by Rynne et al. (2004).

Table 2.1. Composition of centrifugate and pasteurized cheese milks[1]

Compositional parameters	Centrifugate[2]		Cheese milks[3]		
	Before HHT	After HHT	CT	CF	CFHHT
Protein (%, wt/wt)	6.10[A]	5.11[A]	3.38[a]	3.29[a]	3.32[a]
Fat (%, wt/wt)	0.23[A]	0.22[A]	2.98[a]	2.90[a]	2.92[a]
Lactose (%, wt/wt)	4.52[A]	4.05[A]	4.74[a]	4.66[a]	4.64[a]
Protein/Fat	-	-	1.134[a]	1.135[a]	1.137[a]
Casein number	79.22	89.72	79.56	79.16	80.69
NPN (%, wt/wt)	5.91	5.57	6.33	6.57	6.47
Native whey protein (% total)	14.86	4.71	14.11	14.27	12.85
WPD (% total whey protein)	-	68.30	5.81	4.77	14.24

[1]Abbreviations: HHT, high heat treatment, CT, Control milk, CF, centrifuged milk; CFHHT, centrifuged milk containing high heat-treated centrifugate; NPN, non-protein nitrogen; WPD, whey protein denaturation.

[2, 3]Data presented are the mean of data from three replicate trials for protein, fat, lactose and protein/fat; for other parameters, data are from one representative trial. Values within a row not sharing common superscripts differ ($P < 0.05$) in the case of protein, fat, lactose and protein/fat. For parameters without superscripts, statistical analysis was not carried out because the data are from only 1 representative trial.

2.4.2 SDS-PAGE analysis

The individual proteins in raw milk, centrifugate before and after HHT, and cheese milks were analyzed using SDS-PAGE (Figure 2.2). The SDS-PAGE patterns of centrifugate before HHT were different compared to centrifugate after HHT, with a lower intensity of the bands corresponding to α-lactalbumin, β-lactoglobulin and other minor proteins, such as, lactoferrin and bovine serum albumin, in HHT centrifugate. Moreover, some large protein aggregates were observed at the top of the stacking gel in the HHT centrifugate, as denoted by X in Figure 2.2A. Similar to our result, Patel, Singh, Anema, and Creamer (2006) also observed heat-induced large aggregates in HHT milk samples. Heat-denatured whey proteins can form complexes with themselves and with caseins, particularly κ-casein, through disulfide interchange reactions and hydrophobic forces (Jean, Renan, Famelart, & Guyomarc'h, 2006; Patel et al., 2006; Kethireddipalli & Hill, 2015). In reducing SDS-PAGE, the large heat-induced aggregates in HHT centrifugate disappeared when the samples were reduced with dithiothreitol; moreover, the SDS-PAGE patterns in reduced gels appeared similar (Figure 2.2B). This suggests that the protein aggregates in HHT centrifugate may be bonded by various molecular forces, such as, hydrophobic forces and disulphide bonding (Donato et al., 2009).

The SDS-PAGE patterns of raw milk and cheese milks appeared similar, although the level of denatured whey protein was higher in cheese milks, especially CFHHT, than in raw milk. This suggests that the SDS-PAGE analysis is not very sensitive for differentiating between low levels of whey protein denaturation.

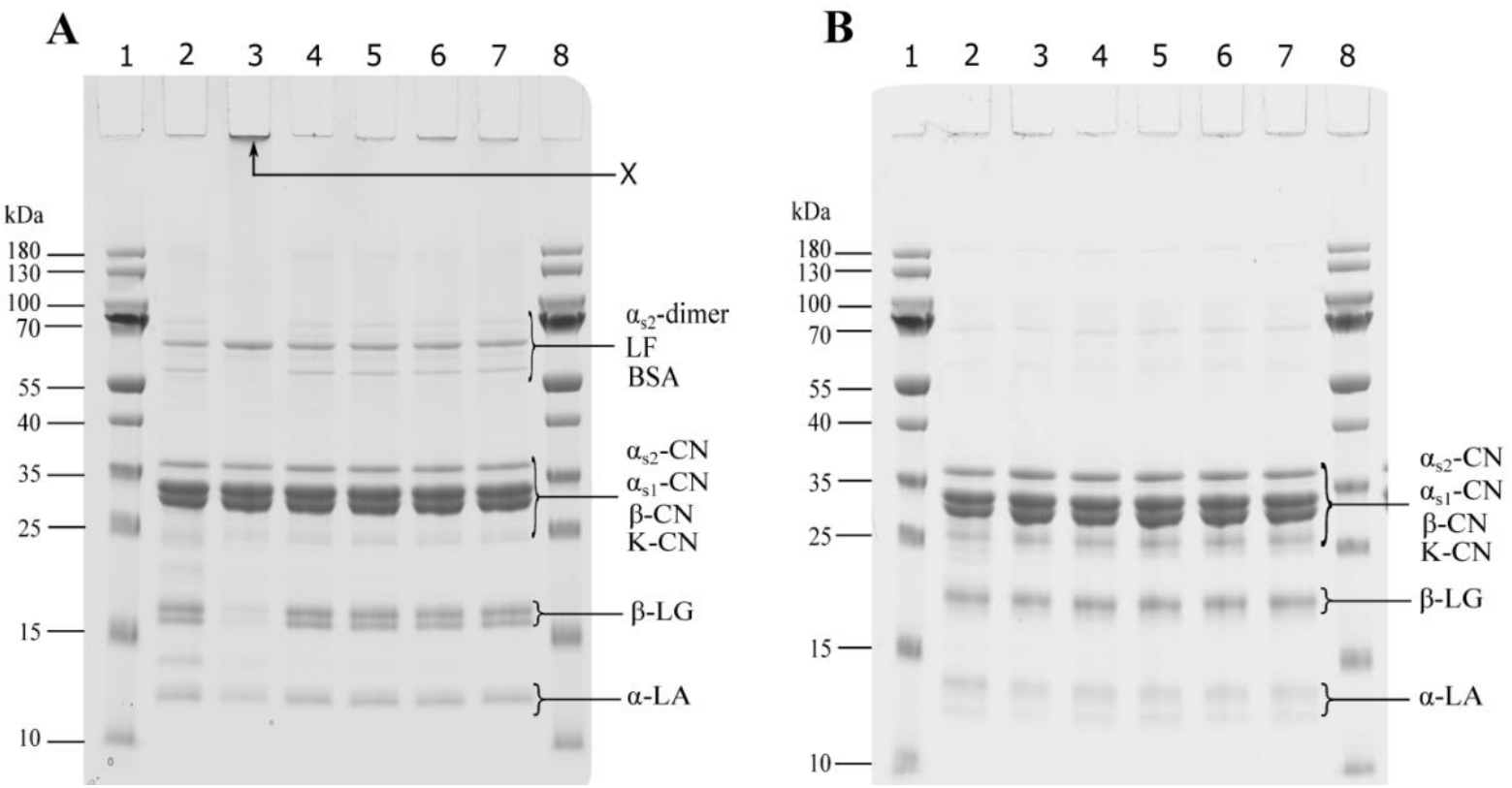


Figure 2.2. SDS-PAGE patterns of milk samples under (A) non-reducing and (B) reducing conditions. Lane 1 and 8 = prestained protein molecular weight marker, lane 2 = centrifugate before high heat treatment, lane 3 = centrifugate after high heat treatment, lane 4 = raw milk, lane 5 = pasteurized (72°C for 15 s) control cheese-milk, lane 6 = pasteurized centrifuged milk containing high heat-treated (HHT) centrifugate, and lane 7 = pasteurized centrifuged milk. Protein aggregates in HHT centrifugate are denoted by X. Abbreviations: LF, lactoferrin; BSA, bovine serum albumin; CN, casein; β-LG, β-lactoglobulin; α-LA, α-lactalbumin. Samples shown are from one representative trial.

2.4.3 Rennet coagulation characteristics

The average time to reach an elastic shear modulus (G′) value of 35 Pa for CFHHT CT and CF milks after rennet addition was 45.1, 38.7 and 40 min, respectively. Several factors can influence the rennet-induced coagulation of milk, such as, milk composition and renneting conditions (e.g., pH, temperature and ionic strength) (Guinee, O'Kennedy, & Kelly, 2006; Ong, Dagastine, Auty, Kentish, & Gras, 2011; Ong, Dagastine, Kentish, & Gras, 2012). Some studies have reported that the heat-induced complexes formed between whey protein and κ-CN on the surface of the casein micelles inhibit the primary phase of rennet coagulation, i.e., decrease the accessibility of enzyme to κ-CN (Van Hooydonk, De Koster, & Boerrigter, 1987). However, more recent studies have suggested that whey protein denaturation has minimal impact on the primary phase of rennet coagulation; instead, whey protein denaturation has more clear effect on the secondary phase of rennet coagulation, i.e., aggregation (fusion) of destabilized casein micelles (Vasbinder et al., 2003). The casein-whey protein and whey protein aggregates (in serum phase) can sterically hinder the aggregation of destabilized casein micelles (Waungana, Singh, & Bennett, 1996; Vasbinder et al., 2003). In the current study, although the level of denatured whey protein of CFHHT was ~2.5-3 fold higher than that of CT and CF milks, the rennet coagulation time of CFHHT milk did not differ statistically significantly to the others. The levels of WPD (as percent of total whey protein) for all experimental cheese milk were below 15%, and its seems that such levels of WPD had no pronounced impact on the rennet-induced coagulation of milk. More pronounced effects were reported previously for severely heat-treated milk (Rynne et al., 2004).

2.4.4 Cheese composition

The composition of the experimental cheeses is shown in Table 2.2. Centrifugation of milk had no significant effect on mean levels of moisture, MNFS, protein, fat, salt, total calcium, lactose and galactose of final cheeses. However, incorporation of HHT centifugate into the centrifuged milk increased ($P < 0.05$) the mean levels of MNFS of the resultant cheeses. The average moisture content of CFHHT cheese was ~3% higher than control and CF cheeses, which is expected and sizeable in magnitude for cheese moisture; however, the difference was not statistically significant ($P = 0.057$). This is explained by a degree of variation in the compositional data between trials, which influenced the statistical analysis of the data. The coefficients of variation of average moisture content of the experimental cheeses between three trials were below 5%, which is considered acceptable (Thomsson, Ström-Holst, Sjunnesson, & Bergqvist, 2014).

Higher moisture and MNFS levels in CFHHT cheeses are partly attributed to the negative effect of HHT centrifugate on syneresis (expulsion of whey) of rennet-induced milk gels. Denatured whey protein present in HHT centrifugate can sterically hinder the aggregation (fusion) of destabilized casein micelles as described before, and thus hinders syneresis (Pearse, Linklater, Hall, & Mackinlay, 1985; Walstra, Van Dijk, & Geurts, 1985; Vasbinder et al., 2003). Moreover, the high water-binding capacity of the denatured whey proteins may increase the level of MNFS in the CFHHT cheeses (Donato et al., 2009). These results are in agreement with the results of Guinee et al. (1998) and Rynne et al. (2004), who observed increased moisture and MNFS of Cheddar cheese with increasing levels of denatured whey protein.

Table 2.2. Compositional parameters and pH at 11 d of ripening in Maasdam cheeses[1]

Compositional factors	Cheese types[2]			SEM	*P*- value
	CT	CF	CFHHT		
Moisture (%, wt/wt)	44.83[a]	44.15[a]	47.83[a]	0.72	0.057
MNFS (%, wt/wt)	58.9[a]	58.14[a]	61.78[b]	0.63	0.013
Protein (%, wt/wt)	24.04[a]	24.57[a]	23.44[a]	0.30	0.348
Fat (%, wt/wt)	23.9[a]	24.07[a]	22.61[a]	0.44	0.376
FDM (%, wt/wt)	43.31[a]	43.07[a]	43.29[a]	0.35	0.966
Salt (%, wt/wt)	1.53[a]	1.50[a]	1.73[a]	0.05	0.183
S/M (%, wt/wt)	3.41[a]	3.40[a]	3.61[a]	0.07	0.450
Total calcium (mg/100 g)	821[a]	800[a]	837[a]	12.19	0.514
pH (11 d)	5.28[a]	5.31[a]	5.27[a]	0.01	0.548
Lactose (mg/100 g)					
1d	54.39[a]	41.82[a]	66.72[a]	16.26	0.861
11 d	20.42[a]	43.72[a]	62.63[a]	15.07	0.585
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	-
Galactose (mg/100 g)					
1 d	28.09[a]	29.57[a]	35.34[a]	4.11	0.798
11 d	27.61[a]	27.26[a]	30.82[a]	4.22	0.947
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	-

[1]Abbreviations: CT, control cheese; CF, cheese made from centrifuged milk; CFHHT, cheese made from centrifuged milk containing high heat-treated centrifugate; MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio.

[2]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean of data from three replicate trials.

Some studies also observed a decrease in the level of total calcium in cheese made from HHT milk (Guinee et al., 1998). However, such results were not observed in the current study. The mean levels of lactose and galactose were very low, with below ~67 and ~36 mg/100 g cheese respectively until 11 d of ripening, and lactose and galactose were not detected after warm-room ripening (Table 2.2). Low lactose and galactose contents within this cheese-type are expected, as the lactose contents of cheese curd were reduced by curd-washing.

2.4.5 Age-related changes in pH

The pH of all experimental cheeses increased ($P < 0.001$) over the 180 d of ripening (Figure 2.3A, Table 2.3), from a mean value of ~5.2 at 1 d to ~5.7 at 180 d. This trend is in agreement with that reported in a previous study of Gouda cheese by Lawrence, Creamer, and Gilles (1987), who also observed increase in pH from ~5.15 at 1 d to ~5.5-5.9 at 150 d of ripening. The increase in the pH is attributed to a number of factors, including the proteolytic liberation of basic compounds, such as ammonia, free basic AA and amines (Fenelon et al., 2000; McSweeney, 2004). A reduction in lactate to protein ratio during maturation of cheese (Figure 2.3B) is known to increase the buffering capacity of cheese (Sheehan et al., 2007a), which may contribute to some extent to the increase in cheese pH during ripening. The pH of the cheese curd was significantly ($P < 0.001$) negatively correlated (adjusted $R^2 = 0.53$) with lactate to protein ratio (Figure 2.3C).

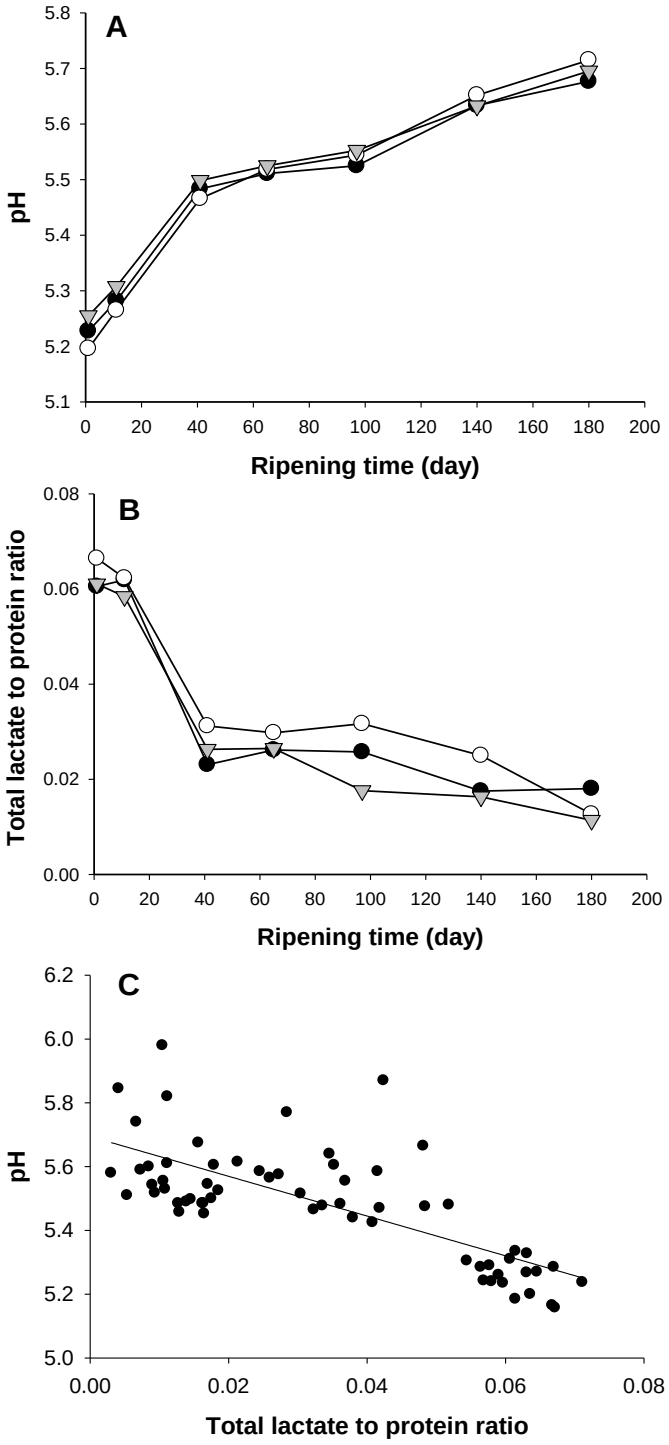


Figure 2.3. The effect of milk pre-treatments on (A) pH and (B) lactate to protein ratio of Maasdam cheese during maturation. Milk pre-treatments: control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data presented are means of data from three replicate trials. (C) Relationship between pH and lactate to protein ratio; data were obtained from all experimental cheeses produced in three replicate trials and analyzed over a 180 d of ripening.

In some brine-salted cheese types, such as Gouda, Edam and Maasdam, the pH after brining is controlled by adjusting the residual curd lactose content by techniques such as curd washing or whey dilution (Lawrence et al., 1987). More recently, membrane separation techniques, such as ultrafiltration, have also been utilized for standardization of lactose content of cheese-milk prior to cheese-making (Moynihan et al., 2016). In the present study, ~34% (wt/wt) of whey was replaced with ~23% (wt/wt) warm reverse-osmosis (RO) water to control the post-manufacture reduction of pH. In contrast, O'Sullivan, McSweeney, Cotter, Giblin, and Sheehan (2016) observed a decrease in pH at the early stages of ripening in Swiss-type cheese (where whey was not replaced by warm water); however, the post-brining pH was higher (~5.6) than that of the cheese in the current study (~5.3). The decrease in the pH at the early stages of ripening has been attributed to continual metabolism of residual lactose and galactose to lactate by starter and non-starter lactic acid bacteria (NSLAB) (O'Sullivan et al., 2016). Shakeel-Ur-Rehman, Waldron, and Fox (2004) also observed a decrease in the pH during ripening when Cheddar cheeses was made from milk supplemented with lactose. Regulation of pH is critical for proper eye development in some eye-forming cheese types, and a reduction in pH can reduce the levels of colloidal calcium, which are considered essential for elastic texture of cheese (Lucey & Fox, 1993), and can also inhibit the growth of PAB (Sheehan, Wilkinson, & McSweeney, 2008). Elastic texture is important in the case of eye-forming cheese types to accommodate gas produced during warm-room ripening for smooth eye formation (Daly, McSweeney, & Sheehan, 2010). No significant effect of treatment on the mean cheese pH during maturation was observed (Table 2.3).

Table 2.3. Summary of the effects of treatment, ripening time and their interactions on properties of Maasdam cheeses[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
Total lactate to protein ratio	NS	***	NS
L-lactate	NS	***	NS
D-lactate	NS	***	NS
Total lactate	NS	***	NS
pH 4.6-SN (% TN)	NS	***	NS
Total FAA	NS	***	NS
Hardness	*	NS	NS
Springiness	NS	*	NS
Cohesiveness	NS	***	NS
Resilience	NS	**	NS

[1]Abbreviations: pH 4.6-SN (% TN), soluble nitrogen at pH 4.6 as percentage of total nitrogen; FAA, free AA

*P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05

2.4.6 Levels of L-, D- and total lactate

The mean level of L-lactate of all experimental cheeses was ~1.5 (%, wt/wt) until 11 d of ripening, which is most likely due to fermentation of glucose, lactose and galactose by starter lactic acid bacteria, including *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus helveticus*, during production of cheese (Beresford et al., 2001). However, the level of L-lactate decreased ($P < 0.001$) over ripening in all experimental cheeses especially during warm room ripening from a mean of ~1.5 (%, wt/wt) at 11 d to ~0.4 (%, wt/wt) at 41 d (Figure 2.4A). This trend is in agreement with that reported in previous studies for different cheese varieties, such as Swiss-style cheese (Sheehan et al., 2008; O'Sullivan et al., 2016), Grevé (eye-forming semi-hard cheese types) (Rehn et al., 2011) and Cheddar (Rynne et al., 2007). This is expected as L-lactate is metabolized by starter and non-starter bacteria to different metabolites, such as, propionate, acetate, butyrate, formate, succinate, DL-lactate, CO_2, H_2 and H_2O (McSweeney, 2004; Agarwal, Sharma, Swanson, Yüksel, & Clark, 2006). D-lactate was virtually absent for all experimental cheeses at 1 and 11 d of ripening.

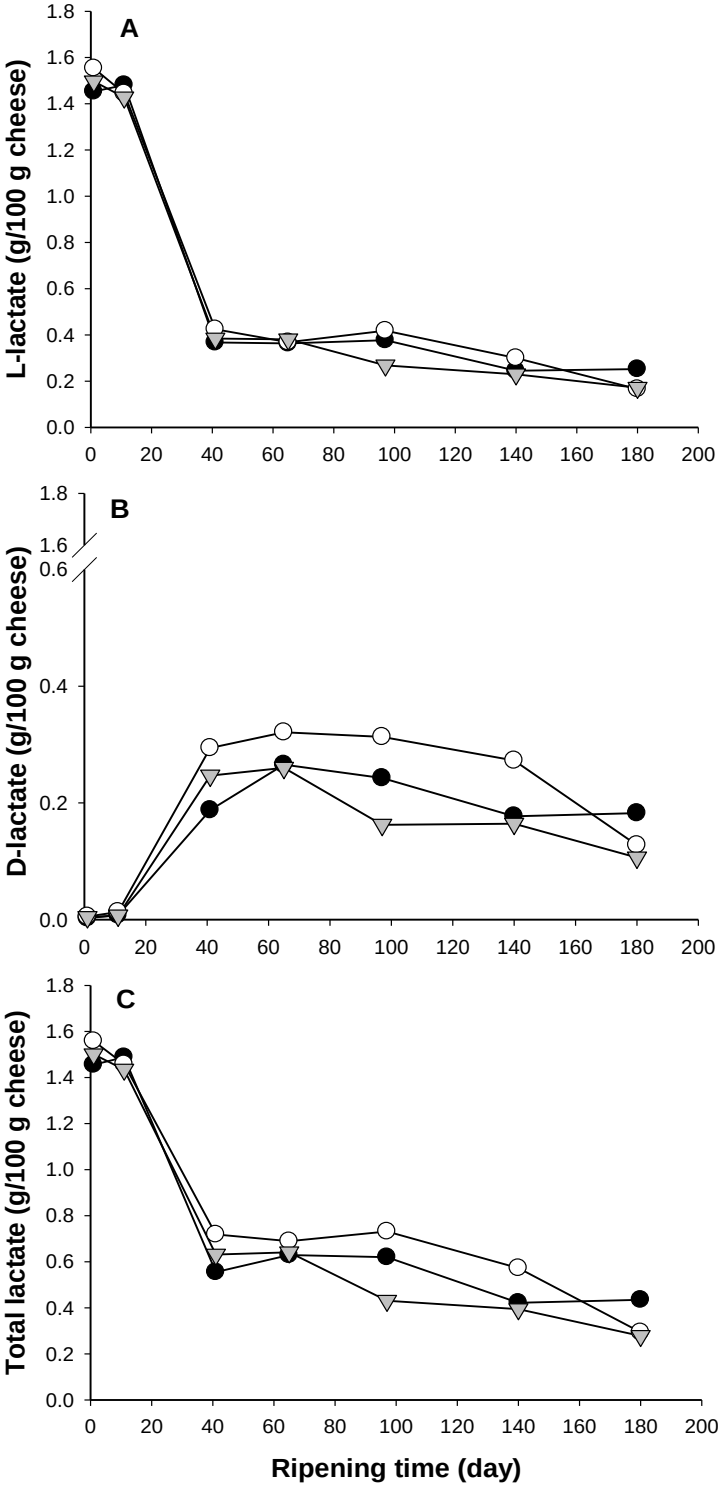


Figure 2.4. The effect of milk pre-treatments on the mean level of (A) L-lactate, (B) D-lactate, (C) total lactate of Maasdam cheeses during ripening. Milk pre-treatments: control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data presented are means of data from three replicate trials.

However, unlike L-lactate, the level of D-lactate increased ($P < 0.001$) during warm room ripening, from a mean of ~0.01 (%, wt/wt) at 11 d to ~0.2-0.3 at 41d (Figure 2.4B). D-lactate within cheese matrix typically arises either from fermentation of glucose, lactose or galactose by microorganisms, including *Lactobacillus helveticus* and some *Leuconostoc* spp. (Beresford et al., 2001), or by racemization of L-lactate by NSLAB (Agarwal et al., 2006). Since the levels of residual lactose and galactose were very low in all experimental cheeses before warm room ripening (below 70 and 40 mg/100 g, respectively), due to curd washing, it may be assumed that the contribution of residual lactose and galactose for formation of D-lactate is minimal. The formation of D-lactate was most likely due to racemization of L-lactate to DL-lactate by NSLAB because the level of NSLAB reached ~10^8 cfu/g in all cheeses at 41 d of ripening (data not shown).

Similar trends have previously been reported in different cheese varieties, such as half-fat Cheddar (Rynne et al., 2007) and Swiss-type cheese (O'Sullivan et al., 2016). However, interestingly, the level of D-lactate decreased gradually after 65 d of ripening, probably due to its metabolism by microorganisms within the cheese matrix such as PAB (O'Sullivan et al., 2016). The degradation pathways of D-lactate in the cheese matrix are not yet fully understood. No significant effect of treatment was observed in the levels of L-lactate, D-lactate and total lactate (Figure 2.4C) throughout ripening (Table 2.3).

2.4.7 Proteolysis

2.4.7.1 Soluble nitrogen at pH 4.6 (% of total nitrogen)

Primary proteolysis in cheeses was assessed by measuring the level of nitrogen soluble at pH 4.6, as a percentage of total nitrogen, which increased ($P <$

0.001) in all cheeses over the 180 d of ripening, especially during warm-room ripening, from ~5% of total nitrogen before warm room ripening (11 d) to ~17% of total nitrogen at the end of warm room ripening (41 d) in all experimental cheeses (Figure 2.5A). The levels in all cheeses reached ~22% at 180 d. The increase was of the same order of magnitude as that previously reported for semi-hard cheeses (Exterkate & Alting, 1995; Sheehan, Oliveira, Kelly, & McSweeney, 2007b; Huc, Challois, Monziols, Michon, & Mariette, 2014). However, no significant effect of treatment on level of nitrogen soluble at pH 4.6, as a percentage of total nitrogen was observed (Table 2.3).

2.4.7.2 Total and individual free amino acids

The mean levels of total FAA increased ($P < 0.001$) during ripening, especially when the cheeses entered the hot-room ripening phase, from ~3000 mg/kg at 11 d to ~7000-8000 mg/kg at 41 d (Figure 2.5B). Enzymes from starter and non-starter microorganisms and somatic cells, such as, proteases and peptidases, where present within the cheese matrix contribute to primary and secondary proteolysis, and thereby to liberation of FAA during ripening (McSweeney, 2004; Kelly, O'Flaherty, & Fox, 2006). No significant effect of treatment was observed (Table 2.3), although it was expected that the centrifugation process can alter secondary proteolysis, as the process can remove ~86-92% of total bacteria and ~95% of somatic cells from cheese milk (Te Giffel et al., 2004; Wieking, 2004). In the current study, the centrifugation process also reduced the SCC by ~95%.

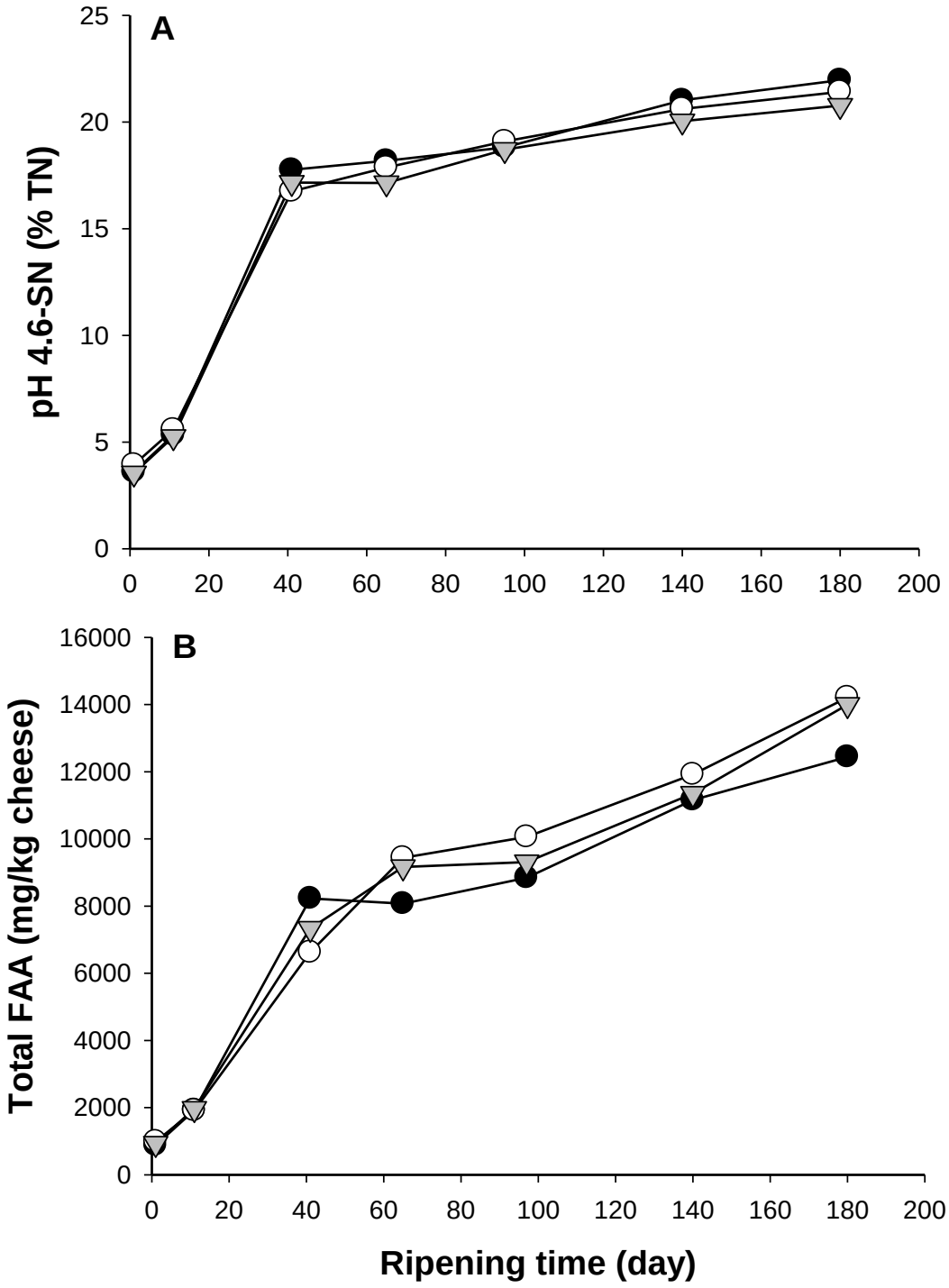


Figure 2.5. The effect of milk pre-treatments on mean level of (A) pH 4.6-soluble nitrogen of percentage of total nitrogen (pH 4.6-SN, % TN), and (B) total free amino acids (FAA) of Maasdam cheeses during ripening. Milk pre-treatments: control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data presented are means of data from three replicate trials.

The concentrations of individual FAA (mg/kg) in cheeses at 140 d of ripening are shown in Figure 2.6. Leucine was the most abundant FAA found in all experimental cheeses, with ~2300 mg/kg at 140 d, followed by Glu, Phe, Val, Lys, Pro and Thr. Similar to this result, O'Sullivan et al. (2016) also observed a high level of Glu, Leu, Val, Lys, and Pro in Swiss-type cheese at 95 d of ripening. In contrast, the concentrations of Asp, Ser, Gly, Cys, Tyr, and Arg were amongst the lowest of the FAA. Free AA are important precursors for the formation of different classes of volatiles, such as, amines, aldehydes, alcohols, acids and sulphur compounds (Engels, Dekker, de Jong, Neeter, & Visser, 1997; Yvon & Rijnen, 2001). No significant effect of treatment on the mean levels of individual FAA at 140 d of ripening was observed.

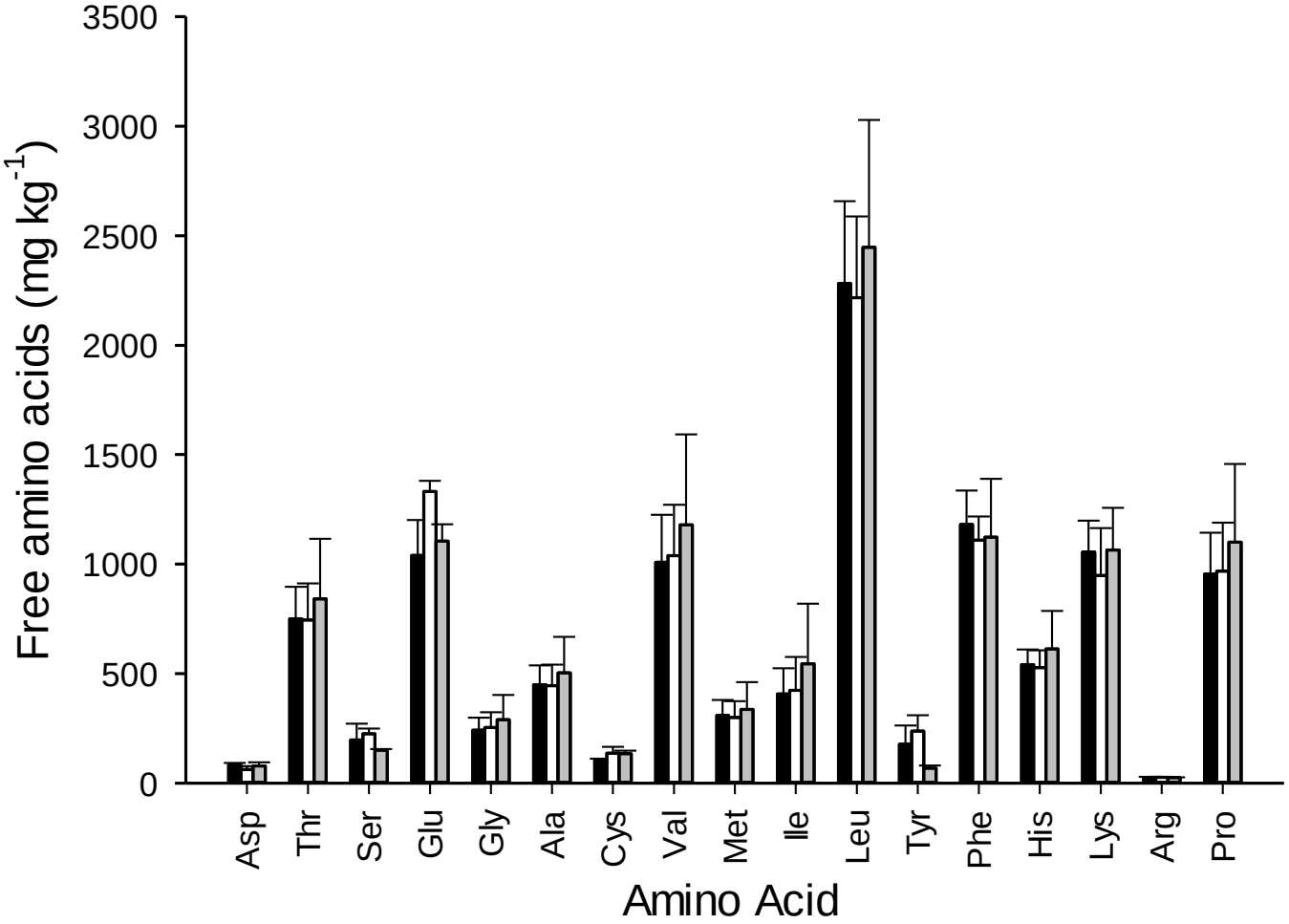


Figure 2.6. The effect of milk pre-treatments on the mean levels of individual free amino acids in pH 4.6-soluble nitrogen extracts from Maasdam cheeses at 140 d of ripening. Milk pre-treatments: control (■), centrifugation (□); and centrifuged milk containing high heat-treated centrifugate (■). Data presented are means of data from three replicate trials. Error bars show the standard error of mean from three replicate trials.

2.4.8 Texture profile analysis

The incorporation of HHT centrifugate into cheese-milk decreased ($P < 0.05$) the mean level of instrumentally measured hardness of the resultant cheeses compared to CT and CF cheeses (Figure 2.7). This was attributed to significantly higher MNFS level in the CFHHT cheeses than CT and CF cheeses; MNFS is considered a good indicator of moisture associated with proteins (Lawrence, Gilles, & Creamer, 1993). Moisture in the cheese matrix acts as a plasticizer between the protein strands, making cheese softer and more flexible. Moreover, during coagulation, the whey protein and whey protein-casein micelle aggregates may hinder the close approach of casein micelles during aggregation (fusion) of destabilized casein micelles; this may result in a weaker gel and curd texture (Waungana et al., 1996). From a materials science perspective, the strength of a material is known to be influenced by factors such as the extent of cross-linking, and the orientation or the structural regularity of the constituents of the material (Pastorino, Hansen, & McMahon, 2003; Lamichhane et al., 2017). It may be assumed that denatured whey protein can alter the extent of cross-linking of casein micelles, and the orientation or the structural regularity of casein networks within the cheese matrix.

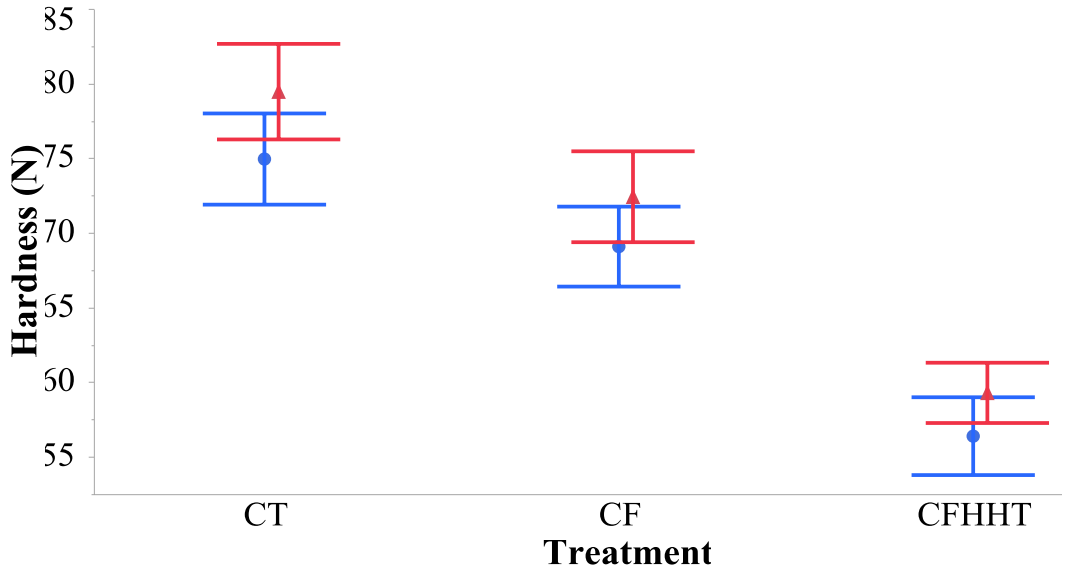


Figure 2.7. The effect of milk pre-treatments on mean levels of hardness between 1 d (●) and 11 d (▲) of ripening. Experimental cheese types: CT = control cheese, CF = cheese made from centrifuged milk, CFHHT = cheese made from centrifuged milk containing high heat-treated centrifugate. Error bars show the standard error of mean from two replicate trials.

No significant effects of treatment on mean levels of cohesiveness, resilience and springiness in the cheeses were observed (Table 2.3); however, the values for these texture parameters decreased ($P < 0.05$) between 1 and 11 d of ripening (data not shown). Although the exact reasons for this are unknown, this may be attributed to solubilization of colloidal calcium during the early stages of ripening (O'Mahony, Lucey, & McSweeney, 2005). O'Mahony et al. (2005) also observed rapid decrease in the value for springiness and cohesiveness of Cheddar cheese between 1 and 21 d of ripening. Levels of insoluble calcium were not determined in cheeses in the present study, and the authors suggest that this should be a focus for future studies in Maasdam-type cheese. There was no significant difference in the mean level of hardness of cheese between 1 and 11 d of ripening, contrary to the results obtained by O'Mahony et al. (2005), who observed rapid decrease in the texture value within first 21 d of ripening of Cheddar cheese; this discrepancy may be attributed to different cheese types and different manufacturing steps. We were unable to analyze the texture profile of cheese after 11 d of ripening due to eye-formation.

2.5 Conclusions

We demonstrated the impact of centrifugation and incorporation of HHT centrifugate on the composition, texture and ripening characteristics of Maasdam cheese. Interestingly, centifugation of cheese-milk prior to cheese-making appeared to have minimal impact on composition and age-related changes on texture, pH, proteolysis and lactate levels of Maasdam cheese. However, incorporation of HHT centrifugate into cheese milk at levels of approximately 6 to 10 % (wt/wt), depending on the protein content of centrifugate, into cheese milk significantly increased MNFS levels and also significantly decreased cheese hardness compared

to control cheeses and cheeses made from centrifuged milk. Composition and strength of curd are considered important for eye-development characteristics of cheese without slits and cracks. In the current study, no clear trend for eye characteristics was observed between the treatments, and thus we are unable to draw a conclusion regarding the impact of the treatments applied on eye quality of cheese. The authors proposed that this should be the focus of further research, possibly requiring analysis of a large number of commercial samples over the course of a manufacture season.

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Chapter 3: Effect of milk centrifugation and incorporation of high heat-treated centrifugate on the microbial composition and levels of volatile organic compounds of Maasdam cheese

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3.1 Abstract

Centrifugation is a common milk pre-treatment method for removal of *Clostridium* spores which, on germination, can produce high levels of butyric acid and gas, resulting in rancid, gassy cheese. The aim of this study was to determine the effect of centrifugation of milk, as well as incorporation of high heat-treated (HHT) centrifugate into cheese milk, on the microbial and volatile profile of Maasdam cheese. To facilitate this, 16S rRNA amplicon sequencing in combination with selective media-based approach were used to study the microbial composition of cheese during maturation, and volatile organic compounds within the cheese matrix were analyzed by HPLC and solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS). Both culture-based and molecular approaches revealed major differences in microbial populations within the cheese matrix between pre- and post-warm room ripening. During warm room ripening, an increase in counts of propionic acid bacteria (by ~$10^{1.5}$ cfu) and non-starter lactic acid bacteria (by ~10^{8} cfu) and a decrease in the counts of *Lactobacillus helveticus* (by ~$10^{2.5}$) were observed. *Lactococcus* species dominated the curd population throughout ripening, followed by *Lactobacillus, Propionibacterium* and *Leuconostoc,* and the relative abundance of these accounted for more than 99% of the total genera, as revealed by high-throughput sequencing. Among sub-dominant microflora, the overall relative abundance of *Clostridium sensu stricto* was lower in cheeses made from centrifuged milk than control cheeses, which coincided with lower levels of butyric acid. Centrifugation as well as incorporation of HHT centrifugate into cheese-milk seemed to have little impact on the volatile profile of Maasdam cheese, except for butyric acid levels. Overall, this study suggests that centrifugation of milk prior to cheese-making is a suitable method for controlling

undesirable butyric acid fermentation without significantly altering the levels of other volatile organic compounds of Maasdam cheese.

3.2 Introduction

Centrifugation at ~9,000 × g is a milk pre-treatment method for removal of *Clostridium* spores. Some *Clostridium* spp., on germination, can produce gas and a high level of butyric acid via butyric acid fermentation, resulting in down-graded cheeses (Su & Ingham, 2000; Le Bourhis et al., 2007). As well as removal of bacterial spores, centrifugation removes bacterial cells present in milk (Te Giffel & Van Der Horst, 2004). Some of these milk microorganisms can survive pasteurization and can grow during ripening of cheese (Grappin & Beuvier, 1997; Jordan & Cogan, 1999; Quigley et al., 2013; Sheehan, 2013). Therefore, it may be assumed that the reduction in microbial load in cheese-milk by centrifugation may influence the microbial composition of cheese during maturation, as the environment would be less competitive, thus further favoring the growth of the most abundant bacteria. The microbial composition within the cheese matrix is known to play an important role in determining biochemical and ripening characteristics, including flavor development through production of enzymes and metabolites, of different varieties of cheese (Beuvier et al., 1997; Beresford, Fitzsimons, Brennan, & Cogan, 2001; Montel et al., 2014; Guarrasi et al., 2017).

Although traditional culture-based approaches are effective for quantifying common starter or non-starter bacteria, these approaches are not sensitive to those microorganisms that are difficult to culture or are present as subdominant populations or both (Quigley et al., 2013; O'Sullivan et al., 2015). Moreover, recent studies based on culture-independent approaches have suggested that some bacterial cells in a highly stressed condition are viable but not culturable (Quigley et al., 2013;

Ruggirello, Dolci, & Cocolin, 2014; Hickey, Fallico, Wilkinson, & Sheehan, 2018). Alternatively, molecular approaches, including high-throughput sequencing, can provide a detailed insight into the composition of both dominant and sub-dominant microflora. More recently, 16S rRNA amplicon sequencing is being increasingly used in the study of microbial composition within fermented food products, including cheese (Quigley et al., 2012; O'Sullivan et al., 2015; Alessandria et al., 2016). For the first time, this study profiled the microbiota of cheese made from centrifuged milk, as well as cheese made from centrifuged milk containing high heat-treated (HHT) centrifugate compared to control cheeses, using high-throughput sequencing.

Maasdam is a washed-curd, brine-salted, large eye forming, semi-hard cheese, which was developed by combining the cultures and technologies of Emmental and Gouda cheese. Apart from thermophilic lactobacilli, mesophilic mixed-strain cultures comprising *Lactococcus* and *Leuconostoc* are used as starters (as in Gouda cheese) and propionic acid bacteria (PAB) are used as secondary starters (as in Emmental cheese). To date, very little has been published regarding the microbial and volatile profile of Maasdam cheese; a better understanding of which will aid manufacturers to consistently achieve the desirable cheese aroma profile (Johnson & Lucey, 2006).

The objective of this study was to investigate the impact of (i) centrifugation and (ii) the incorporation of the HHT centrifugate into cheese-milk on microbial composition and levels of volatile organic compounds (VOC) of Maasdam cheese during maturation. In this study, centrifugation refers to the separation of bacteria and spores at a centrifugal force of ~9,000 × g [in some studies this is also referred

as bactofugation; Te Giffel et al. (2004)], whereas centrifugal separation refers to separation of milk into cream and skim milk. A parallel study was conducted investigating the effect of milk centrifugation and incorporation of HHT centrifugation on the composition, texture and ripening characteristics of Maasdam cheese (Lamichhane et al., 2018).

3.3 Materials and methods

3.3.1 Cheese manufacture

Cheese milks were prepared as described by Lamichhane et al. (2018) and in Supplementary Figure 3.1. In summary, raw milk from a local dairy company was divided into two portions. One portion of the raw milk was separated into skim milk and cream using a cream separator. Control milk (CT) was prepared by adding a portion of cream and skim milk obtained from cream separator to achieve a protein to fat ratio of 1.13: 1. Another portion of the raw milk was centrifuged at 9,000 × g resulting in centrifuged whole milk and centrifugate. Centrifuged whole milk was separated into cream and skim milk while high heat treatment (120°C for 26 s) was applied to centrifugate. A second cheese milk type (i.e., centrifuged milk, CF) was prepared by adding a portion of cream and skim milk obtained from separation of centrifuged whole milk, whereas a third cheese milk type (CFHHT) was prepared by mixing a portion of cream and skim milk obtained from separation of centrifuged whole milk and HHT centrifugate (at a level of 6 to 10%, wt/wt, depending on the protein content of centrifugate). The protein to fat ratio of all cheese milks were standardized to 1.13: 1. All cheese milks were pasteurized prior to Maasdam cheese manufacture. Maasdam cheeses were manufactured as per Lamichhane et al. (2018). Three experimental Maasdam cheese types, i.e., cheese made from control milk (CT

cheese), centrifuged milk (CF cheese) and centrifuged milk containing HHT centrifugate (CFHHT cheese), were each manufactured on three different occasions in replicate cheese-making trials over a 3 month period as per Lamichhane et al. (2018). Starters and secondary starters (frozen direct vat inoculate, Chr. Hansen Ltd., Cork, Ireland) used for the manufacture of Maasdam cheese were: (1) mesophilic mixed-strain (C950, 18 mg/kg milk), consisting of *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Leuconostoc*; (2) *Lactobacillus helveticus* (LH-B01, 4.8 mg/kg milk); and (3) PAB (PS-60, 7.0 mg/kg milk).

3.3.2 Enumeration of starter lactic acid bacteria, propionic acid bacteria and non-starter lactic acid bacteria

Samples were aseptically removed from cheese wheels using a cheese trier, at 1, 11, 41, 65, 97, 140 and 180 d of ripening. The cheese samples (10 g) were placed in a sterile stomacher bag (Grade, Leicestershire, UK), diluted (10-fold) with 2% (wt/vol) trisodium citrate buffer (VWR, Dublin, Ireland) and stomached for 10 min using a stomacher (Iul Instruments, Barcelona, Spain). Serial dilutions of 10-fold diluted cheese samples were made using maximum recovery diluent, containing low levels of peptone (1 g/L) and sodium chloride (8.5 g/L). Total numbers of non-starter lactic acid bacteria (NSLAB) cells were enumerated on *Lactobacillus* selection agar (BD, Oxford, UK), with an overlay, after aerobic incubation for 5 d at 30°C. Viable cells of PAB were enumerated on sodium lactate agar, supplemented with kanamycin sulphate (Sigma-Aldrich, Arklow, Ireland) at a level of 4 mg/100 mL sodium lactate agar, after anaerobic incubation for 7 d at 30°C, and only light brown colonies were counted as PAB (Rehn et al., 2011). *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa and Sharpe agar (BD, Oxford, UK) pH 5.4

after anaerobic incubation for 3 d at 42°C (Hickey, Auty, Wilkinson, & Sheehan, 2017). Anaerobic conditions were maintained through the use of anaerobic gas jars (Oxoid, Basingstoke, UK) and AnaeroGen system (Oxoid, Basingstoke, UK).

3.3.3 Study of microbial composition using high-throughput sequencing

3.3.3.1 Sampling and nucleic acid extraction

Aseptic samples were removed using a cheese trier, at 1, 11, 41, 65, 97 and 180 d of ripening. Cheese samples (5 g) were homogenized in 45 mL of Ringer's solution (¼ strength, Sigma-Aldrich, Arklow, Ireland) in a stomacher (BagMixer 400P, Interscience, Saint Nom, France). Enzymatic lysis of homogenized cheese samples was conducted prior to DNA extraction and included treatment with lysozyme (1 mg/mL, EC 3.2.1.17, Sigma-Aldrich, Arklow, Ireland) and proteinase K (5 mg/mL, EC 3.4.21.64, Sigma-Aldrich, Arklow, Ireland) followed by incubation at 37°C for 30 min, and 55°C for 15 min respectively. DNA was extracted using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, USA).

3.3.3.2 PCR amplification of the microbial 16s rRNA gene

Extracted DNA was amplified using primers targeting the V3 and V4 regions of the bacterial 16S rRNA gene. Illummina adapter overang nucleotide sequences were added to the primers. Therefore, the primer set used included the 16S Amplicon PCR Forward Primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and the 16S Amplicon PCR Reverse Primer (5'-GTCTCGTGGGCTCGGAGATGTGTAAGAGACAGGACTACHVGGGTATCTAATCC). Identification of individual sequences from the pooled samples was

achieved by incorporating dual indexing strategy where two unique pairs of 8 base indices were attached to each sample. Prepared samples were purified by using AMPure XP purification system (Beckman Coulter, Takeley, UK) prior to sequencing.

Amplicon PCR reactions contained 25 µL of 2x KAPA HiFi HotStart ReadyMix (Roche Diagnostics, West Sussex, England), 10 µL of each of the primers, and 5 µL of the DNA template. Therefore, the total volume of the reaction mix was 50 µL. PCR amplification was carried out using a 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA). The amplification parameters were as follows: initial denaturation at 95°C for 3 min followed by 30 cycles consisting of three 30 s steps including denaturation at 95°C, annealing at 55°C, and extension at 72°C. The process was completed by final elongation stage at 72°C for 2 min. Obtained amplicons were quantified by using Quan-It dsDNA High Sensitivity Assay Kit (Invitrogen, USA). Additionally, samples were normalized by dilution to equimolar concentrations prior to library preparation and sequencing.

3.3.3.3 High-throughput sequencing

16S rRNA amplicons from the V3 and V4 regions were sequenced on a MiSeq platform in the Teagasc sequencing facility, in accordance with standard Illumina sequencing protocols. Paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Denoising, chimera detection and clustering into operational taxonomic units were performed using USEARCH (Version 7.0-64 bit). Taxonomy was assigned using BLAST against the SILVA database release 123. Alpha diversity was calculated in

QIIME (v1.9.0) (http://qiime.org). Further data analysis was carried out using Phyloseq package in R (www.r-project.org).

3.3.4 Analysis of acetic, propionic and butyric acid

Acetic, propionic and butyric acids were recovered from cheese matrix by steam distillation and subsequently quantified by ligand exchange, ion-exclusion HPLC as described by Kilcawley, Wilkinson, and Fox (2001), with slight modifications. Briefly, 5 g of grated cheese samples, 10 mL of 10% (wt/vol) H_2SO_4 (Sigma-Aldrich, Arklow, Ireland), 1 mL of valeric acid (Sigma-Aldrich, Arklow, Ireland) of concentration 1 mg/mL, one drop of silicon antifoaming agent (Sigma-Aldrich, Arklow, Ireland), and 10 mL of distilled water were added to a distillation tube prior to distillation (2100 Kjeltec Distillation unit, Foss). The first 100 mL of distillate was collected into a flask, mixed gently and representative distillate samples were filtered using 0.2-µm nylon syringe filters (Agilent Technologies, Germany) into HPLC vials (Agilent Technologies, Germany). Recovery of short-chain carboxylic acids from cheese matrix by steam distillation was checked based on recovery of valeric acid, which was added as an internal standard.

The filtered samples were then analyzed for acetic, propionic and butyric acid content using HPLC (1260 Infinity, Agilent Technologies) equipped with Rezex RHM-Monosaccharide H+ (8%) column (Phenomenex, Cheshire, UK) under the following working conditions: sample injection volume, 40 µL; mobile phase, 0.01 *N* sulphuric acid (isocratic); flow rate, 0.7 mL/min; column temperature, 50°C; run time, 50 min; detection at 220 nm (UV detector). The quantification of analytes was based on the external standard method as described by Kilcawley et al. (2001). Results were expressed as mg/kg of cheese samples.

3.3.5 Analysis of volatiles

Volatiles in cheese at 140 d of ripening were determined using gas chromatography–mass spectrometry (GC-MS). Grated cheese samples (4 g) were placed in a 20 mL screw capped amber SPME vial (Apex Scientific, Maynooth, Ireland) and equilibrated to 40°C for 10 min with pulsed agitation of 5 s at 500 rpm. Sample introduction was accomplished using a Shimadzu AOC 5000 Autosampler. A single 50/30 µm CarboxenTM/divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS; Agilent Technologies) fiber was used. The SPME fiber was exposed to the headspace above the samples for 20 min at depth of 1 cm at 40°C. The fiber was retracted and injected into the GC inlet and desorbed for 2 min at 250°C. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60 m × 0.32 mm ×1.8 µm) column using a split/splitless injector with a 1/10 split. A merlin microseal was used as the septum. The temperature of the column oven was set at 40°C, held for 5 min, increased at 5°C/min to 230°C, then increased at 15°C/min to 260°C, yielding total GC run time of 50 min. The carrier gas was helium held at a constant flow of 1.2 mL/min. The detector was a Shimadzu TQ8030 mass spectrometer detector, run in single quadrupole. The ion source temperature was 220°C and the interface temperature was set at 260°C. The MS mode was electronic ionization (70 V) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, a commercial flavor and fragrance library (FFNSC 2, Shimadzu Corporation, Kyoto, Japan) and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound. Linear retention indices were calculated as per van Den Dool and Kratz (1963). Spectral deconvolution was also performed to confirm identification of

compounds using AMDIS. Batch processing of samples was carried out using metaMS (Wehrens, Weingart, & Mattivi, 2014). An auto-tune of the GC-MS was carried out prior to the analysis, to ensure optimal GC-MS performance. All analyses were performed in triplicate.

3.3.6 Statistical analysis

Three experimental cheese types (CT, CF and CFHHT) were each manufactured on three different occasions in replicate cheese-making trials. Analysis of variance (ANOVA), using IBM SPSS software version 24 (IBM Corp., 2016), was applied to determine the effect of treatment on formation of VOC. A split-plot design was used to determine the effect of treatment, ripening time and their interactions on *Lactobacillus helveticus*, PAB and NSLAB count and levels of short-chain carboxylic acids (acetate, propionate and butyrate). Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple-comparison test was used for paired comparison of treatment means at a 5% level of significance.

3.4 Results and discussion

3.4.1 Cheese composition

The compositional parameters of cheeses were described in detail by Lamichhane et al. (2018) and in Supplementary Table 3.1. Briefly, except for levels of moisture in non-fat substance, all other compositional parameters of experimental cheeses were not statistically different. Cheeses made from cheese milk containing HHT centrifugate had higher ($P < 0.05$) levels of moisture in non-fat substance than cheeses made from centrifuged milk or control cheeses. This was attributed to the

negative effect of HHT centrifugate (incorporated at levels of approximately 6 to 10 % of the total cheese milk weight, depending on the protein content of centrifugate) on syneresis of rennet-induced milk gels. The mean moisture content of CFHHT cheese was ~3% higher than that of CT and CF cheeses; however, the data did not differ statistically ($P = 0.057$).

3.4.2 Growth and viability of *Lactobacillus helveticus*, PAB, and NSLAB

Mean viable counts of *Lactobacillus helveticus* in all experimental cheeses decreased ($P < 0.001$) from ~10^7 cfu/g at 1 d and 11 d to ~$10^{4.5}$ cfu/g at 41 d (Figure 3.1A), indicating lysis during warm room ripening. Similar trends have previously been reported in Swiss-type cheese (White, Broadbent, Oberg, & McMahon, 2003; Sheehan, Wilkinson, & McSweeney, 2008; O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016). As the increased number of NSLAB would influence the accuracy of *Lactobacillus helveticus* counts (O'Sullivan et al., 2016), we did not enumerate *Lactobacillus helveticus* beyond 41 d. As expected, the mean viable count of PAB increased ($P < 0.001$) in all experimental cheeses during the warm room ripening, from ~$10^{6.5}$ cfu/g at 11 d to ~10^8 cfu/g at 41 d (Figure 3.1B). The increase in PAB count during the warm room ripening stage was consistent with previous results for Grevé (Rehn et al., 2011) and Swiss-type cheeses (O'Sullivan et al., 2016). The count decreased slightly thereafter to ~10^7 cfu/g during cold storage (4°C).

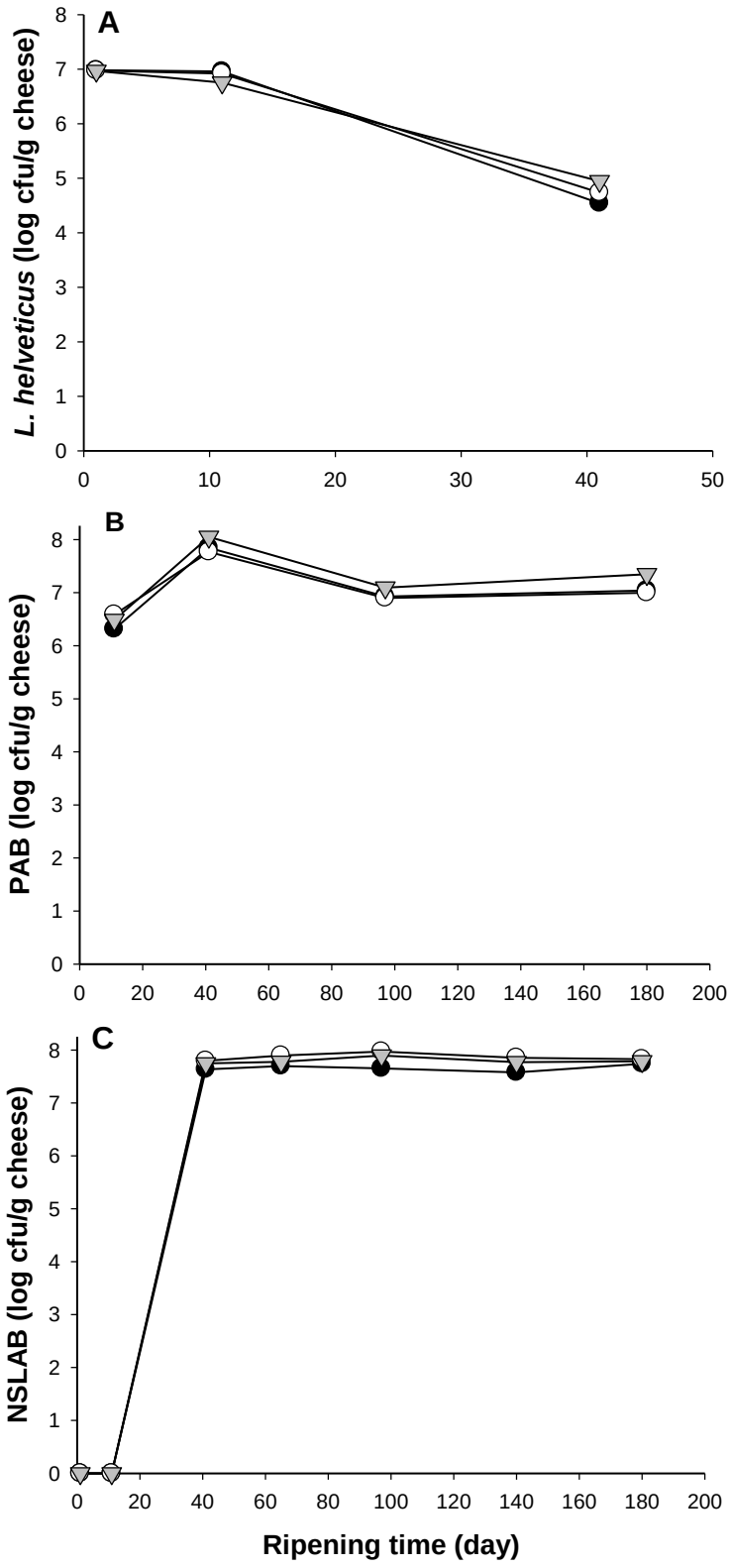


Figure 3.1. The effect of milk pre-treatments on average count of (A) *Lactobacillus helveticus*, (B) propionic acid bacteria (PAB), and (C) non-starter lactic acid bacteria (NSLAB) of Maasdam cheeses during ripening. Milk pre-treatments: control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data are means of data from three replicate trials.

Although counts of NSLAB were very low at 1 and 11 d of ripening, their levels in all cheeses increased to ~10^8 cfu/g at 41 d of ripening and leveled-off during further storage (Figure 3.1C). Sheehan et al. (2008) also observed a similar trend in Swiss-style cheeses. NSLAB in cheese can originate from milk, processing equipment or the processing environment, and counts are reported to be less than 10^2 cfu/g in young cheese made under good sanitary conditions with high quality milk (Steele, Budinich, Cai, Curtis, & Broadbent, 2006). Pasteurization of milk drastically reduces the number of milk microorganisms; however, some of these milk microorganisms can survive pasteurization and can grow during ripening of cheese (Grappin et al., 1997; Jordan et al., 1999; Johnson, 2001; Quigley et al., 2013). Contrary to our result, O'Sullivan et al. (2016) observed high levels of NSLAB (~10^6 cfu/g) at 1 d of ripening in Swiss-type cheese, and the authors speculated that they might have originated from the processing environment during cheese manufacture. The rapid increase in numbers of NSLAB during warm room ripening is attributed to elevated temperature (23°C), which accelerates the metabolic activities of microorganisms (Beresford et al., 2001; De Filippis, Genovese, Ferranti, Gilbert, & Ercolini, 2016), and availability of substrates, such as sugars, nucleic acids and lactate, from metabolism of starters and their cell lysate (Steele, Broadbent, & Kok, 2013; Ortakci, Broadbent, Oberg, & McMahon, 2015). NSLAB contribute to cheese maturation through production of enzymes and metabolites (Settanni & Moschetti, 2010). No significant effect of treatment was observed for mean counts of NSLAB, *L. helveticus*, and PAB during ripening (Table 3.1).

Table 3.1. Summary of the effects of treatment, ripening time and their interactions on microbiology and short-chain carboxylic acids profile of Maasdam cheeses[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
L. helveticus	NS	***	NS
PAB	NS	***	NS
NSLAB	NS	***	NS
Acetic acid	NS	***	NS
Propionic acid	NS	***	NS
Butyric acid	***	***	NS

[1]Abbreviations: PAB, Propionic acid bacteria; NSLAB, non-starter lactic acid bacteria

***$P < 0.001$, NS = $P > 0.05$

3.4.3 Microbial composition of Maasdam cheese

Post-DNA extraction, amplicons of the bacterial 16S rRNA gene were generated by PCR. These amplicons were then subjected to next-generation sequencing, generating an average of 253,870 good quality reads per sample. Alpha-diversity was calculated for each sample to analyze species richness and diversity within each sample. Chao 1 values, which represent species richness, ranged from 16 to 65 while the Shannon index ranged from 0.20 to 2.66. Analysis of these data revealed that the bacterial diversity fluctuated throughout ripening ; however, an overall increase in diversity was observed, in contrast to a similar study conducted by O'Sullivan et al. (2015) in Swiss-type cheese with thermophilic starters *Streptococcus thermophilus* and *Lactobacillus helveticus*.

Differences in microbial taxa and shifts in relative abundance of the population were revealed between CT, CF and CFHHT cheeses. Phylogenetic assignment of the sequences revealed presence of bacteria belonging to 7 phyla: *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, and *Saccharibacteria*. As expected, *Firmicutes* dominated across all samples, with relative abundance ranging between 78.72-99.96% in the CT cheeses, 67.83-99.86% in the CF cheeses and 76.63-99.89% in the CFHHT cheeses. The second most abundant phylum was *Actinobacteria*, followed by *Proteobacteria*. In addition, a rapid increase in abundance of the bacteria belonging to *Actinobacteria* phylum can be observed after 41d of ripening (post warm room stages) in all experimental cheese types.

Lactococcus, *Lactobacillus*, *Propionibacterium* and *Leuconostoc* were the dominant genera of Maasdam cheese throughout ripening, accounting for more than

99% relative abundance altogether (Figure 3.2). Before warm room ripening (i.e., until 11 d post-production), the relative abundance of major microorganisms (at genus level) were similar between treatments; *Lactococcus* spp. (ranged from 86.8 to 94.5%) dominated the curd population followed by *Lactobacillus* (4.8-12.4%). *Leuconostoc* (0.15-0.4%) and *Propionibacterium* (0.14-0.35%) were detected in very small proportions during this period. These results were expected as all these genera were added as starters or secondary starters at a similar proportion for all experimental cheeses.

In agreement with the results from the culture-based approach, the molecular approach also revealed major differences in microbial populations within the cheese matrix between pre- and post-warm room ripening. As expected, the relative abundance of *Propionibacterium* was higher after warm room ripening than before warm room ripening in all experimental cheeses. An increase in the level of PAB by ~$10^{1.5}$ cfu/g during warm room ripening was also observed in the culture-based method. Although the overall relative abundance of *Lactococcus* decreased after warm room ripening, it was still the most dominant genus in all experimental cheeses; *Lactobacillus* and *Propionibacterium* were the second most abundant genera followed by *Leuconostoc.* During maturation, in general, some lactic acid bacteria (LAB) within the starter cultures die off, and their metabolites (e.g., lactate) and carbon sources from cell lysate (e.g., ribose) favor the growth of secondary starter, such as PAB, and NSLAB (Ortakci et al., 2015). Moreover, the elevated temperature (23°C) during warm room ripening accelerates the metabolic activity of microorganisms (Beresford et al., 2001; De Filippis et al., 2016).

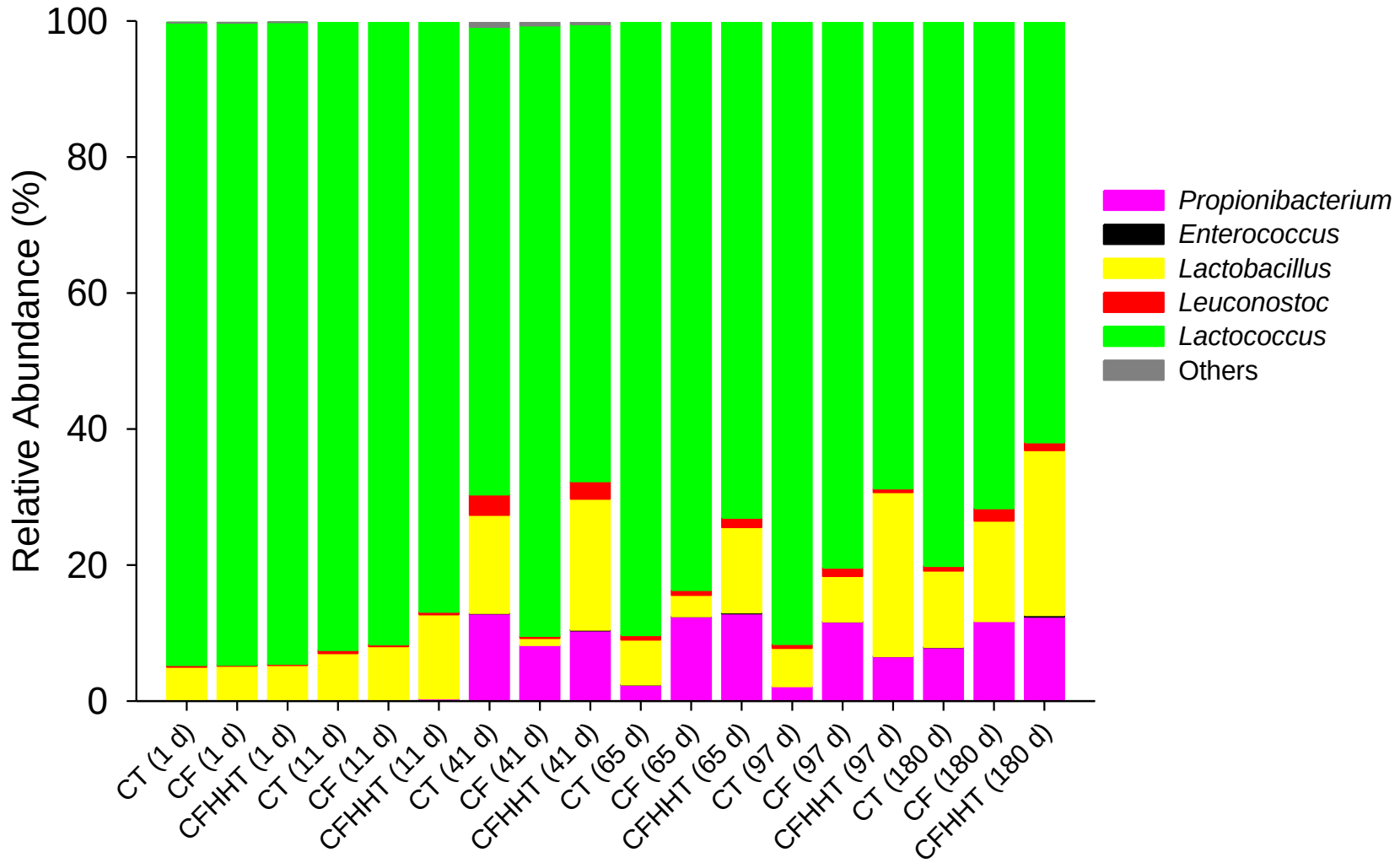


Figure 3.2. Relative abundance of bacteria at genus level within the three experimental cheese types during maturation, i.e., CT = control cheese, CF = cheese made from centrifuged milk, CFHHT = cheese made from centrifuged milk containing high heat-treated centrifugate. Data are means of data from three replicate trials.

Subtle differences were observed in the composition of dominant microflora (i.e., *Lactococcus*, *Lactobacillus*, *Propionibacterium* and *Leuconostoc*) between treatments; however, the differences were not consistent throughout ripening. This suggests that milk centrifugation as well as incorporation of HHT centrifugate into cheese milk had minimal impact in the composition of major genera of Maasdam cheese, which is consistent with the results from selective-media based approach.

Apart from major microflora, many (~40) other genera were also detected; however, their relative abundances were very low, altogether ranging from 0.04 to 0.95%. Among these subdominant genera, *Enterococcus*, *Stenotrophomonas*, *Paenibacillus*, *Pseudomonas*, and *Acinetobacter* were detected in relatively higher abundance. The presence of *Enterococcus* was detected at all-time points in CT and CF cheeses and its population increased rapidly from 41 d of ripening. This genus was not detected in CFHHT cheeses at 1 d of ripening; however, it was detected thereafter. Enterococci have been previously isolated from traditional cheeses produced with raw or pasteurized milk and are considered to originate from bulk tank, milking machine, processing equipment or the processing environment (Gelsomino, Vancanneyt, Cogan, Condon, & Swings, 2002; Nieto-Arribas et al., 2011). Although enterococci have shown a potential role in ripening and flavor development in some artisanal cheeses and cheeses made from raw milk (Beuvier et al., 1997; Beresford et al., 2001; Nieto-Arribas et al., 2011), the significance of the presence of this genus within Maasdam cheese matrix is not yet fully understood, and requires further investigation.

Pseudomonas, *Stenotrophomonas*, and *Acinetobacter* were present in all experimental cheese types but in different proportions. The highest abundance of *Pseudomonas* was observed in CT cheeses, particularly at 1, 11 and 97 d of ripening.

Pseudomonas spp. (psychrotrophic bacteria) have previously been isolated from cheese matrix (O'Sullivan et al., 2015), which can cause flavor and texture defects in cheese if they are present in high numbers (Champagne et al., 1994). *Stenotrophomonas* was present uniformly across the sample groups but not across time points. Although *Acinetobacter* has frequently been detected in several types of cheese, such as Camembert (Addis, Fleet, Cox, Kolak, & Leung, 2001) and Swiss-style (O'Sullivan et al., 2015), its role on ripening of cheese is not fully understood.

Clostridium sensu stricto is a subset of the species of *Clostridium* that form a distinct cluster in the 16S rRNA tree (cluster I) (Gupta & Gao, 2009). Nearly all species within this genus produce butyric acid as a major fermentation product (Wiegel, 2009). *Clostridium* spp. associated with late blowing defect (LBD) of cheese, including *Clostridium tyrobutyricum* and *Clostridium butyricum*, also fall within this group (Collins et al., 1994; Brändle, Domig, & Kneifel, 2016). Although the overall percentage relative abundance of *Clostridium sensu stricto* was very low (below 0.05%), the overall percentage relative abundance in CT cheeses (ranged between 0.00 and 0.02% throughout ripening) was relatively higher than those in CF (0.00 to 0.002%) and CFHHT (0.00 to 0.003 %) cheeses. This may be explained by the removal of *Clostridium* spores from milk by centrifugation. It is well known that the centrifugation can remove more than 97% of *Clostridium* spores from milk (Su et al., 2000; Te Giffel et al., 2004).

3.4.4 Levels of acetic, propionic and butyric acids

Short-chain carboxylic acids contribute to the aroma profile of most cheese varieties (Kilcawley et al., 2001). Starter, secondary starter and non-starter bacteria present in the cheese matrix can produce short-chain volatile carboxylic acids,

including propionic, acetic and butyric acids. Propionic acid is one of the major products of lactate metabolism by PAB, and hence it was detected in all experimental cheeses at high levels (Figure 3.3A), particularly during warm room ripening, with a mean level of ~50 mg/kg cheese at 11 d (at start of warm ripening) and ~4,000 mg/kg cheese at 41 d (at end of warm room ripening). Similar trends have been reported in other studies (Huc, Challois, Monziols, Michon, & Mariette, 2014; O'Sullivan et al., 2016). No significant effect of treatment was observed (Table 3.1). In Swiss-type cheese, propionic acid contributes to "sweet" or "nutty" notes characteristic of these varieties (Kilcawley et al., 2001).

The production of acetic acid followed a similar trend to that of propionic acid during ripening of cheese. Acetic acid in cheese can be formed from several pathways, including propionic acid fermentation by PAB, and metabolism of lactate and citrate by LAB (Sheehan et al., 2008; Huc et al., 2014). The mean level of acetic acid at 1 and 11 d of ripening was only ~200 mg/kg cheese. However, the level increased rapidly during warm room ripening to ~2,200 mg/kg cheese at 41 d (Figure 3.3B). The production of acetate in all experimental cheeses during warm room ripening was most likely due to activity of starter LAB, NSLAB and PAB. No significant effect of treatment was observed (Table 3.1).

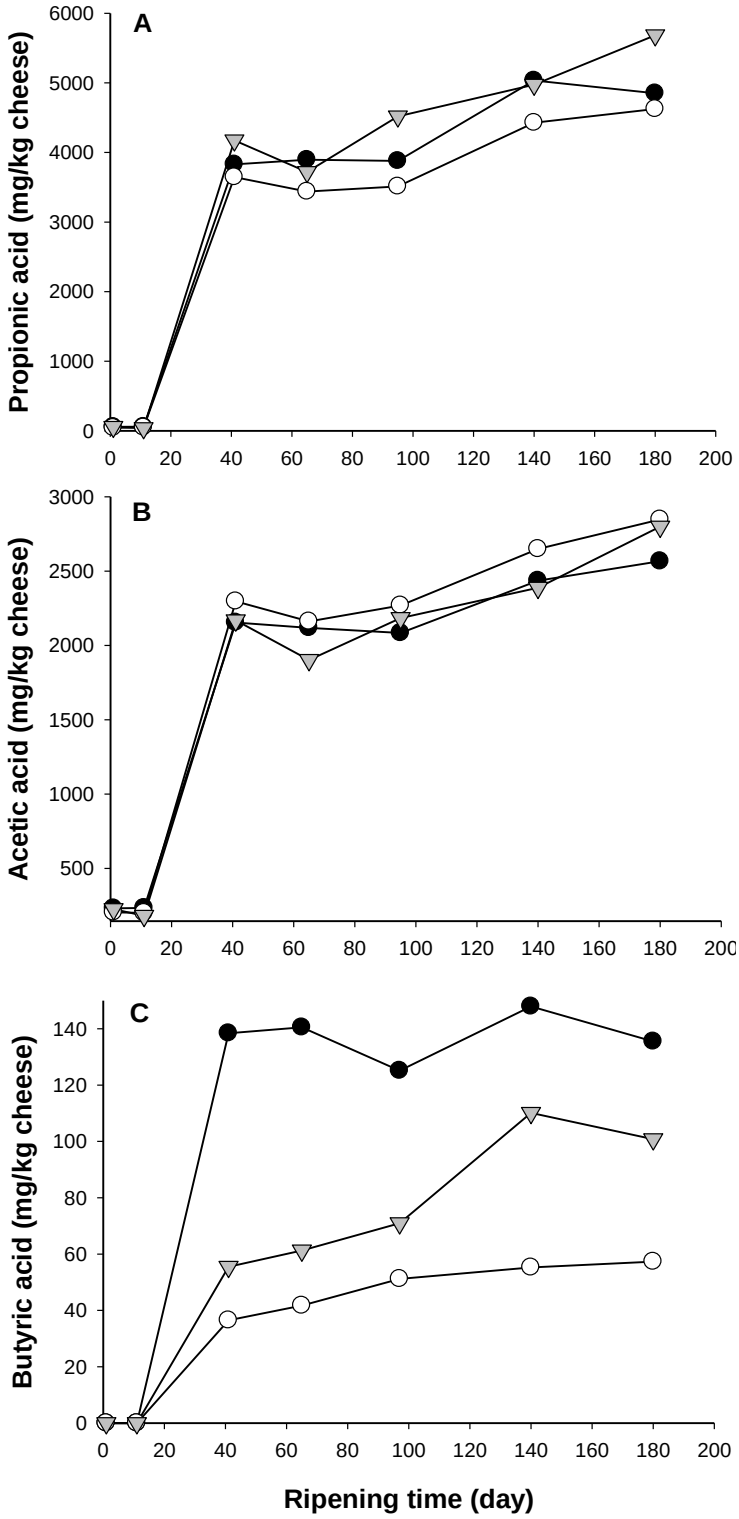


Figure 3.3. The effect of milk pre-treatments on the mean level of (A) propionic acid, (B) acetic acid, (C) butyric acid during ripening of Maasdam cheeses. Milk pre-treatments: control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data are means of data from three replicate trials.

Significant effects of treatment and time were observed for mean levels of butyric acid during maturation (Table 3.1). The mean levels of butyric acid in CT cheeses were higher ($P < 0.05$) than in CF and CFHHT cheeses (Figure 3.3C), which coincided with the higher relative abundance of *Clostridium sensu stricto* in CT cheeses than CF and CFHHT cheeses, as revealed by high-throughput sequencing. Higher levels of butyric acid in the CT cheeses were most probably due to butyric acid fermentation by *Clostridium*, which may be removed during centrifugation in CF and CFHHT milks.

A high level of butyric acid can influence the flavor profile of cheese and may result in down-graded cheese. Some species of *Clostridium* produces carbon dioxide and hydrogen via butyric acid fermentation, which can impair the quality of eyes and also increase the risk of slits and crack formation in cheese (Sheehan, 2011; Gómez-Torres, Garde, Peirotén, & Ávila, 2015). The level of butyric acid in late-blown cheeses varies among studies; in semi-hard (Bogovič Matijašić, Koman Rajšp, Perko, & Rogelj, 2007) and Gouda cheese (Klijn, Nieuwenhof, Hoolwerf, van der Waals, & Weerkamp, 1995), butyric acid contents higher than 200 mg/kg have been found to be associated with the LBD, and the severity of this defect was greater when the level of butyric acid was higher. However, it should be noted that butyric acid within the cheese matrix can also originate from lipolysis during ripening (Le Bourhis et al., 2007; Garde, Ávila, Gaya, Arias, & Nuñez, 2012). Although the level of butyric acid was significantly higher in CT cheeses than CF and CFHHT cheeses, we did not observe LBD in the CT cheeses. In the current study, the level of butyric acid was below 200 mg/kg in all experimental cheeses over six months of ripening. These results are in agreement with the studies of Le Bourhis et al. (2007) and Beuvier et al. (1997), who also observed butyric acid contents of less than 200

mg/kg in normal Swiss-type cheeses. The comparatively low level of butyric acid in the CT cheeses was most probably due to low levels of *Clostridium* spores in the cheese milk obtained from a local dairy company during spring-summer (May-July); during this period, contamination of milk with *Clostridium* is less likely, since the milk supply was from spring-calving herds fed on pasture grass rather than silage [the main source of clostridial spore contamination in milk; Sheehan (2011)]. The levels of butyric acid between CF and CFHHT cheeses were not statistically different.

3.4.5 Volatile profile of Maasdam cheese

In total, 28 major volatile compounds were identified at 140 d of ripening, consisting of 8 ketones, 7 acids, 4 alcohols, 4 esters, 2 aldehydes, 2 sulphur compounds and a hydrocarbon (Table 3.2). The volatile flavor compounds in cheese are the result of complex biochemical reaction during maturation, such as, proteolysis, lipolysis and glycolysis (McSweeney, 2004). The correct balance and concentration of a wide range of flavor compounds gives the characteristic flavor of different cheese varieties. Centrifugation of cheese milk and incorporation of HHT centrifugate into centrifuged milk had virtually no impact on the formation of volatile compounds at 140 d of ripening of Maasdam cheese. However, as expected, the mean relative abundance of butanoic acid (butyric acid) was lower ($P < 0.05$) in CF and CFHHT cheeses than in CT cheeses (Table 3.2), attributed to the removal of butyrate-fermenting *Clostridium* spores from cheese-milk by the centrifugation process. Although butanoic acid at low levels contributes positively to the aroma of the cheese, it gives an undesirable rancid note at high concentration (Curioni & Bosset, 2002). Propionic and acetic acid were also detected in higher abundance in

all experimental cheeses, as expected. The presence of hexanoic and octanoic acids within the experimental cheeses was attributed to lipolytic activity of enzymes (Delgado, González-Crespo, Cava, García-Parra, & Ramírez, 2010).

Only two aldehydes, i.e., benzaldehyde and 2-methylbutanal, were detected in all experimental cheeses, and these are derived from Phe and Ile, respectively (Yvon & Rijnen, 2001) via α-keto acids by the transaminase pathway (Smit, Smit, & Engels, 2005). Branched chain aldehydes, including 2-methylbutanal, are generally detected in high levels in cheese containing PAB (Thierry, Maillard, Richoux, Kerjean, & Lortal, 2005), and are responsible for dark chocolate/malty aroma notes (Singh, Drake, & Cadwallader, 2003; Bertuzzi et al., 2017). Aldehydes are relatively unstable compounds and thus can further catabolize to other groups of volatile compounds, such as, alcohols or carboxylic acids. It has been reported that 3-methylbutanal and 2-methylbutanal can oxidize to 3-methylbutanoic acid and 2-methylbutanoic acid, respectively, and these acids contribute to cheesy/sweaty/rancid notes in cheese (Yvon et al., 2001). Alcohol dehydrogenase from LAB can convert 2-methylbutanal to 2-methyl-1-butanol which has fruity/waxy/sweaty-fatty acid aroma notes (Singh et al., 2003).

Ketone flavor compounds, such as, 2,3-butanedione (responsible for creamy/buttery aroma note), acetoin and 2-butanone, are considered important volatile compounds in Maasdam cheese, and are likely generated from metabolism of citrate by *Lactococcus lactis* and *Leuconostoc* spp. (Engels, Dekker, de Jong, Neeter, & Visser, 1997; Le Bars & Yvon, 2008). Detection of these ketone flavor compounds in all experimental cheeses at higher abundance is not surprising because *Lactococcus* and *Leuconostoc* spp. were added as starter culture, and these genera were also detected within the cheese matrix throughout ripening, as revealed by

high-throughput sequencing. Interestingly, 2,3-pentanedione was only detected in CF and CFHHT cheeses, whereas 2-hexanone was only detected in CT cheeses; however, these compounds were detected in only one trial out of three trials. Pentane-2,3-dione has been suggested to be produced from an intermediate of Ile metabolism (Imhof, Glättli, & Bosset, 1995). Heptan-2-one, one of the important methyl ketones in Parmigiano-Reggiano cheese types (Qian & Reineccius, 2002), derived from the oxidation of octanoic acid, was also present in all experimental cheeses and likely contributes to cheesy/fruity aroma notes.

Ethanol and 2-butanol were the two most abundant alcohols detected in all experimental cheeses. Ethanol may be produced by the heterofermentative LAB present within the cheese matrix (Thierry, Maillard, Richoux, & Lortal, 2006). The abundance of ethanol and short-chain acids, such as, propionic, butyric and hexanoic acids, inevitably results in ethyl esters via esterification or alcoholysis from microbial activity (Hong et al., 2018). Ethanol is considered as the limiting factor for ethyl esters formation in different cheese types, including Swiss cheese (Thierry et al., 2006). Therefore, modulation of ethanol level can potentially alter the fruity flavor of Swiss (Thierry et al., 2006) and Camembert (Hong et al., 2017) cheeses.

Esters, especially ethyl esters, are responsible for fruity aroma notes in some cheese varieties, such as Parmesan and Swiss-type cheeses (Engels et al., 1997; Thierry et al., 2006). Ethyl propanoate was one of the most abundant esters in all experimental cheeses, in agreement with the results previously reported by other authors for cheeses containing PAB (Thierry et al., 2005; Thierry et al., 2006). Ethyl butanoate, propyl propanoate and ethyl hexanoate were also detected.

Volatile sulphur compounds are considered to be an important contributor to the flavor of different cheese types, and these compounds are reported to have very low-odor threshold values (Martínez-Cuesta, Peláez, & Requena, 2013). Dimethyl sulfide [responsible for rotten cabbage/cheese/vegetative/sulphur aroma notes; Smit et al. (2005)] and carbon disulfide were the two sulphur compounds present in all experimental cheeses at 140 d of ripening, which are considered important flavor compounds of Swiss cheese released from metabolic activity of PAB (Adda, Gripon, & Vassal, 1982). These sulphur-containing compounds are derived from the catabolism of sulphur AA (Met and Cys) by microorganisms during ripening (Smit et al., 2005; Liu, Prakash, Nauta, Siezen, & Francke, 2012).

Toluene was detected in all experimental cheeses, and may originate from the degradation of β-carotene (Verzera et al., 2010; O'Callaghan et al., 2017). Some studies have observed high levels of toluene in milk of cows fed on pasture grass (Villeneuve et al., 2013) and also in cheese made from milk of cows fed on pasture grass (O'Callaghan et al., 2017).

Table 3.2. Mean volatile compound peak areas from Maasdam cheese samples at 140 d of ripening[1]

Volatile compounds	LRI	Ref. LRI[3]	Experimental cheese groups[2] CT	CF	CFHHT	SEM	*P*-value
Acids							
Acetic acid	690	720	937,050[a]	892,939[a]	863,495[a]	42,910	NS
Propanoic acid	784	813	1,780,403[a]	1,796,587[a]	1,574,464[a]	138,686	NS
Butanoic acid	864	883	507,815[a]	200,039[b]	187,989[b]	63,928	*
3-Methylbutanoic acid	917	924	17,441[a]	19,946[a]	15,435[a]	2,355	NS
2-Methylbutanoic acid	924	945	34,971[a]	38,769[a]	27,595[a]	5,155	NS
Hexanoic acid	1,052	1,074	109,023[a]	102,471[a]	93,513[a]	10,345	NS
Octanoic acid	1,244	1,264	9,755[a]	10,307[a]	9,382[a]	811	NS
Alcohols							
Ethanol	506	-	328,148[a]	277,956[a]	295,035[a]	30,099	NS
1-Propanol	612	611	11,263[a]	11,044[a]	25,842[a]	3,461	NS
2-Butanol	648	624	229,554[a]	69,075[a]	614,349[a]	179,338	NS
2-Methyl-1-butanol	789	794	22,266[a]	32,545[a]	25,350[a]	2,937	NS
Aldehydes							
2-Methylbutanal	700	700	8,163[a]	12,268[a]	10,572[a]	1,239	NS
Benzaldehyde	1,032	1,016	151,680[a]	101,440[a]	90,813[a]	32,465	NS
Esters							
Ethyl propanoate	737	744	44,917[a]	38,659[a]	42,970[a]	4,493	NS
Ethyl butanoate	826	830	24,229[a]	18,161[a]	17,146[a]	2,645	NS
Propyl propanoate	835	-	10,310[a]	6,582[a]	8,226[a]	1,274	NS
Ethyl hexanoate	1,024	1,028	6,041[a]	5,502[a]	4,455[a]	615	NS
Ketones							
Acetoin	778	782	2,423,512[a]	2,345,157[a]	1,829,257[a]	370,152	NS
Acetone	533	529	20,569[a]	12,568[a]	7,144[a]	3,736	NS
2,3-Butanedione	631	632	339,191[a]	308,814[a]	246,050[a]	44,948	NS
2-Butanone	639	630	1,705,995[a]	1,780,078[a]	4,570,336[a]	857,979	NS

Table 3.2 continue

2-Pentanone	730	733	18,254[a]	22,489[a]	20,644[a]	1,033	NS
2,3-Pentanedione	736	740	0.00[a]	5,465[a]	17,682[a]	5,943	NS
2-Hexanone	834	834	10,146[a]	0.00[a]	0.00[a]	3,378	NS
2-Heptanone	936	933	58,171[a]	38,408[a]	37,236[a]	6,968	NS
Sulphur compounds							
Dimethyl sulfide	538	532	593[a]	1,099[a]	814[a]	195	NS
Carbon disulfide	546	538	10,454[a]	10,360[a]	6,091[a]	1,212	NS
Hydrocarbon							
Toluene	794	788	42,593[a]	48,265[a]	41,576[a]	4,741	NS

[1]LRI = linear retention index; Ref. LRI = reference linear retention index

[2]Values within a row not sharing common superscripts differ significantly; data presented are the means of data from three replicate trials. CT, control cheese; CF, cheese made from centrifuged milk; CFHHT, cheese made from centrifuged milk containing high heat-treated centrifugate

[3]Reference LRI for ethanol or propyl propanoate were not found; however, they have been identified correctly.

*$P < 0.05$, NS = $P > 0.05$

It is well known that heat treatment changes the flavor profile of milk through production of volatile compounds from proteins (e.g., sulphur compounds from heat denaturation of whey protein), carbohydrates (via the non-enzymatic browning reactions) and lipids (e.g., formation of methyl ketones, lactones and aldehydes from degradation of milk fat; Calvo and de la Hoz, 1992). However, the volatile profile of cheese made from CF and CFHHT was not statistically different, suggesting that the VOC solely generated by the heat-treatment given to the centrifugate have minimal impact on the volatile profile of final cheese.

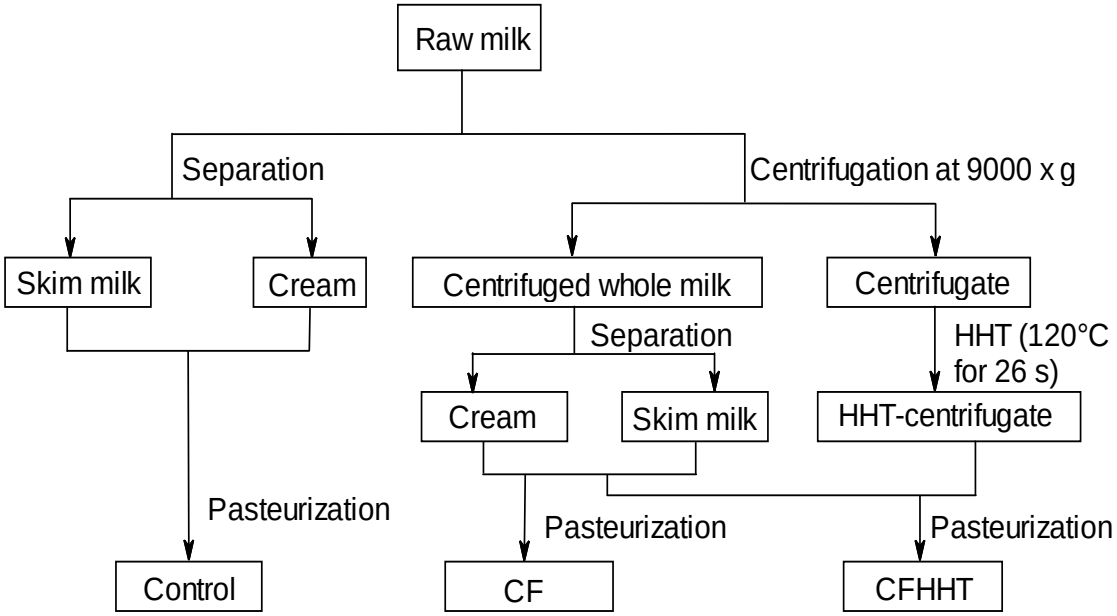
Overall, the treatments applied to the milk had minimal impact on the volatile profile of Maasdam cheese, except for butyric acid levels, suggesting that centrifugation was a suitable method for controlling undesirable butyric acid fermentation without significantly altering the other VOC in Maasdam cheese. The level of butyric acid in the current study was not high (below 200 mg/kg cheese) in all experimental cheeses, suggesting that the milk was contaminated by low levels of *Clostridium* spores. Although levels of *Clostridium* spores may be higher in milk supplies other than those studied, this would not influence the findings of the current study, as it was focused on the influence of the milk pre-treatments (i.e., centrifugation and high heat treatment of centrifugate) on the microbial and volatile profile of the cheese and not on the influence of clostridia *per se*.

Based on the relative abundance of volatiles present in the current study and the previous study of Engels et al. (1997), acetoin, 2-butanone, propionic acid, acetic acid, 2,3-butanedione, and butyric acid (at low levels) can be considered as key aroma compounds of Maasdam cheese. Sensory analysis of the cheeses would complement the volatile results, and this could be the focus for future studies.

3.5 Conclusions

High-throughput sequencing in combination with a selective media-based approach revealed distinct differences in the composition of microbiota between pre- and post-warm room ripening. High-throughput sequencing facilitated a more detailed insight into the complexity of microbes within the Maasdam cheese matrix, and revealed subtle changes in both dominant and subdominant microbiota between treatments. Interestingly, except for butyric acid, treatments applied had minimal impact on other VOC in the fully ripened Maasdam cheeses. Overall, high-throughput sequencing proved to be a useful method to profile the complex microbial population structure of Maasdam cheese during maturation; moreover, the centrifugation of milk prior to cheese-making can potentially control the level of butyric acid in Maasdam cheese without significantly altering the levels of other VOC.

3.6 Supplementary materials



Supplementary Figure 3.1. Flow charts of the preparation of cheese milks, i.e., control, centrifuged (CF) and centrifuged milk containing high heat-treated centrifugate (CFHHT). Abbreviations: HHT, high heat treatment, HHT-centrifugate, high heat-treated centrifugate.

Supplementary Table 3.1. Compositional parameters and pH at 11 d of ripening in Maasdam cheeses[1]

Compositional factors	Cheese types[2]			SEM	*P*- value
	CT	CF	CFHHT		
Moisture (%, wt/wt)	44.83[a]	44.15[a]	47.83[a]	0.72	0.057
MNFS (%, wt/wt)	58.9[a]	58.14[a]	61.78[b]	0.63	0.013
Protein (%, wt/wt)	24.04[a]	24.57[a]	23.44[a]	0.30	0.348
Fat (%, wt/wt)	23.9[a]	24.07[a]	22.61[a]	0.44	0.376
FDM (%, wt/wt)	43.31[a]	43.07[a]	43.29[a]	0.35	0.966
Salt (%, wt/wt)	1.53[a]	1.50[a]	1.73[a]	0.05	0.183
S/M (%, wt/wt)	3.41[a]	3.40[a]	3.61[a]	0.07	0.450
Total calcium (mg/100 g)	821[a]	800[a]	837[a]	12.19	0.514
pH (11 d)	5.28[a]	5.31[a]	5.27[a]	0.01	0.548
Lactose (mg/100 g)					
1d	54.39[a]	41.82[a]	66.72[a]	16.26	0.861
11 d	20.42[a]	43.72[a]	62.63[a]	15.07	0.585
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	-
Galactose (mg/100 g)					
1 d	28.09[a]	29.57[a]	35.34[a]	4.11	0.798
11 d	27.61[a]	27.26[a]	30.82[a]	4.22	0.947
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	-

[1]Abbreviations: CT, control cheese; CF, cheese made from centrifuged milk; CFHHT, cheese made from centrifuged milk containing high heat-treated centrifugate; MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio.

[2]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean of data from three replicate trials.

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Chapter 4: Microstructure and fracture properties of semi-hard cheese: Differentiating the effects of primary proteolysis and calcium solubilization

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4.1 Abstract

The individual roles of hydrolysis of α_{S1}- and β-caseins, and calcium solubilization on the fracture properties of semi-hard cheeses, such as, Maasdam and other eye-type cheeses, remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low temperature (8 °C) decreased the hydrolysis of α_{S1}-casein by ~95%, ~45%, or ~30%, respectively, after 90 d of ripening, whereas ~35% of β-casein was hydrolysed in that time for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of insoluble calcium as a percentage of total calcium decreased significantly from ~75% to ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to the expectation that shortness of cheese texture is associated with α_{S1}-casein hydrolysis, fracture strain was significantly positively correlated with the level of intact β-casein and insoluble calcium content, indicating that the cheeses with low levels of intact β-casein or insoluble calcium content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this study suggests that the fracture properties of cheese can be modified by selective hydrolysis of caseins, altering the level of insoluble calcium or both. Such approaches could be applied to design cheese with specific properties.

4.2 Introduction

Knowledge of fracture properties of cheese is important for understanding breakdown properties of cheese during mastication, in designing cheese texture suitable for size reduction operations (e.g., slicing, dicing or grating), and in understanding the reasons for formation of undesirable texture defects within the cheese matrix, such as, slits and cracks (Luyten, 1988).

Development of undesirable slits and cracks within the cheese matrix is an international problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to downgrading of the product, resulting in lost revenue to manufacturers (Grappin, Lefier, Dasen, & Pochet, 1993; White, Broadbent, Oberg, & McMahon, 2003; Guggisberg et al., 2015). To date, the exact causes for development of such defects are not known. However, excessive production of gas, an unsuitable cheese texture, or both, have been considered as root causes for occurrence of this defect (Daly, McSweeney, & Sheehan, 2010; Rehn et al., 2011). If the cheese texture is short or brittle (i.e., fracturing of cheese matrix at a relatively small deformation), the cheese matrix is no longer able to withstand increased gas pressure during eye-formation or storage, leading to formation of cracks and splits. Although the exact reasons for a cheese to become short or brittle during ripening are not yet fully understood, proteolysis, partial solubilization of colloidal calcium phosphate associated with *para*-casein matrix of the curd during ripening, or both, have been considered as possible reasons (Lucey, Johnson, & Horne, 2003; Daly et al., 2010). However, the role of primary proteolysis and level of insoluble calcium on fracture behaviour of brine-salted semi-hard cheese has not yet been fully elucidated.

From a structural perspective, α_{S1}-casein and β-casein are the two important caseins within the cheese matrix, and these undergo varying degree of hydrolysis during ripening in different cheese varieties through the action of residual coagulant and plasmin, respectively (Sheehan, O'Sullivan, & Guinee, 2004b; Kelly, O'Flaherty, & Fox, 2006; Lamichhane, Kelly, & Sheehan, 2018b). Studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N- and C-terminal, respectively (Lucey et al., 2003). Thus, these caseins are held together by various molecular forces within the cheese matrix. Moreover, calcium associated with casein enhances the cross-linking of casein within the cheese matrix. Therefore, it is reasonable to assume that both hydrolysis patterns of casein and solubilization of colloidal calcium during ripening alter the casein interactions, which may in turn influence the textural, rheological and fracture behaviour of cheese. A better understanding of the individual contribution of such factors may allow the development of specific strategies to design cheese with specific properties.

Unlike high maximum scald temperatures (~55°C) in Emmental cheese manufacture, cheese curds are cooked only to ~40°C during manufacture of most semi-hard cheeses, such as Maasdam and Jarlsberg (Fröhlich-Wyder et al., 2017), which is not sufficient to inactivate or reduce the residual chymosin activity, resulting in extensive breakdown of α_{S1}-casein during ripening (McGoldrick & Fox, 1999). The role of chymosin-mediated proteolysis on texture properties of Cheddar cheese has previously been studied by inhibition of the residual chymosin by the addition of a chymosin inhibitor to the curd-whey mixture (O'Mahony, Lucey, & McSweeney, 2005). However, little is known about the role of chymosin-mediated

proteolysis on the fracture behavior of semi-hard Swiss, Dutch and related eye-type cheeses. Some semi-hard eye-type cheeses are ripened in a warm room (~23°C) for 4-6 weeks for the development of eyes. However, the effect of such elevated ripening temperature on solubilization of calcium and hydrolysis of casein is also not fully understood.

The aim of this study was to decouple and explore the individual role of primary proteolysis (both of α_{S1}- and β-casein) and insoluble calcium on the fracture properties of washed-curd brine-salted semi-hard cheese.

4.3 Materials and methods

4.3.1 Milk supply and cheese manufacture

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52 % (w/w) and 3.21 % (w/w), respectively. The standardized cheese-milks were then pasteurized at 72°C for 15 sec (MicroThermics, Raleigh, NC, USA) and stored at 4°C overnight prior to cheese manufacture.

Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk, for each replication. Each vat contained automated variable speed cutting and stirring equipment. All cheese milks were inoculated at 32°C with frozen direct vat inoculation cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactococcus lactis* ssp. *cremoris*,

Lactococcus lactis ssp. *lactis*; and (2) LH-B02 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*. Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-formation during ripening of cheese which would not permit measurement of texture parameters.

All cheese milks were pre-acidified to 6.55 using 4% (w/v) lactic acid (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) prior to rennet addition. After 40 min of pre-ripening, the coagulant, fermentation-produced bovine chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 2 mL/11 kg cheese milk in 3 out of 4 vats, whereas fermentation-produced camel chymosin (FPCC; CHY-MAX M, ~200 IMCU/mL; Chr. Hansen Ltd., Cork, Ireland), was added at a level of 1.5 mL/11 kg cheese milk in the fourth vat. Coagulants were diluted ~1:10 with deionized water prior addition. The addition rates of both FPBC and FPCC to milk were predetermined through a series of rheological experiments where the levels of the coagulants were adjusted to achieve coagula of similar gel strength (35 Pa) after a set period of ~45 min.

All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments, New Castle, DE, USA) and the resultant curd/whey mixture was allowed to heal for 5 min before being stirred continuously for another 10 min. Stirring was then stopped and a portion of whey (0.35 kg/kg cheese milk) was removed. Just after whey removal, in one vat out of four vats, Pepstatin A (synthetic; Enzo life science, Colleton Cres, Exeter, UK) was added to the curd/whey mixture at a rate of 10.0 μmol/kg cheese milk and evenly distributed by continuous stirring during cooking. Pepstatin A is an inhibitor of aspartic proteases, including chymosin, pepsin, and cathepsin D

(Marciniszyn, Hartsuck, & Tang, 1976). After whey removal, reverse osmosis water at ~50°C (0.25 kg/kg cheese milk) was added to each cheese vat to cook the curd to 37°C at a rate of 0.2°C/min with continuous stirring.

Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 hours. When the pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a saturated brine solution (23%, w/w, NaCl, 0.56%, w/w, $CaCl_2$, and pH 5.2) for 7.5 h at 8°C. After brining, cheese wheels were vacuum-packed (Falcon 52, Original Henkelman vacuum system, 's-Hertogenbosch, the Netherlands), and transferred to the ripening room. Cheese wheels were ripened at 8°C for 20 d (pre-ripening), at 23°C for 28 d (warm-room ripening) or 8°C for 28 d (without warm room ripening), and finally stored at 4°C for 42 d. A summary of the experimental plan is shown in Table 4.1.

4.3.2 Milk and cheese composition

The composition of raw and pasteurized (72°C for 15 s) cheese milks were analyzed as described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed at 20 d of ripening in duplicate for moisture, fat, protein and salt as described by Hickey et al. (2018b). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon, Wilkinson, and McSweeney (2007).

Table 4.1. General overview of the treatments and ripening regimens used in the study[1]

Treatment	Cheese type[2]			
	Control	noWR	CC	PepA
Rennet type	FPBC	FPBC	FPCC	FPBC
Chymosin inhibitor	Not added	Not added	Not added	Added
Ripening regimen	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 8 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d

[1]FPBC, fermentation-produced bovine chymosin; FPCC, fermentation-produced camel chymosin

[2]noWR, cheese without warm room ripening; CC, cheese made using fermentation-produced camel chymosin as a coagulant; PepA, cheese containing chymosin inhibitor, i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

4.3.3 Enumeration of starter and nonstarter lactic acid bacteria

Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of ripening. Cheese samples were prepared as described by Lamichhane et al. (2018c). Viable *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium, supplemented with 0.5% (w/v) lactose, after aerobic incubation at 25°C for 3 d (Ruggirello et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42°C (Lamichhane et al., 2018c). Nonstarter lactic acid bacteria (NSLAB) cells were enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation for 5 d at 30°C (Lamichhane et al., 2018c).

4.3.4 Proteolysis

4.3.4.1 pH 4.6-soluble nitrogen (% of total nitrogen)

The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 was measured after 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

4.3.4.2 Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd., Watford, Herts, UK), as described by Sheehan and Guinee (2004a). Briefly, grated cheese samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55°C for 10 min and each sample was loaded at a level of 12 µL per well. Sodium caseinate powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples initially ran through the stacking

gel at 280 V and then through the separating gel at 300 V. The resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee (2017). Densitometry analysis was performed on the scanned images using image analysis software, i.e., ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Eight major bands corresponding to caseins or its breakdown products were used for calculation: 1, β-CN (f106–209) (γ2); 2, β-CN (f29–209) (γ1); 3, β-CN (f108–209) (γ3); 4, β-casein; 5, β-CN (f1–192); 6, α_{S1}-casein; 7, α_{S1}-CN (f102–199); 8, α_{S1}-CN (f24–199). The area of each protein band was expressed as a percentage of total band area of these eight major bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage of level at 1 d.

4.3.5 Determination of total and insoluble calcium content

The total calcium content of milk and cheese samples (after 20 d) was determined using atomic absorption spectroscopy (IDF, 2007). The cheese insoluble calcium contents, expressed as percentage of total calcium, were determined after 1, 20, 48, and 90 d of ripening using an acid-base titration method as described by Hassan, Johnson, and Lucey (2004).

4.3.6 Fracture properties

Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples were wrapped in tin foil to prevent moisture loss; half of the cylindrical cheese samples were stored at 4°C and the remainder was stored at 23°C for at least 4 hours. These specific temperatures were chosen to mimic the warm-room ripening (23°C) and subsequent cheese storage temperature (4°C). Cheese samples (at 4°C or 23°C) were compressed at a rate of 60 mm/min until fracture.

True stress (σ; Equation 4.1) and Hencky strain ($\varepsilon_{H;}$ Equation 4.2) were calculated, assuming a constant volume deformation (Rehn et al., 2011):

$$\sigma = \frac{FH_t}{A_0 H_0} \qquad (4.1)$$

$$\varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \qquad (4.2)$$

where F is a load applied, H_t is the sample height at time t, and A_0 and H_0 are the initial cross-sectional area and height of sample, respectively. Fracture stress (σ_f) and fracture strain (ε_f) values of cheese samples were determined from the inflection point of the stress-strain curve (Rehn et al., 2011).

4.3.7 Visualization of cheese microstructure

Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission, Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto 2500, Gatan, UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (-200°C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high vacuum cryo-preparation chamber at -185°C, etched at -95°C over a period of 15 min, sputter-coated at -125°C and finally transferred onto the SEM cold stage at -125°C. Cryo-SEM images were acquired at -125°C.

The microstructure of cheese samples was also visualised using confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany). Rectangular cheese samples (5 mm × 5 mm × 2 mm) were

removed from cheeses using a sharp scalpel. Solutions of the protein specific dye Fast Green (Sigma Aldrich) and fat specific dye Nile Red (Sigma Aldrich) were prepared at a concentration of 0.01% (w/v) in deionized water and 1,2-propanediol (Sigma Aldrich), respectively, which were then mixed at a ratio of 3:1. The prepared dye mixture (40 μL) was applied to the surface of cheese samples; a cover slip was gently placed on top and the sample was held at 4°C for 10 min prior to imaging. The protein and fat phases of the cheese samples were visualised by exciting the Fast Green dye (using a He–Ne laser; excitation wavelength of 633 nm and emission wavelength range of 650-700 nm) and Nile Red dye (using an Argon laser; excitation wavelength of 488 nm and emission wavelength range of 500-580 nm) respectively as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica Microsystems, Baden-Württemberg, Germany).

4.3.8 Statistical analysis

One way ANOVA, using SPSS software version 24 (IBM Corp., Armonk, NY), was performed to determine the effect of treatment on cheese composition. A split-plot design was used to determine the effect of treatment, ripening time, and their interactions on pH, counts of *Lactococcus lactis* and *Lactobacillus helveticus*, levels of pH 4.6-SN (% TN), insoluble calcium (% of total calcium) and fracture properties (stress and strain at fracture) of cheese. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple comparison tests was used for paired comparison of treatment means at a 5% level of significance. Pearson correlation analysis was performed between fracture parameters, pH 4.6-SN (% TN), insoluble

calcium (% of total calcium), intact β-casein level and intact α_{S1}-casein level using SPSS software version 24 (IBM Corp., Armonk, NY).

4.4 Results and discussion

4.4.1 Milk and cheese composition

The average fat, protein, and lactose contents of the standardized and pasteurized cheese-milk used for the three replicate cheese-making trials were 3.21, 3.52, and 4.87 % (w/w), respectively. The composition of the experimental cheeses at 20 d of ripening is shown in Table 4.2. The cheeses had a composition similar to those of Maasdam-type cheese reported by Lamichhane et al. (2018a). The treatments applied had no significant effect on the mean levels of moisture, moisture in non-fat substance, protein, fat, fat-in-dry matter, salt, salt-in-moisture and pH (at 1 d of ripening) of the experimental cheeses.

4.4.2 pH

The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 4.3) during ripening from 5.18-5.23 at 1 d to 5.35-5.40 at 90 d (Figure 4.1a). The pH trend during ripening is consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane et al., 2018a). No significant effect of treatment was observed for the mean value of pH during ripening.

Table 4.2. Compositional parameters at 20 d and pH at 1 d of ripening in semi-hard cheeses[1]

Compositional factors	Cheese types[2]				*P*-value
	Control	noWR	CC	PepA	
Moisture (%, w/w)	40.83 ± 1.61[a]	40.89 ± 2.47[a]	40.95 ± 3.36[a]	41.84 ± 2.51[a]	0.96
MNFS (%, w/w)	56.24 ± 1.54[a]	56.11 ± 2.20[a]	56.26 ± 3.11[a]	57.05 ± 2.28[a]	0.96
Protein (%, w/w)	25.32 ± 0.90[a]	25.57 ± 1.47[a]	25.44 ± 1.56[a]	25.39 ± 1.48[a]	0.98
Fat (%, w/w)	27.42 ± 1.06[a]	27.16 ± 1.77[a]	27.29 ± 1.98[a]	26.70 ± 1.65[a]	0.95
FDM (%, w/w)	46.33 ± 0.91[a]	45.92 ± 1.46[a]	46.18 ± 0.85[a]	45.88 ± 1.28[a]	0.96
Salt (%, w/w)	1.34 ± 0.12[a]	1.38 ± 0.14[a]	1.38 ± 0.09[a]	1.36 ± 0.08[a]	0.96
S/M (%, w/w)	3.28 ± 0.31[a]	3.39 ± 0.51[a]	3.38 ± 0.41[a]	3.25 ± 0.08[a]	0.95
Total calcium (mg/100 g cheese)	867 ± 30[a]	861 ± 30[a]	837 ± 34[a]	842 ± 27[a]	0.60
pH (1 d)	5.18 ± 0.03[a]	5.21 ± 0.03[a]	5.19 ± 0.03[a]	5.23 ± 0.03[a]	0.28

[1]MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio; Control, control cheeses; noWR, cheeses without warm room ripening; CC, cheeses made using fermentation-produced camel chymosin; PepA, cheeses containing chymosin inhibitor i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

[2]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean ± standard deviation of data from three replicate trials.

4.4.3 Growth and viability of *Lactococcus lactis*, *Lactobacillus helveticus* and NSLAB

A significant effect of ripening time and treatment was observed for the counts of *Lactococcus lactis* (Table 4.3). The counts of *Lactococcus lactis* decreased in all cheeses during ripening from $10^{9.4}$-$10^{9.7}$ cfu/g at 1 d to $10^{7.4}$-10^{9} cfu/g at 90 d, indicating cell death and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses, suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the warm room ripening.

No significant effect of treatment and ripening time was observed for counts of *Lactobacillus helveticus* until 20 d of ripening, at which time the average count was 10^{5}-$10^{6.5}$ cfu/g. After warm-room ripening (48 d), the typical colonies of *Lactobacillus helveticus* were not observed, suggesting that either the cells were in a stressed condition, which may be viable but not culturable, or may have lysed due to changes in the cheese-ripening environment, such as, microbial composition, depletion of energy sources (e.g., low residual lactose), production of metabolites (Steele, Broadbent, & Kok, 2013) or inward diffusion of salt (Hickey, Fallico, Wilkinson, & Sheehan, 2018a).

NSLAB counts were variable between trials, although one trial did show that the average counts of NSLAB increased during ripening from $10^{4.3}$-10^{5} cfu/g at 20 d (before warm room ripening) to $10^{6.7}$-$10^{7.7}$ cfu/g at 48 d (after warm-room ripening). Moreover, the average count of NSLAB was ~1 log lower in noWR cheeses than for the other cheeses at 48 d of ripening.

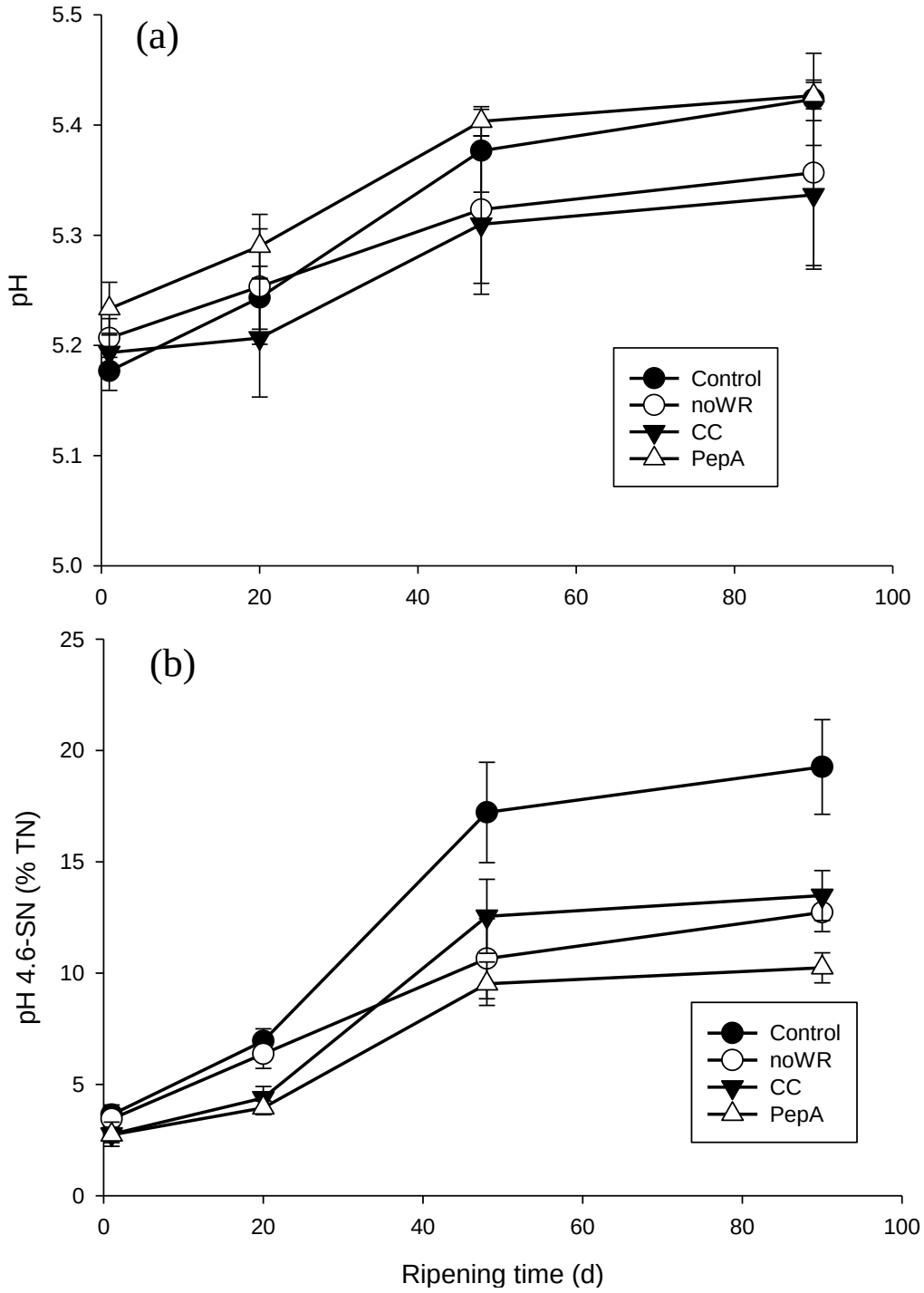


Figure 4.1. Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6, expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from three replicate trials; error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

4.4.4 Proteolysis

4.4.4.1 Nitrogen soluble at pH 4.6 (% of total nitrogen)

A significant ($P < 0.001$, Table 4.3) interaction was observed between the effect of treatment and ripening time for levels of nitrogen soluble at pH 4.6 [% of total nitrogen; pH 4.6-SN (% TN)] in all experimental cheeses. The mean levels of pH 4.6-SN (% TN) increased with increasing ripening time in all experimental cheeses (Figure 4.1b). However, the extent of the increase in pH 4.6-SN (% TN) level during ripening was higher in control cheeses than for other experimental cheese variants, which increased from 6.95 at 20 d to 19.27 at 90 d. The level of pH 4.6-SN (% TN) in control cheeses is in close agreement with that previously reported for semi-hard (Huc, Challois, Monziols, Michon, & Mariette, 2014) and Maasdam (Lamichhane et al., 2018a) cheeses.

Although propionic acid bacteria were not inoculated into the cheese milks of the current study, the levels and trend of pH 4.6-SN (% TN) during ripening of cheeses were found to be similar to semi-hard cheeses with propionic acid bacteria, suggesting that propionic acid bacteria play only a minor role in the proteolysis of washed-curd brine-salted semi-hard cheese (Gagnaire, Thierry, & Léonil, 2001). Moreover, the autolysis of propionic acid bacteria and the release of proteases from their cell have been shown to be limited in cheese (Valence, Richoux, Thierry, Palva, & Lortal, 1998).

Table 4.3. Summary of the effects of treatment, time and their interactions on properties of semi-hard cheeses[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
Lactococcus lactis count	**	***	NS
Lactobacillus helveticus count	NS	NS	NS
pH 4.6-SN (% TN)	***	***	***
Insoluble Ca (% of total Ca)	NS	***	NS
Fracture stress (kPa, measured at 4 °C)	***	**	NS
Fracture stress (kPa, measured at 23 °C)	***	***	NS
Fracture strain (measured at 4 °C)	**	***	NS
Fracture strain (measured at 23 °C)	**	**	NS

[1]pH 4.6-SN (% TN), soluble nitrogen at pH 4.6 as percentage of total nitrogen.

***$P < 0.001$; **$P < 0.01$; NS, $P > 0.05$

As expected, the mean level of pH 4.6-SN (% TN) in PepA cheeses was approximately two-fold lower that of control cheeses at 90 d; O'Mahony et al. (2005) has previously reported a similar trend for Cheddar cheese. The low level of proteolysis in the PepA cheeses was due to inhibition of residual chymosin by pepstatin A (which was added to the curd-whey mixture at a level of 10 μmol/L). The level of pH 4.6-SN (% TN) in PepA cheese was found similar to that reported for Emmental cheese at 90 d of ripening (O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016); in Emmental, residual coagulant is largely or wholly inactivated by use of a high cook temperature during cheese manufacture.

The mean levels of pH 4.6-SN (% TN) in noWR and CC cheeses were 12.73 and 13.49, respectively, after 90 d of ripening, which were significantly lower than in the control cheeses. A higher average level of proteolysis in control cheeses compared to the noWR cheeses was attributed to an increase in the rate of proteolysis due to elevated ripening temperature (Sheehan et al., 2004b; Soodam, Ong, Powell, Kentish, & Gras, 2017). The lower levels of pH 4.6-SN (% TN) in CC cheese compared to control cheeses was attributed to the lower general proteolytic activity of FPCC compared to FPBC (Kappeler et al., 2006; Bansal et al., 2009).

4.4.4.2 Urea-polyacrylamide gel electrophoresis

During ripening, α_{S1}- and β-caseins were progressively hydrolyzed to an extent dependent on the treatment applied and ripening temperature, while breakdown products simultaneously accumulated (Figure 4.2 and Supplementary Figure 4.1). Extensive hydrolysis of α_{S1}-casein was observed for control cheeses during ripening (i.e., more than 90 % of levels at 1 d), with the rate of hydrolysis being most rapid during warm room ripening stages, whereas the hydrolysis of α_{S1}-

casein was ~30% and ~45% less in noWR and CC cheeses at 90 d, respectively, compared to control cheeses (Figure 4.2b).

Less hydrolysis of α_{S1}-casein in noWR cheeses compared to control cheeses was attributed to the influence of temperature on the residual coagulant activity (Sheehan et al., 2004b). Less extensive breakdown of α_{S1}-casein in CC cheeses compared to control cheese was attributed to the lower proteolytic activity of FPCC compared to FPBC (Bansal et al., 2009; McCarthy et al., 2017).

Limited breakdown of α_{S1}-casein, i.e., ~5%, was observed in PepA cheeses in agreement with the previous studies (Shakeel-Ur-Rehman, Feeney, McSweeney, & Fox, 1998; O'Mahony et al., 2005), suggesting that the addition of chymosin inhibitor, i.e., pepstatin A, to the curd/whey mixture during cheese manufacture was an effective means for greatly reducing the chymosin-mediated hydrolysis of α_{S1}-casein within the semi-hard cheese during ripening.

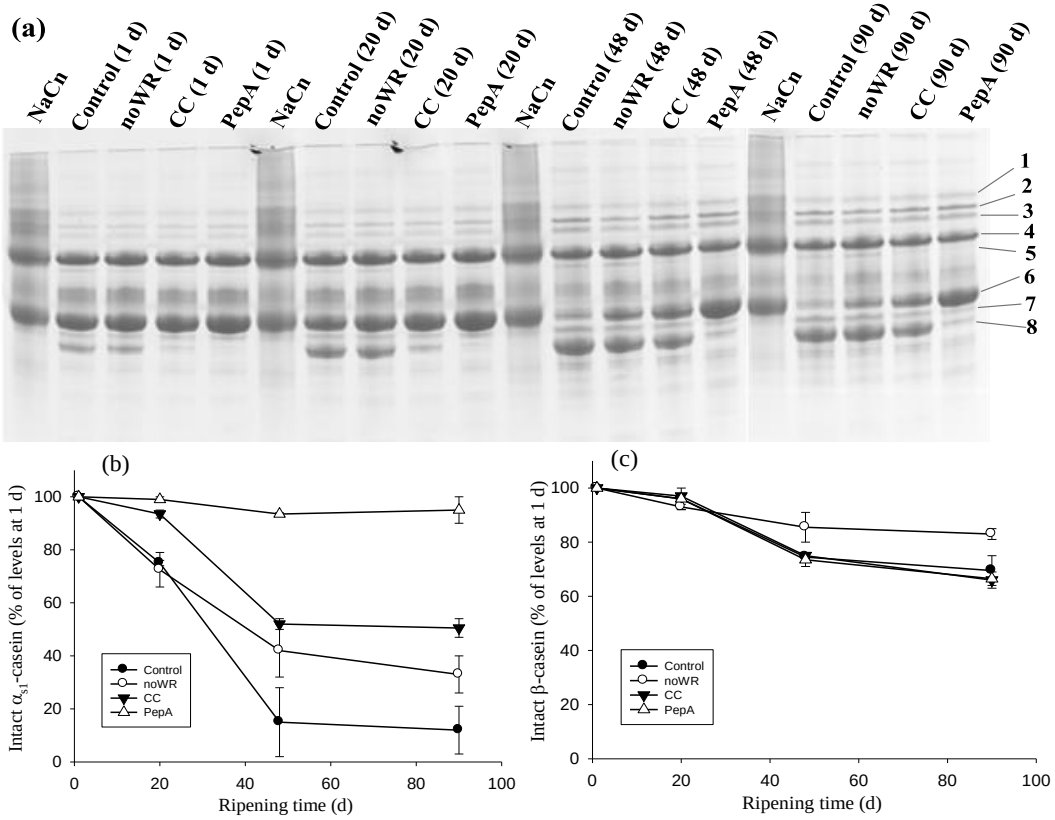


Figure 4.2. (a) Urea-polyacrylamide gel electrophoretograms of semi-hard cheeses after 1, 20, 48 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands were identified according to McCarthy et al. (2017): 1, β-CN (f106–209) (γ2); 2, β-CN (f29–209) (γ1); 3, β-CN (f108–209) (γ3); 4, β-casein; 5, β-CN (f1–192); 6, α_{S1}-casein; 7, α_{S1}-CN (f102–199); 8, α_{S1}-CN (f24–199). Level of (b) intact α_{S1}-casein and (c) intact β-casein as a percentage of the level at 1 d. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Hydrolysis of β-casein was observed in all cheeses during ripening (Figure 4.2c), most likely due to plasmin activity (Kelly et al., 2006). The extent of hydrolysis of β-casein was similar for control, CC and pepA cheeses (i.e., ~35 % of levels at 1 d), suggesting that neither the substitution of FPBC with FPCC nor addition of chymosin inhibitor to the curd/whey mixture influenced the hydrolysis of β-casein in agreement with the previous studies (O'Mahony et al., 2005; Bansal et al., 2009). However, the extent of breakdown was relatively lower in noWR cheeses (i.e., less than 20% of levels at 1 d) than other cheeses, suggesting that warm room ripening accelerates the degradation of β-casein. Overall, these results suggest that the various hydrolysis patterns of casein can be achieved by using different coagulant types, modulating ripening temperature or inhibiting residual chymosin activity, although inhibition of the latter using pepstatin A is obviously not commercially viable.

4.4.5 Insoluble calcium contents of cheeses

The mean level of insoluble calcium (percentage of total calcium) decreased significantly ($P < 0.001$, Table 4.3) during ripening (Figure 4.3), especially at the early stage of ripening, from ~75% at 1 d to ~66% at 20 d. After 20 d of ripening, the rate of decrease in the level of insoluble calcium was slower than at the early stages of ripening, which is in agreement with the previous studies in different cheese types (O'Mahony et al., 2005; Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010).

The effect of warm-room ripening on solubilization of colloidal calcium in brine-salted cheese varieties has not previously been studied. Therefore, the rate of calcium solubilization was compared between cheeses subjected to warm room ripening (control cheeses) and without warm room ripening (noWR cheeses).

Interestingly, the mean insoluble calcium content of noWR cheeses was ~3% higher than that of the control cheese after 48 d of (after warm room ripening); however, the difference observed was not statistically significant, suggesting that, at best, the warm room ripening had only a minor effect on the solubilization of calcium. Hydrolysis of β-casein is known to release phosphopeptides (Gagnaire, Mollé, Herrouin, & Léonil, 2001), which could contribute to decreases in the level of casein-bound calcium. As expected, substitution of FPBC with FPCC as a coagulant or addition of pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on insoluble calcium content.

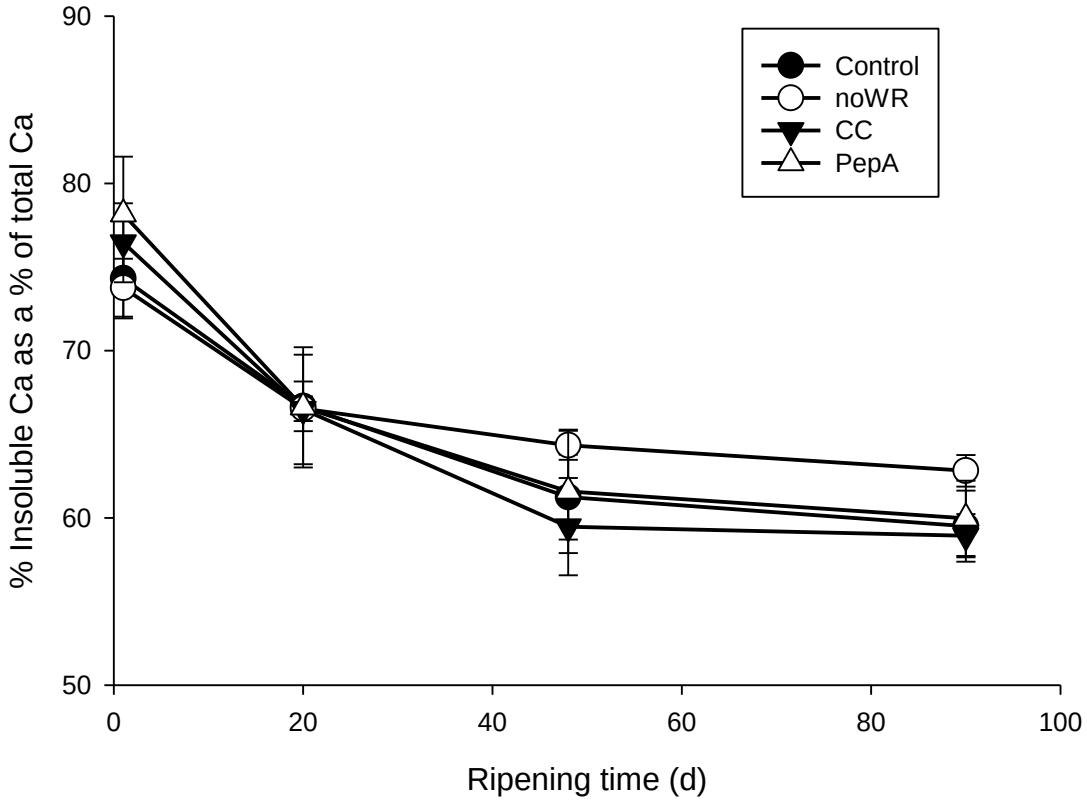


Figure 4.3. Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca) as a function of ripening time in semi-hard cheeses. Data are the mean of data from three replicate trials and error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

4.4.6 Fracture properties

The fracture properties of experimental cheeses were studied at two different temperatures, i.e., 4°C or 23°C (Figure 4.4), to mimic the temperature conditions during cold storage or warm-room ripening, respectively. The stress at fracture (σ_f) and strain at fracture (ε_f) were significantly influenced by treatment and ripening time (Table 4.3).

Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength, or rigidity, of the cheese matrix. The σ_f measured at 4°C or 23°C significantly decreased (Figure 4.4a-b; Table 4.3) in all cheeses over maturation. However, the σ_f was significantly higher ($P < 0.05$) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control cheeses compared to other experimental cheese types was attributed to higher levels of protein breakdown in the control compared to PepA, noWR and CC cheeses (Figure 4.1b). A significant negative correlation (Table 4.4) between pH 4.6-SN (% TN) and σ_f was observed for the experimental cheeses, which was in agreement with previous studies on Cheddar cheese (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly positively (Table 4.4) correlated with intact α_{S1}-casein. Intact β-casein level was also significantly positively correlated with the value of σ_f; however, the correlation coefficient (r) value was lower for intact β-casein (Table 4.4) as compared to intact α_{S1}-casein. This suggested that the intact α_{S1}-casein was the principal load-bearing protein within the semi-hard cheese matrix. No significant correlation was found between the σ_f and insoluble calcium content (Table 4.4), indicating that the extent of solubilization of calcium after 20 d of ripening had no pronounced influence on the strength of the cheese matrix.

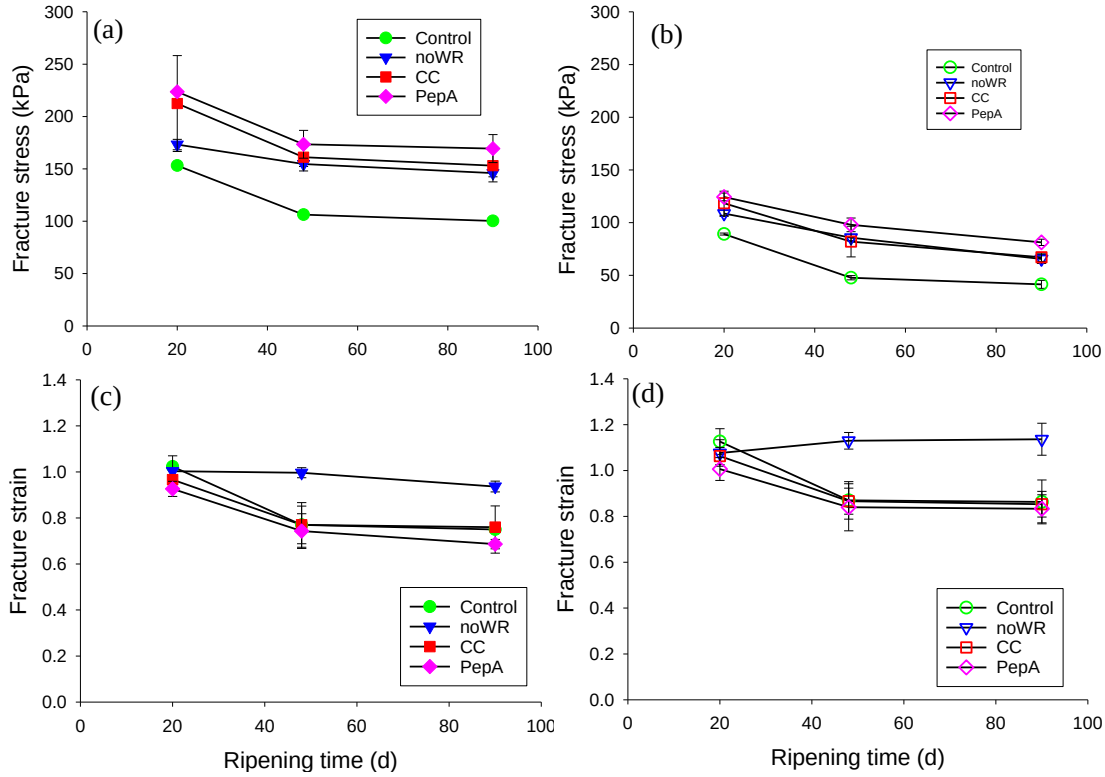


Figure 4.4. Changes in (a-b) fracture stress (σ_f, n = 2) and (c-d) fracture strain (ε_f, n = 3), measured at 4 °C (closed symbols) and 23 °C (open symbols), in semi-hard cheese during ripening. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fracture strain (ε_f) represents the shortness or brittleness of cheese texture; cheeses with a lower fracture strain value are susceptible to fracture at small deformation (Grappin et al., 1993; Sharma, Munro, Dessev, Wiles, & Foegeding, 2018). The ε_f measured at both 4°C or 23°C significantly decreased for control, CC and PepA cheeses, especially during warm room ripening, from 1.0-1.2 at 20 d to 0.75-0.8 at 48 d (Figure 4.4c-d).

Although α_{S1}-casein was hydrolyzed to varying degrees among the control, CC and PepA cheeses after 48 d of ripening (ranging from ~5% in PepA to ~90% in control cheeses; Figure 4.2), no significant difference in ε_f was observed among these cheeses. In the current study, hydrolysis of α_{S1}-casein mainly occurred at Phe_{23}-Phe_{24} during ripening, yielding peptides α_{S1}-CN (f1-23) and α_{S1}-CN (f24-199). The former peptide may be hydrolyzed rapidly by proteinases of the starter micro-organisms (Shakeel-Ur-Rehman et al., 1998), whereas the latter peptide accumulated during ripening (Figure 4.2a). Therefore, the results from this study suggest that the primary breakdown of α_{S1}-casein into the large peptide fragment, i.e., α_{S1}-CN (f24-199) had no pronounced effect on the ε_f in semi-hard cheese during ripening. Since the peptide fraction α_{S1}-CN (f24-199) is so large, it is likely that this fraction may remain attached to the protein network rather than becoming part of the serum phase (Luyten, 1988; Lucey et al., 2003). Further breakdown of α_{S1}-CN (f24-199) (secondary breakdown) into small peptides may decrease the ε_f of cheese (Luyten, 1988). In the current study, no noticeable breakdown of α_{S1}-CN (f24-199) was observed during 90 d of ripening (Figure 4.2a); therefore, the role of secondary breakdown of α_{S1}-CN (f24-199) on shortness of cheese could not be elucidated. Similar to the current study, Luyten (1988) also did not observe a clear link between the primary breakdown of α_{S1}-casein and ε_f in Gouda cheese. A significant decrease

in ε_f in control, CC and PepA cheeses during warm-room ripening may be due to other age-related changes within the cheese matrix rather than primary breakdown of α_{S1}-casein.

Interestingly, the ε_f for the noWR cheeses remained almost the same or decreased slightly over the ripening period (Figure 4.4c-d). Moreover, the ε_f for noWR cheeses was significantly higher ($P < 0.05$) at 48 and 90 d as compared to control, PepA and CC cheeses (which were subjected to warm room ripening stage). Similarly, Luyten (1988) also observed considerably lower ε_f in Gouda cheeses ripened at higher temperature (i.e., 18 °C) than ripened at lower temperature (i.e., 8 °C) during ripening. Furthermore, similar to the current study, ε_f of the Gouda cheeses ripened at 8 °C decreased slightly from 1.3 at 14 d to 1.2 at 42 d of ripening, whereas ε_f of the Gouda cheese ripened at 18 °C decreased considerably from 1.3 to 0.8 over the same ripening period. Although the exact reasons for such an influence of ripening temperature on fracture behaviour of cheese are unknown, it can be assumed that temperature-induced changes within the cheese matrix, such as, rate of solubilization of colloidal calcium, specific hydrolysis patterns of casein and the resultant peptide profiles, could be possible reasons.

In the current study, insoluble calcium (expressed as a percentage of total calcium) and intact β-casein were significantly positively correlated with ε_f (Table 4.4). Furthermore, levels of intact β-casein (Figure 4.2c) and insoluble calcium (Figure 4.3) were on average ~15% and ~3% higher, respectively, in noWR cheeses than in the other cheeses after 48 d of ripening. This suggested that the breakdown of intact β-casein, solubilization of colloidal calcium during ripening, or both may have contributed to the shorter texture (i.e., lower ε_f) observed in control, CC and PepA than noWR cheeses. Therefore, the results from this study suggested that the

influence of varying degrees of hydrolysis of β-casein, or level of colloidal calcium, on shortness of cheese texture merits further research.

It is now well established that the calcium associated with casein is an important structural component, which enhances the cross-linking of caseins within the cheese matrix (Lucey et al., 2003; O'Mahony et al., 2005; Lamichhane et al., 2018b). Thus, it was reasonable to assume that the solubilization of colloidal calcium during ripening within the cheese matrix was one of the possible reasons for shorter texture of cheese. Moreover, studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N and C termini, respectively (Lucey et al., 2003). Therefore, it is likely that the specific hydrolysis of caseins during ripening may alter their molecular interactions within cheese matrix, which in turn may influence the texture, rheological and fracture behaviour of cheese. For example, Bogenrief and Olson (1995) observed that degree of melt of Cheddar cheese was more closely related to the extent of β-casein hydrolysis than the hydrolysis of α_{S1}-casein.

Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein, modulation of colloidal calcium associated with casein, or both. Such knowledge is particularly important for designing cheese with desired texture profiles or for designing cheese texture suitable for withstanding increased gas pressures during ripening in some eye-type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is higher for cheeses with lower ε_f (short or brittle texture) (Grappin et al., 1993; Rehn et al., 2011). However,

it should be noted that unsuitable cheese texture is one possible contributing factor, amongst other factors, for the development of undesirable splits or cracks, such as; rate and extent of gas production and its behavior (e.g., solubility and diffusivity) within the cheese matrix; late gas production; and the presence of micro-defects within the cheese matrix (Daly et al., 2010).

Table 4.4. Pearson correlation coefficients between fracture parameters, primary proteolysis, insoluble calcium, and intact β-casein and α_{S1}-casein[1]

	σ_f (measured at 4 °C)	ε_f (measured at 4 °C)	σ_f (measured at 23 °C)	ε_f (measured at 23 °C)
pH 4.6-SN (% TN)	-0.77**	-0.62**	-0.88**	-0.54**
Insoluble Ca (% of total Ca)	0.23^{NS}	0.66**	0.33^{NS}	0.53**
Intact β-casein	0.50*	0.75**	0.68**	0.60**
Intact α_{S1}-casein	0.79**	0.25^{NS}	0.83**	0.19^{NS}

[1] σ_f, stress at fracture; ε_f, strain at fracture; pH 4.6-SN (% TN), soluble nitrogen at pH 4.6 as percentage of total nitrogen; data were obtained from all experimental cheeses over a 90 d of ripening.
** $P < 0.01$; * $P < 0.05$; $^{NS}P > 0.05$

The σ_f of cheeses measured at 4 °C (Figure 4.4a) was considerably higher as compared to same cheeses measured at 23 °C (Figure 4.4b) at all stages of ripening, which was attributed to the temperature-induced changes on the components of cheese and their interactions (Lamichhane et al., 2018b). At low temperature (~4 °C), more than half of the milk fat present within the cheese matrix is in a crystallized form, and acts as a reinforcing filler, contributing to the elastic texture of cheese (Lopez, Briard-Bion, Camier, & Gassi, 2006; Lamichhane et al., 2018b). However, the test temperature (4 °C or 23 °C) had no pronounced effect on the ε_f of cheeses at all stages of ripening.

4.4.7 Microstructure

The microstructure of cheese (at 90 d of ripening) observed by cryo-SEM was shown in Figure 4.5. The microstructure of the control cheese was clearly different from that of the other experimental cheese types; the microstructure observed for the control cheese was more open than that of the other experimental cheeses. The open structure may be attributed to significantly higher levels of proteolysis in the control cheeses compared to the other cheese types. For other experimental cheeses, the microstructure looked visually similar. During proteolysis, the intact caseins, which are responsible for network formation, breakdown into small and medium size peptides and free amino acids and these peptides and amino acids are released into the serum fraction of the cheese (Sousa, Ardö, & McSweeney, 2001). Soodam, Ong, Powell, Kentish, and Gras (2015) also observed a less open structure of cheese with low levels of primary proteolysis than in cheeses with high levels.

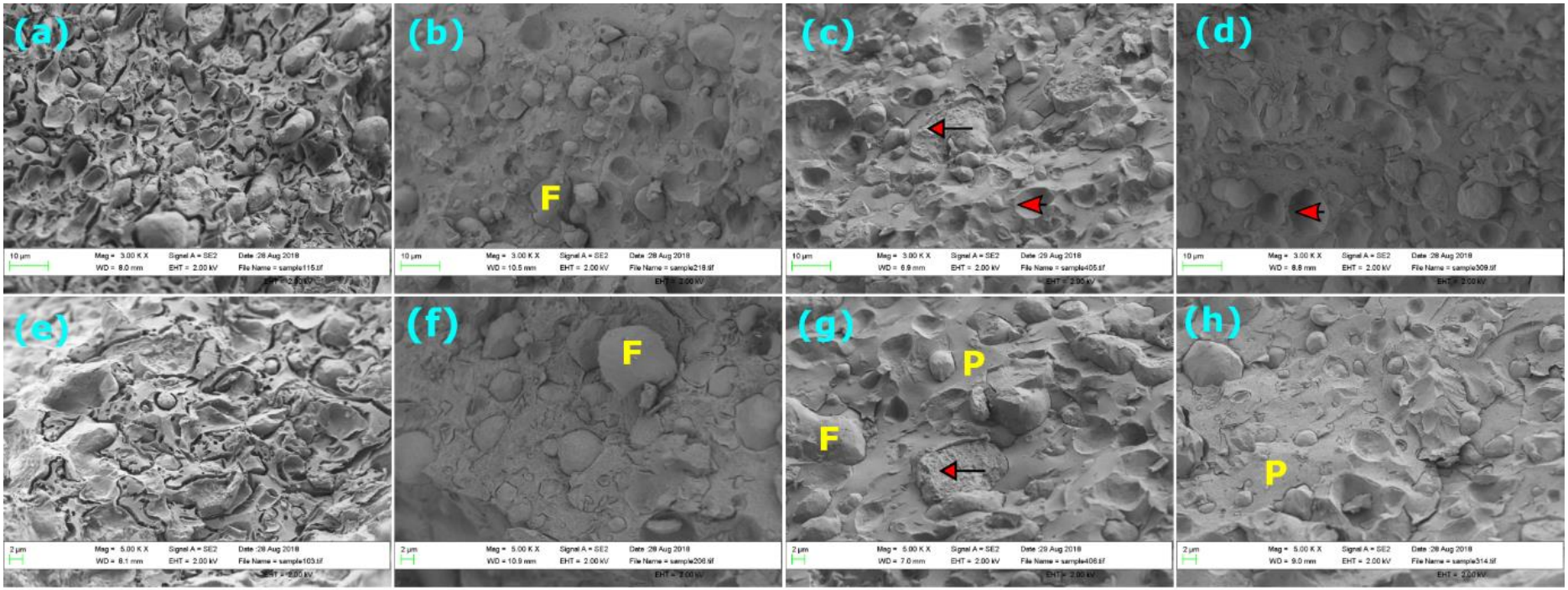


Figure 4.5. Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h) PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows = spherical imprints in the protein matrix left by fat globules that were removed during sample preparation, and long arrows = remnant fat from globules partially removed during sample preparation.

The microstructure of the cheeses (at 90 d ripening) was also visualized using CLSM (Supplementary Figure 4.2). In agreement with the previous studies (Lopez, Camier, & Gassi, 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese matrix, which was attributed to the aggregation, coalescence, and disruption of the fat globules due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al., 2007). The microstructures of all experimental cheeses were visually similar.

4.5 Conclusions

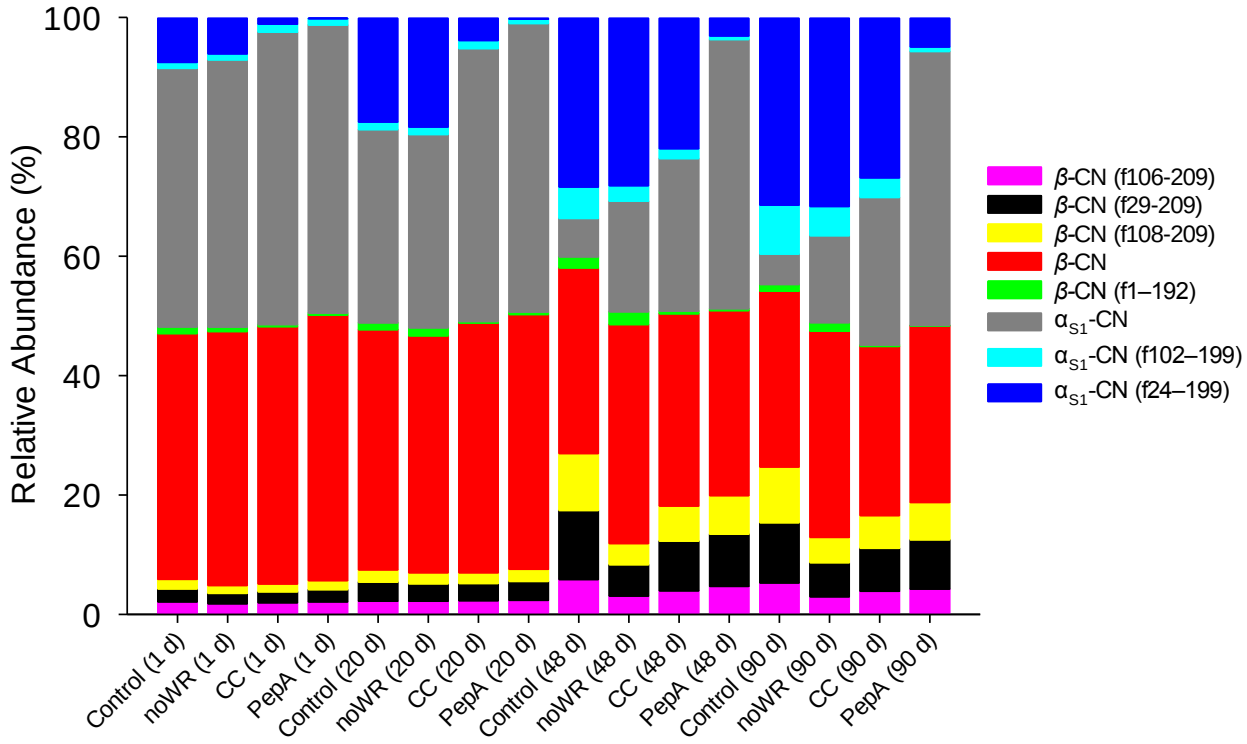
The roles of primary proteolysis and calcium solubilization on the fracture properties of washed-curd brine-salted semi-hard cheese were investigated. Addition of a chymosin inhibitor (i.e., pepstatin A) to the curd/whey mixture during cheese manufacture, substitution of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in all cheeses during ripening.

The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to expectation, shortness or brittleness (as indicated by lower strain at fracture) of cheese texture was negatively associated particularly with the level of intact β-casein and also with insoluble calcium content.

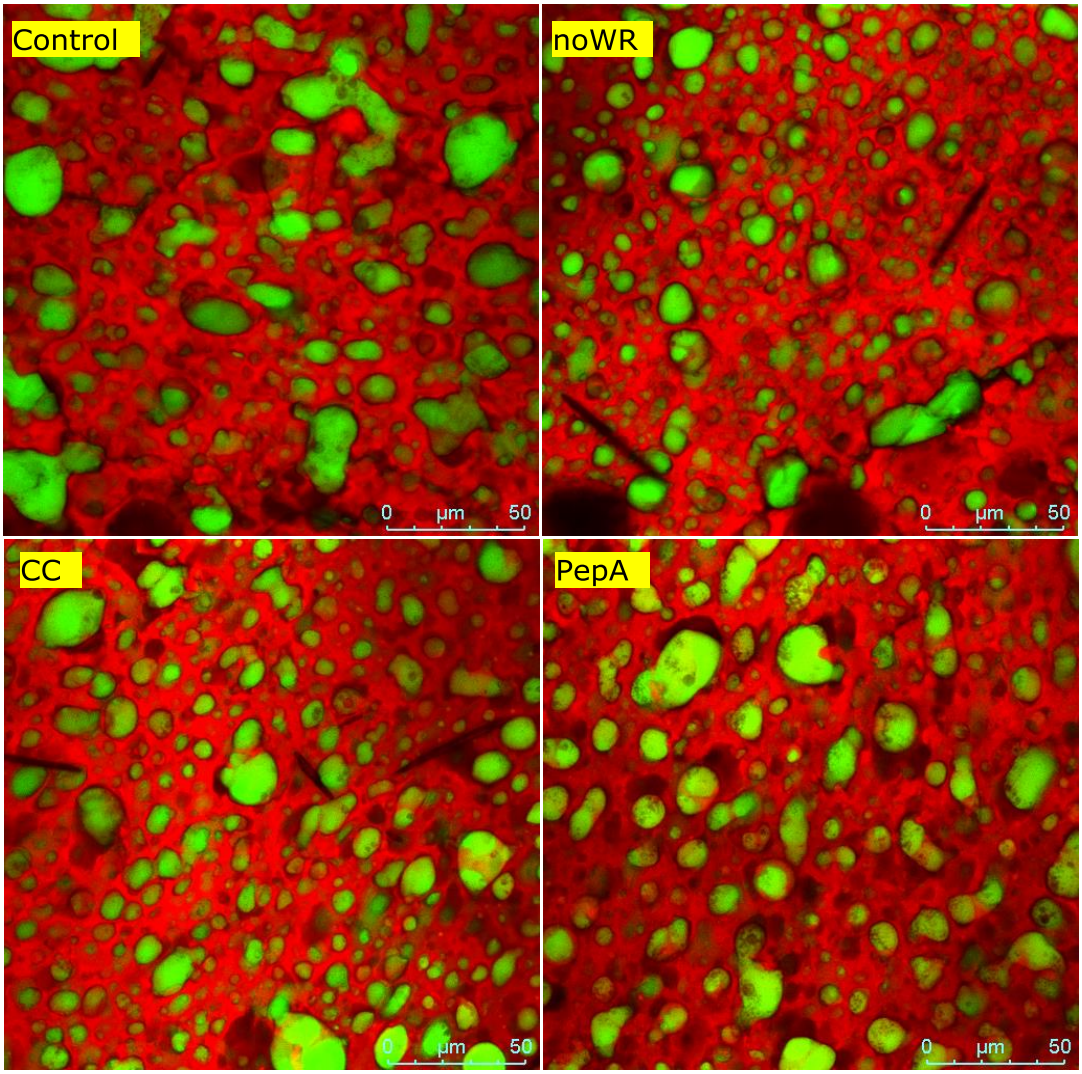
The results from this study suggested that modulation of hydrolysis of α_{S1}-casein was an effective means for maintaining the strength of the cheese matrix during ripening. This could be achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or modulating ripening temperature. However,

shortness or brittleness of cheese texture could potentially be altered by maintaining higher levels of intact β-casein or insoluble calcium content, or both, within the cheese matrix. Shortness or brittleness of cheese has previously been associated with undesirable slits or cracks. Therefore, the role of intact β-casein or insoluble calcium content on fracture behaviour, especially fracture strain, merits further research.

4.6 Supplementary materials



Supplementary Figure 4.1. Average relative abundance (%, n = 2) of caseins or their breakdown products of experimental cheeses at 1, 20, 48 and 90 d. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).



Supplementary Figure 4.2. Confocal laser scanning micrographs of cheese samples after 90 d of ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A). The protein phase appears red while the fat phase appears green.

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Chapter 5: Dynamic *in situ* imaging of semi-hard cheese microstructure under large-strain tensile deformation: Understanding structure-fracture relationships

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5.1 Abstract

Changes in the microstructure of semi-hard cheeses were observed *in situ* under tensile deformation by placing a microtensile stage directly under a confocal scanning laser microscope, and recording force/displacement data simultaneously. On tensile deformation, detachment of fat globules and their subsequent release from the cheese matrix were observed, suggesting that they were weakly bonded to, or entrapped, within the cheese matrix. Moreover, an inherent micro-defect was observed at a curd granule junction within the cheese matrix, which fractured along the curd granule junction under tensile deformation, suggesting that such micro-defects could be a key to the formation of undesirable slits or cracks. Furthermore, the fracture behaviour of semi-hard cheese varied with ripening temperature, coagulant type, and inhibition of residual chymosin activity. Overall, this study demonstrated the potential of dynamic *in situ* imaging of cheese microstructure for developing a greater understanding of the breakdown behaviour of cheese matrices.

5.2 Introduction

Food matrices go through a series of structure formation (e.g., gel formation) and breakdown (e.g., during mastication) processes during manufacture, storage and consumption. Understanding these processes from a structural point of view is of growing interest to researchers and food producers, as many of the desirable properties/functionalities of foods are determined by their structure (Lamichhane, Kelly, & Sheehan, 2018a).

To date, considerable success has been achieved in visualization of food microstructure at multiple scales by utilising various microscopy techniques (Everett & Auty, 2008; Everett & Auty, 2017). More recently, several studies have shown the potential of dynamic *in situ* imaging of food microstructure for comprehensive understanding of the physical changes to food structure when subjected to various conditions relevant to processing, storage or consumption, such as heating/cooling, shearing, compression and tension. For example, Auty, Fenelon, Guinee, Mullins, and Mulvihill (1999) studied the milk gelation and cheese melting process using a hot-stage placed under a confocal scanning laser microscope (CSLM). Other studies investigated the structural and mechanical changes under large deformation of pure, mixed and emulsion-filled gels (Brink, Langton, Stading, & Hermansson, 2007; Abhyankar, Mulvihill, & Auty, 2011; Abhyankar, Mulvihill, & Auty, 2014) and meat (James & Yang, 2011) using a microtensile stage coupled with CSLM or environmental scanning electron microscopy (ESEM). Boitte, Hayert, and Michon (2013) visualized the microstructure of dough under shear (relevant to dough processing condition) using a rheo-optical device. Liu, Stieger, van der Linden, and

van de Velde (2015) observed the microstructure of gels under shear to better understand the role of fat droplet characteristics on fat-related sensory perception.

Although a number of studies have monitored the structure of model food systems or complete foods, there is no published data on the physical changes to the structure of cheese under large-strain deformation. Cheese is an inhomogeneous composite material, in which individual curd granules bind together, and where granule junctions remain between individual curd granules. Visualization of cheese microstructure under large-strain deformation may help to understand sensory perception, flavour and nutrient release, and the mechanisms of formation of undesirable slits and cracks within the cheese matrix. It is believed that texture perception is largely determined by structural rearrangements during the deformation process (Brink et al., 2007). Moreover, dynamic *in situ* imaging may help to identify weak spots within the cheese matrix, which are seeds for the development of slits/cracks within the cheese matrix. Development of undesirable slits and cracks is a major problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to downgrading of the product and lost revenue to manufacturers (White, Broadbent, Oberg, & McMahon, 2003; Guggisberg et al., 2015).

The breakdown properties of cheese depends on composition, manufacturing procedures, maturation profile, environmental conditions (e.g., pH, temperature, and solvent quality/ionic strength), and the presence of defects (such as, mechanical holes, slits and cracks), among other factors (Luyten, 1988; Visser, 1991). Extensive hydrolysis of α_{S1}-casein has been reported in some semi-hard eye-type cheeses (McGoldrick & Fox, 1999), especially during the warm-room ripening stage, mainly due to residual chymosin activity. Intact α_{S1}-casein is considered important for

maintaining the elastic texture of eye-type cheeses, which is vital to accommodate gas produced by microorganisms (Daly, McSweeney, & Sheehan, 2010). Previous studies have shown that the substitution of fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC) reduced the primary hydrolysis of α_{S1}-casein during ripening in different cheese types (Bansal et al., 2009; Soodam, Ong, Powell, Kentish, & Gras, 2015).

Although previous studies have investigated the influence of factors, such as, maturation, chymosin-mediated proteolysis, ripening temperature and coagulant types, on texture and fracture properties of different cheese varieties (O'Mahony, Lucey, & McSweeney, 2005; Soodam et al., 2015; McCarthy, Wilkinson, & Guinee, 2017; Lamichhane, Sharma, Kennedy, Kelly, & Sheehan, 2019), there is limited understanding on the influence of such factors on changes to the microstructure of cheese under large-strain deformation.

The primary objective of this study was to achieve dynamic *in situ* imaging of cheese microstructure under tensile deformation. The secondary objective of this study was to investigate the effect of maturation, ripening temperature, coagulant type and chymosin-mediated proteolysis on changes in microstructure and fracture behaviour of cheeses under tensile deformation, using a microtensile stage and CSLM. The current study was undertaken as an element of a larger study to differentiate the effects of primary proteolysis and calcium solubilization on the fracture properties of semi-hard cheese (Lamichhane et al., 2019). Levels of primary proteolysis, pH, breakdown patterns of caseins, and insoluble calcium content of the cheeses of the current study have been characterized therein.

5.3 Materials and methods

5.3.1 Cheese manufacture

Four variants of a washed-curd, brine-salted semi-hard cheese, i.e., control cheese, cheese without warm-room ripening (noWR), cheese made using FPCC as a coagulant (CC), and cheese containing a chymosin inhibitor, i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture (PepA), were manufactured in three replicate trials as reported in Lamichhane et al. (2019). Briefly, all cheese milks used in this study were standardized (protein to fat ratio of 1.1: 1.0) and pasteurized (72 °C for 15 s). Frozen direct vat inoculation cultures (Chr. Hansen Ltd., Cork, Ireland) were used as a starter cultures: (1) R-604 (180 mg kg^{-1} milk), containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02 (9 mg kg^{-1} milk), containing *Lactobacillus helveticus*. For control, noWR and PepA cheeses, FPBC (CHY-MAX Plus, ~200 international milk clotting units (IMCU) mL^{-1}; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 0.18 mL kg^{-1} cheese milk, whereas FPCC (CHY-MAX M, ~200 IMCU mL^{-1}; Chr. Hansen Ltd.) was added at a level of 0.14 mL kg^{-1} cheese milk for CC cheeses. Curd granules were washed by removing 35% of whey with subsequent addition of 23% warm (50 °C) reverse-osmosis water as a percentage of the total milk weight. Chymosin inhibitor, i.e., pepstatin A, was added at a rate of 10.0 μmol kg^{-1} cheese milk into the curd/whey mixture for PepA cheese manufacture. Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds and pressed vertically. After pressing, each cheese wheel (600 g) was placed in a saturated brine solution for 7.5 h at 8 °C prior to vacuum packaging (Falcon 52, Original Henkelman vacuum system, 's-Hertogenbosch, the Netherlands). A

summary of the experimental plan, including ripening regimens, is shown in Supplementary Table 1.

5.3.2 Cheese composition

Grated cheese samples were analysed in duplicate for content of moisture, fat, protein, salt, and total calcium and for pH as reported in Lamichhane et al. (2019).

5.3.3 Tensile testing and in situ imaging of cheese microstructure

A schematic of the experimental set-up for tensile testing is shown in Figure 5.1. Cylindrical samples of diameter 30 mm were removed from each experimental cheese using a stainless steel borer. From each cylindrical sample, thin cheese discs (thickness ~4 mm and diameter 30 mm) were prepared using a 4-mm thick plastic ring and sharp scalpel. Cheese samples of dimensions 25 mm × 10 mm × 4 mm were removed from these thin cheese discs, and a small indentation (~1.5 mm), also called a notch, was made in the centre of the each test piece using a sharp scalpel. The exact thickness of samples was determined afterwards using a vernier caliper. The test samples were then clamped between the grips of a micro-tensile stage (Deben, UK), equipped with 2 N load cell and motorised gear box. The gap between the clamps was 10 mm. Fast Green (protein specific dye; Sigma Aldrich, Arklow Co Wicklow, Ireland) and Nile Red (fat specific dye; Sigma Aldrich) of concentration 0.01% (w/v), prepared in deionized water and in 1,2-propanediol, respectively, were mixed in a ratio of 1:3 (Le Tohic et al., 2018). The prepared dye (10 μL) was poured on the cheese samples and a cover slip (diameter = 9 mm, VWR International Limited, Dublin, Ireland) was gently placed on the clamped test samples to avoid the entrapment of air bubbles. The micro-tensile stage was placed directly under the

CSLM (Leica Microsystems, Baden-Württemberg, Germany). Protein and fat phases in the cheese samples were visualised by exciting the Fast Green (using a helium-neon laser) and Nile Red (using an argon laser) dyes, respectively, as described by Abhyankar et al. (2014). The test samples were elongated at a constant tensile speed of 2 mm min^{-1} and images of cheese microstructure were simultaneously taken using air objectives of magnification of 5× or 10× (zoom factor 2 or 3). Load and displacement data of the test samples were recorded every 500 ms, which were then converted to true stress (σ; Equation 5.1) and Hencky strain (ε_H; Equation 5.2; Abhyankar et al., 2011):

$$\sigma = \frac{F \times (L_0 + \Delta L)}{A \times L_0} \qquad (5.1)$$

$$\varepsilon_H = \ln\left(\frac{L_0 + \Delta L}{L_0}\right) \qquad (5.2)$$

where F is the extensional force, A is original cross sectional area of the sample, L_0 is original length of the sample and ΔL is change in length of sample. The crack length of test samples during tensile deformation was measured as described by Abhyankar et al. (2011), using the Leica Application Suit X software (Leica Microsystems, Baden-Württemberg, Germany). Crack length was defined as the perpendicular distance between the tip of an artificial notch (at time zero) and the tip of the new fracture surface (at time t) during tensile deformation. Young's modulus was calculated from the slope of the initial linear region of stress-strain curve. Fracture stress and fracture strain values of cheese samples were determined from the inflection point of the stress-strain curve. At least four samples, from two independent replicate trials, of each cheese were tested on the microtensile unit to

determine the effect of maturation and treatment on the structural breakdown behaviour of experimental cheeses. All measurements were conducted at room temperature (~20 °C).

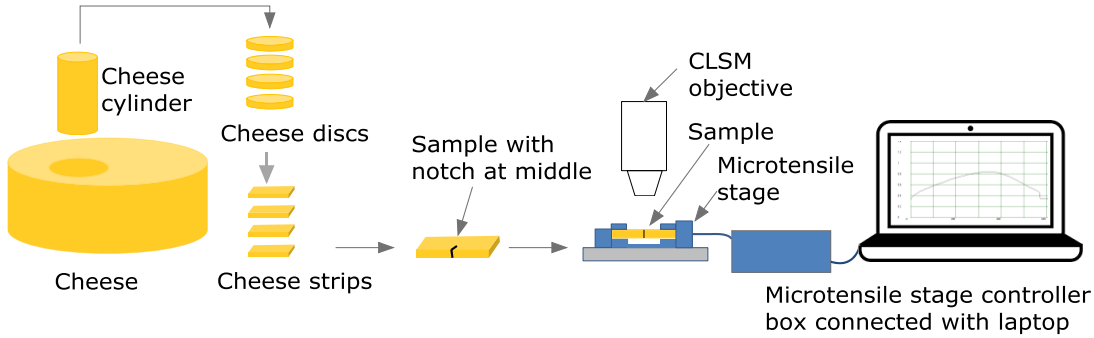


Figure 5.1. Schematic of experimental set-up for microtensile testing, showing sample preparation and accessories related to microtensile testing.

5.3.4 Statistical analysis

Statistical analyses of the data were performed using SigmaPlot version 14 (Systat Software, Inc., San Jose, California, USA). The effect of maturation and treatment on cheese fracture parameters was determined performing one way ANOVA followed by *posthoc* Tukey tests. Before ANOVA evaluation, data were checked for normality and homoschedasticity by performing Shapiro–Wilk and Brown-Forsythe tests, respectively. When these assumptions were not verified, appropriate non-parametric and *post hoc* tests were applied. The level of significance was set at $P \leq 0.05$.

5.4 Results and discussion

5.4.1 Cheese composition

The compositional parameters of the cheeses analysed here were described in detail by Lamichhane et al. (2019) and in Supplemental Table 2. The cheeses had a composition typical of that of Maasdam-type cheese (Lamichhane, Kelly, & Sheehan, 2018; Lamichhane et al., 2018b; Panthi et al., 2019). Treatment had no significant effect on mean levels of moisture, moisture-in non-fat substance, protein, fat, fat-in-dry matter, salt, salt-in-moisture, total calcium and pH (at 1 d of ripening) of the cheeses.

5.4.2 Effect of maturation on structural breakdown behaviour of cheese

To determine the effect of maturation on structural breakdown behaviour of cheese, control cheese samples at 20 d (before warm-room ripening), 48 d (after warm-room ripening) and 90 d of ripening were tested on the microtensile unit. The stress-strain relationships of cheese samples before warm-room ripening (20 d) were

found to be different compared to those of cheese samples after warm-room ripening (48 d and 90 d) (Figure 5.2a).

Changes to the microstructure of cheeses before warm-room ripening (20 d) and after warm-room ripening (48 d) were similar under tensile deformation (micrographs not shown), i.e., widening of the notch, stretching of the protein network near the leading point of notch and propagation of notch. However, the stress profile during notch propagation was different between cheeses before warm-room ripening and after warm-room ripening, which can be visualized by plotting stress versus crack length (a perpendicular distance between the tip of an artificial notch and the tip of the new fracture surface during tensile deformation as measured from the CSLM time-series micrographs). Such an approach has previously been applied to study the breakdown behaviour of different kinds of gels, such as, whey protein, mixed biopolymer or emulsion-filled gels (Öhgren, Langton, & Hermansson, 2004; Brink et al., 2007; Abhyankar et al., 2011).

For cheese samples at 48 d or 90 d of ripening, true stress increased gradually until the cheese sample started to fracture from the notch tip. Once the cheese started to fracture from the notch tip, the stress required to further break-down cheese samples leveled off, or decreased, with further increase in the crack length (Figure 5.2b). However, the stress required to break down cheese at 20 d of ripening continued to increase until the crack length of cheese reached ~4 mm, followed by a decrease in the stress with further increases in the crack length (Figure 5.2b). This suggests that, as expected, the young semi-hard cheeses can resist fracture to a greater extent compared to mature cheeses, which was further supported by the fracture parameters obtained from stress-strain curve; Young's modulus (Figure

5.2c), fracture stress and fracture strain (Figure 5.2d) values were significantly higher ($P < 0.05$) for cheeses before warm-room ripening than after warm-room ripening. Young's modulus measures the stiffness of the material; a higher value of Young's modulus corresponds to a higher material stiffness (Vandenberghe, Choucharina, De Ketelaere, De Baerdemaeker, & Claes, 2014). Fracture stress measures the rigidity of the material whereas the fracture strain measures the brittleness or shortness of the material.

Overall, semi-hard cheese structure becomes weak, brittle and less stiff (as indicated by lower fracture stress, fracture strain and Young's modulus values) during maturation, especially during warm-room (23 °C) ripening, which is attributed to age-related structural changes on the cheese matrix, such as proteolysis and partial solubilization of colloidal calcium (O'Mahony et al., 2005). During ripening, intact caseins (which are responsible for network formation) are hydrolyzed by proteolytic enzymes into small and medium-sized peptides. Moreover, the calcium associated with the casein (which enhances the cross-linking of casein within the cheese matrix) partially solubilizes during ripening (O'Mahony et al., 2005). The level of insoluble calcium of the cheeses of the current study significantly decreased whereas the level of primary proteolysis increased, especially during warm-room ripening (Lamichhane et al., 2019).

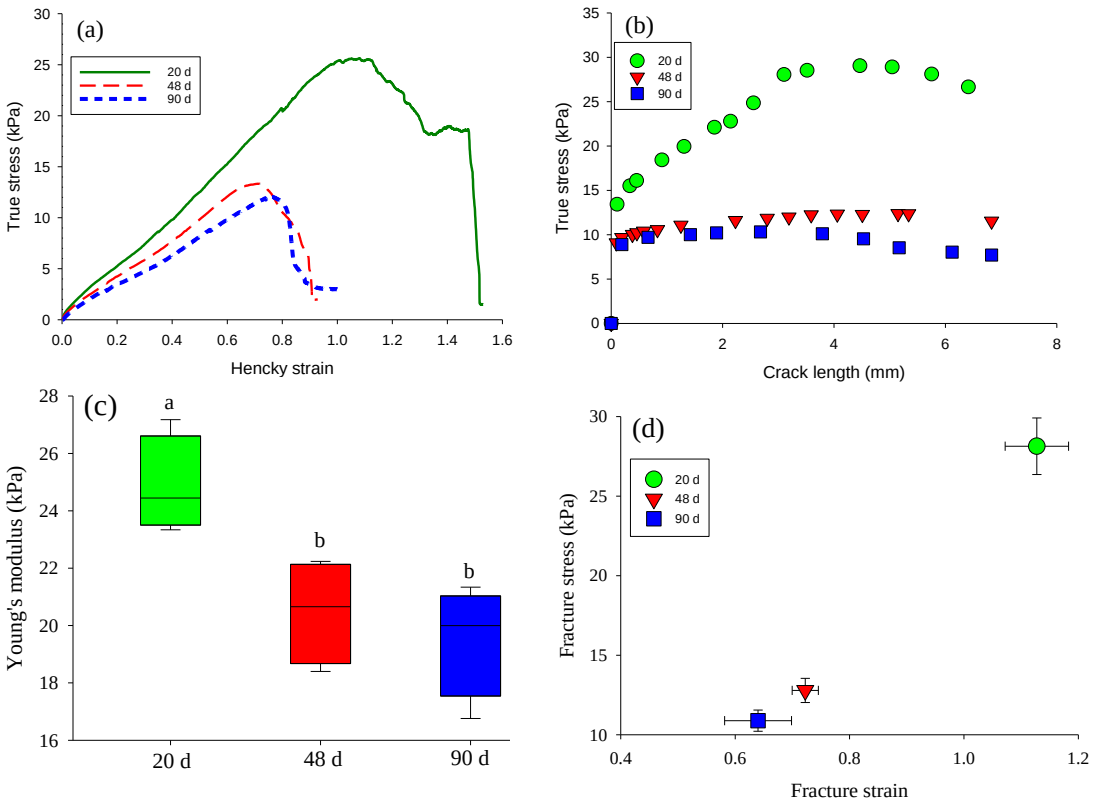


Figure 5.2. Effect of maturation on the fracture behaviour of control cheese: (a) typical true stress and Hencky strain relationships; (b) stress profile as a function of crack length; (c) Young's modulus; and (d) fracture stress and strain profile for cheese at 20, 48 and 90 d of ripening. Data presented are means of data from two replicate trials. Error bars represent standard errors of means.

5.4.3 Effect of treatment on structural breakdown behaviour of cheese

The effect of three different treatments, i.e., (1) warm-room ripening, (2) chymosin-mediated proteolysis, and (3) coagulant type, on structural breakdown behaviour of the semi-hard cheeses at 48 d of ripening was studied. Typical true stress and Hencky strain relationships for each experimental cheese types were shown in Figure 5.3a. The CC, noWR and PepA cheeses showed similar stress-strain relationships. However, the stress-strain relationships for control cheeses were different from the other cheeses. Moreover, the fracture parameters obtained from strain-stress curve was found to be significantly different between experimental cheese variants; Young's modulus (Figure 5.3c) and fracture stress (Figure 5.3d) values of CC, noWR and PepA cheeses were significantly higher than the control cheese at 48 d of ripening. In agreement with the fracture strain results obtained from uniaxial compression test in a parallel study (Lamichhane et al., 2019), the mean fracture strain value of noWR cheeses obtained from the tension test was relatively higher than for the other cheese variants (Figure 5.3d). However, the difference observed was not statistically significant.

Although changes to the microstructure of experimental cheeses variants at 48 d of ripening were observed to be similar under tensile deformation (micrographs not shown), stress profile as a function of crack length was found to be different between these cheeses (Figure 5.3b). Stress increased until the control cheese sample started to fracture from the notch tip during tensile deformation, followed by a levelling off, or decrease, in the stress with further increase in the crack length.

However, for CC, noWR and PepA cheeses, the stress required for breakdown of cheese samples continued to increase until the crack length reached ~2

mm, followed by a decrease in the stress with further increase in the crack length. Fracture may occur once the stress exceeds the cohesive strength of the material (Foegeding et al., 2011). This suggests that the cohesive strength of the CC, noWR and PepA cheeses was higher as compared to the control cheeses. The different fracture behaviour of the control cheeses compared to the noWR cheeses was attributed to temperature-induced biochemical changes, mainly proteolysis, in the cheese matrix (Soodam, Ong, Powell, Kentish, & Gras, 2017), as the latter cheeses were not subjected to warm-room ripening.

The different fracture behaviour of the control and CC cheeses at 48 d of ripening was attributed to the different proteolytic activities of residual coagulants; FPBC was used as a coagulant in control cheeses, whereas in CC cheeses FPCC was used. Studies have reported that the proteolytic activity of FPCC was much lower as compared to FPBC (Kappeler et al., 2006). The extent of primary proteolysis has been found to be lower in different cheese types made with FPCC than in cheeses made with FPBC, such as Cheddar (Bansal et al., 2009; McCarthy et al., 2017), Mozzarella (Moynihan et al., 2014), and Italian soft cheese (Alinovi et al., 2018). In the current study, substitution of FPBC with FPCC as coagulant reduced the primary proteolysis of cheese during ripening (Lamichhane et al., 2019).

The different fracture behaviour of PepA cheeses as compared to control cheeses is attributed to the inhibition of residual chymosin activity (Shakeel-Ur-Rehman, Feeney, McSweeney, & Fox, 1998; O'Mahony et al., 2005) in the former cheeses where a chymosin inhibitor, i.e., pepstatin A, was added to the curd/whey mixture. This suggested that the activity of residual chymosin may have an important influence on the fracture behaviour of semi-hard cheese.

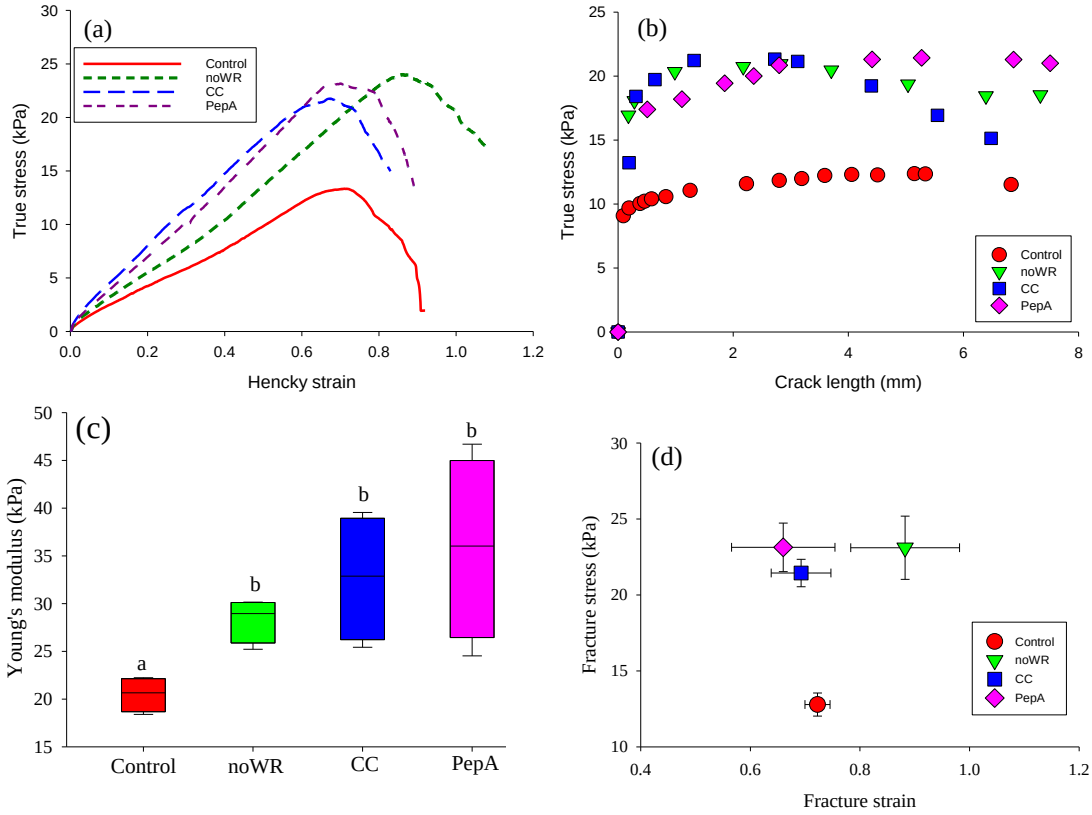


Figure 5.3. Effect of treatment on fracture behaviour of semi-hard cheese at 48 d of ripening: (a) typical true stress and Hencky strain relationships; (b) stress profile as a function of crack length; (c) Young's modulus; and (d) fracture stress and strain profile for experimental cheese variants: Control, control cheeses; noWR, cheese without warm-room ripening; CC, cheese made using fermentation-produced camel chymosin as a coagulant; PepA, cheese containing chymosin inhibitor, i.e., pepstatin A, which was added to the curd-whey mixture during cheese manufacture. Data presented are means of data from two replicate trials. Error bars represent standard errors of means.

5.4.4 Dynamic visualization of cheese microstructures under tensile deformation

Changes to the microstructures of cheese during tensile deformation were obtained using CSLM. Widening of the notch (Figure 5.4a), stretching of the protein network (in the direction of tensile force) near the notch tip (Figure 5.4b-c) and initiation of fracture from the leading point of notch (Figure 5.4d) were all observed prior to propagation (i.e., rapid growth of fracture surface) of a notch (Figure 5.4e-f) during tensile deformation, in all experimental cheeses, regardless of maturation level or applied treatment. Stretching of the protein network near the notch tip is due to stress concentration (Luyten, 1988).

Moreover, on tensile deformation, the fat globules near the notch tip appeared disconnected from the stretched protein networks (Figure 5.4c-f), suggesting that the fat globules within the semi-hard cheese matrix were unbound to the protein matrix or weakly held (entrapped) within the protein matrix. Furthermore, fracture propagated through protein network in all cheeses leaving fat globules/pools intact. Abhyankar et al. (2014) also reported a similar observation in a particulate gel, i.e., whey-protein-emulsion-filled gels prepared at pH 5.4.

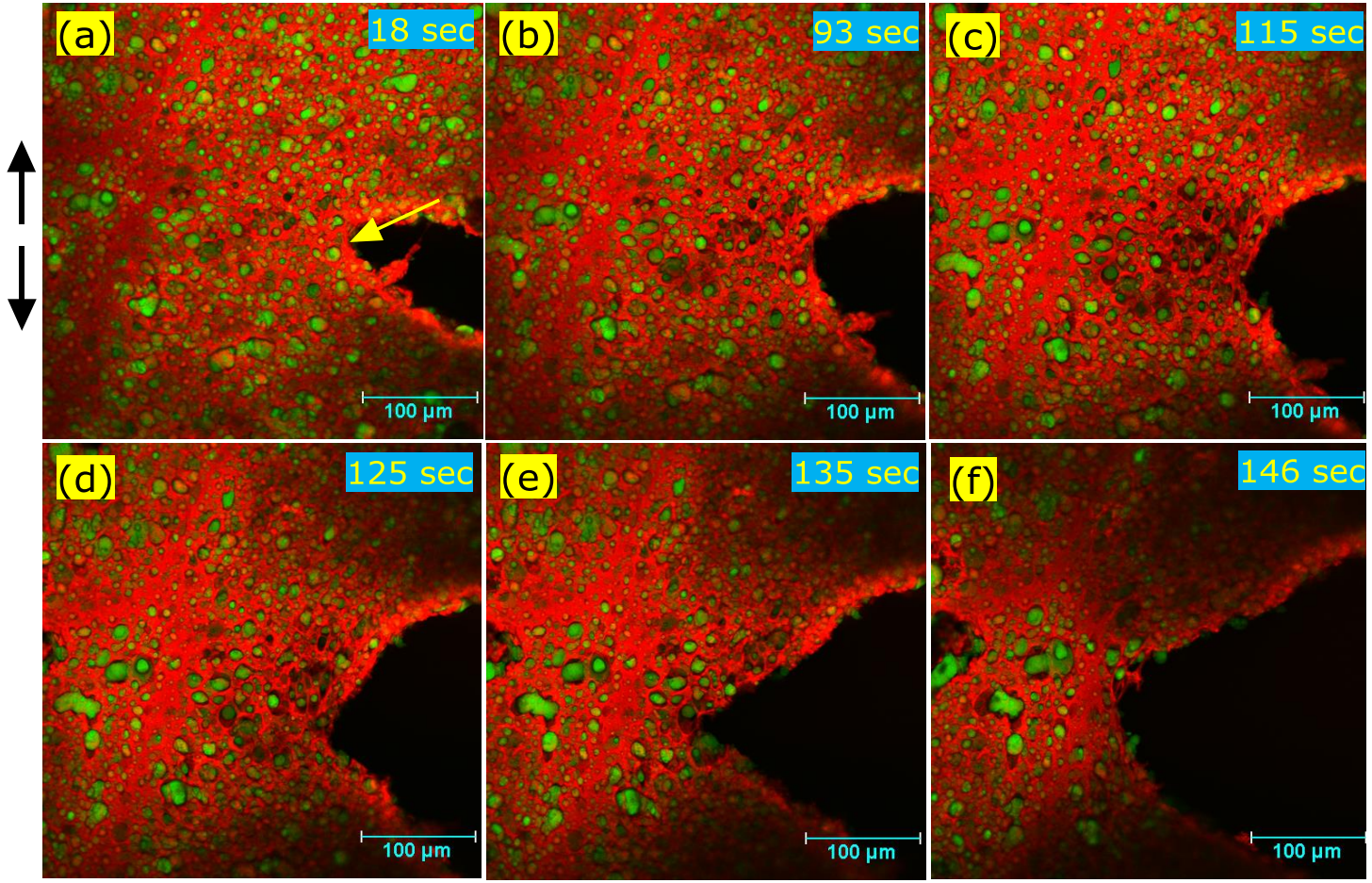


Figure 5.4. Selected confocal scanning laser microscopy (CSLM) micrographs of cheese obtained during tensile deformation of a notched sample, illustrating; (a) opening of the notch, (b-c) stretching of the protein network near the notch tip and (d-f) fracturing from notch tip. The arrow in the micrograph (a) shows the artificial notch tip. The protein phase appears red while the fat phase appears green. Arrows beside the micrographs indicate the direction of tensile deformation.

Selected CSLM micrographs taken during tensile deformation of an artificially notched sample are shown in Figure 5.5. The time-series micrographs clearly show release of fat from the cheese matrix. The fat pool within the circle (Figure 5.5a-c) is the same fat pool observed at different time points during tensile deformation. Utilizing image analysis, the diameter of the fat pool within the circle (Figure 5.5a), was found to be ~20 µm, which was higher than the average diameter of native milk fat globules, i.e., 4 µm (Lopez, 2005), suggesting that the fat pool was either coalesced fat (resulting from fusion of individual fat globules) or non-globular fat (Lopez, Camier, & Gassi, 2007). Cheese manufacture steps, such as, cooking and pressing, can cause aggregation, coalescence and disruption of fat globules, with the formation of non-globular fat or free fat (Lopez et al., 2007). Release of fat from the protein matrix was not only observed at the initial stages of notch propagation, but was also observed at the advanced stages of notch propagation. Furthermore, the release of fat from the cheese matrix was observed at all stages of ripening (micrographs not shown). These results suggest that the applied technique is suitable for understanding fat-protein interactions within semi-hard cheese matrix.

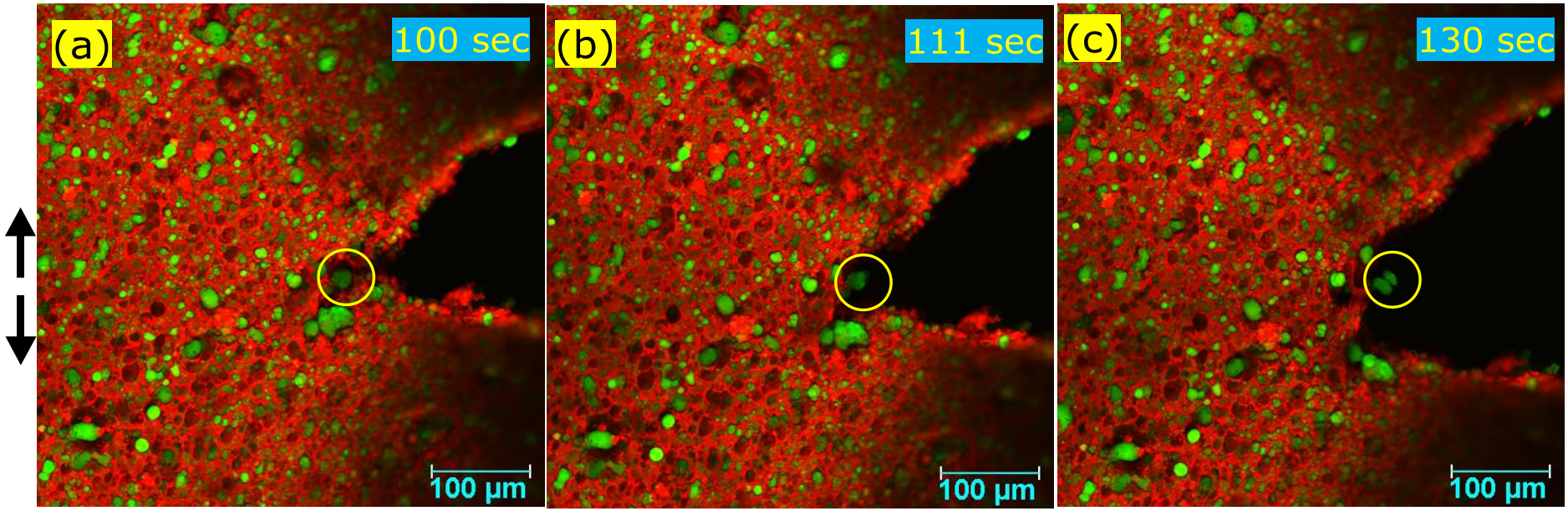


Figure 5.5. Selected confocal scanning laser microscopy (CSLM) micrographs of cheese (90 d of ripening) during tensile deformation of a notched sample, illustrating release of fat globules from the cheese matrix. The fat globule within the circle represents the same globule at different time points during tensile deformation. The protein phase appears red while the fat phase appears green. Arrows beside the micrographs indicate the direction of tensile deformation.

From a materials science perspective, cheese can be viewed as a 2-phase composite material containing fat globules as a filler in a protein gel matrix (Barden, Osborne, McMahon, & Foegeding, 2015; Lamichhane et al., 2018a). The sensory perception of foods is largely determined by properties of the filler, the extent of filler-matrix interactions, distribution of the filler, and characteristics of the gel matrix (Lamichhane et al., 2018a). A better understanding of interactions between the protein matrix and fat globules is desirable to understand the release patterns of fat globules in the mouth during mastication, which in turn will influence texture and potentially flavour perception. Studies have shown that emulsion-filled gels with unbound fat droplets exhibit stronger fat-related sensory perceptions than bound fat droplets (Sala, van de Velde, Cohen Stuart, & van Aken, 2007; Liu et al., 2015).

To better understand the fracture behaviour at a macroscopic level, changes in cheese structure were also observed at low magnification during tensile deformation. The fracture initiated from the leading point of notch and then propagated in an irregular zig-zag manner, probably due to the inhomogeneous nature of the cheese matrix. Similar fracture behaviour has previously been reported in whey-protein-emulsion-filled gels prepared at pH 5.4 (Abhyankar et al., 2014).

It is possible to observe, in real time, whether the fracture goes through individual curd granule or along curd granule junctions by observing cheese microstructure at low magnification. The density of the protein network at the curd granule junction (Figure 5.6, short arrow) is higher compared to the interior of curd granules, due to leaching of the fat from the curd granule surface to whey during cheese manufacture. Selected CSLM micrographs (Figure 5.6) taken during tensile deformation of an artificially notched sample clearly show the fracturing of cheese

sample partly along a curd granule junction. This suggested the presence of localized weak zones along the curd granule junctions within the cheese matrix, which wasprobably due to the strength and types of bond formed between the networks of curd granules.

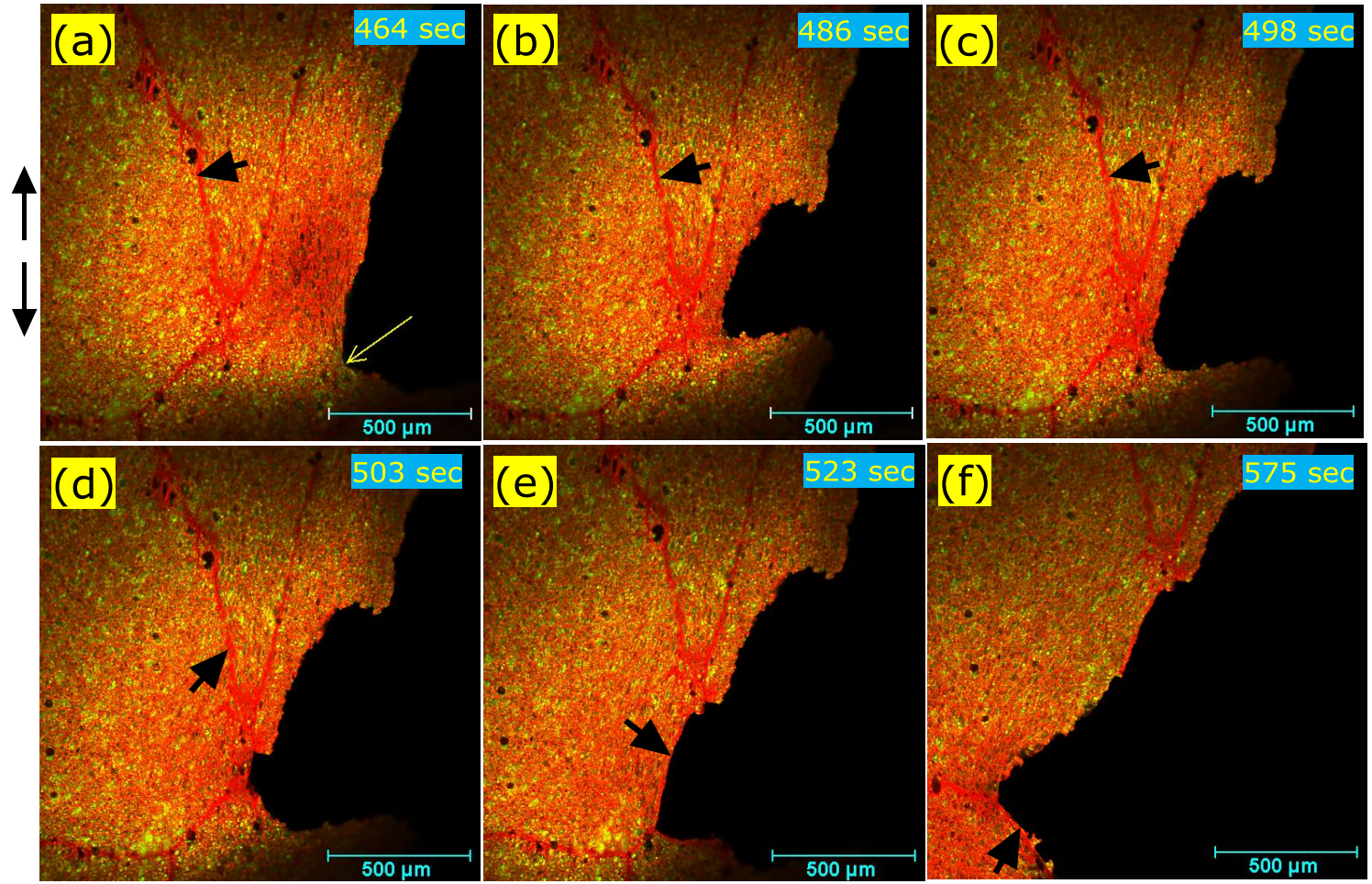


Figure 5.6. Sequence of confocal scanning laser microscopy (CSLM) images of cheese obtained during tensile deformation of a notched cheese sample after 20 d of ripening, illustrating the growth of a crack partly along a curd granule junction. Long and short arrows show the crack tip and curd granule junction respectively. The protein phase appears red while the fat phase appears green. Arrows beside the micrographs indicate the direction of tensile deformation.

An inherent micro-defect (~32 µm; Figure 5.7a) located at a curd granule junction was observed within the cheese matrix. During tensile deformation, the cheese sample not only fractured from the artificial notch but also fractured from the inherent defect located at this curd granule junction (Figure 5.7b-e), and finally both cracks merged together (Figure 5.7f). Interestingly, the inherent defect fractured along the curd granule junction. This suggested that defect within the semi-hard cheese matrix was most likely to be present at the curd granule junction, in agreement with previous reports (Huc, Moulin, Mariette, & Michon, 2013; Huc et al., 2014). Moreover, defects at curd granule junctions tend to fracture along curd granule junction under deformation. This leads us to hypothesize that the presence of micro-cracks within the cheese matrix could be precursors to splits and cracks observed in some eye-type cheeses; micro-cracks may grow gradually during ripening due to the stress exerted by the gas produced by micro-organisms, especially during warm-room ripening, when diffusion of gas at nuclei may create a tensile force within the cheese matrix.

Other studies have also observed micro-cracks (size ranging from 50-200 µm) in eye-type cheeses (Huc et al., 2013; Huc et al., 2014). The actual reasons for occurrence of inherent defects (micro-cracks) within the cheese matrix are not fully understood. However, entrapment of whey pockets, air bubbles or free fat between curd granules are considered as possible reasons, which may inhibit the formation of bonds between the network of curd granules (Akkerman, Walstra, & Van Dijk, 1989; Luyten & Van Vliet, 1996). It may also be possible that temperature variations between cheese curd granules during pressing and localized differences in composition and pH within the cheese matrix (Burdikova et al., 2015) could be contributory factors.

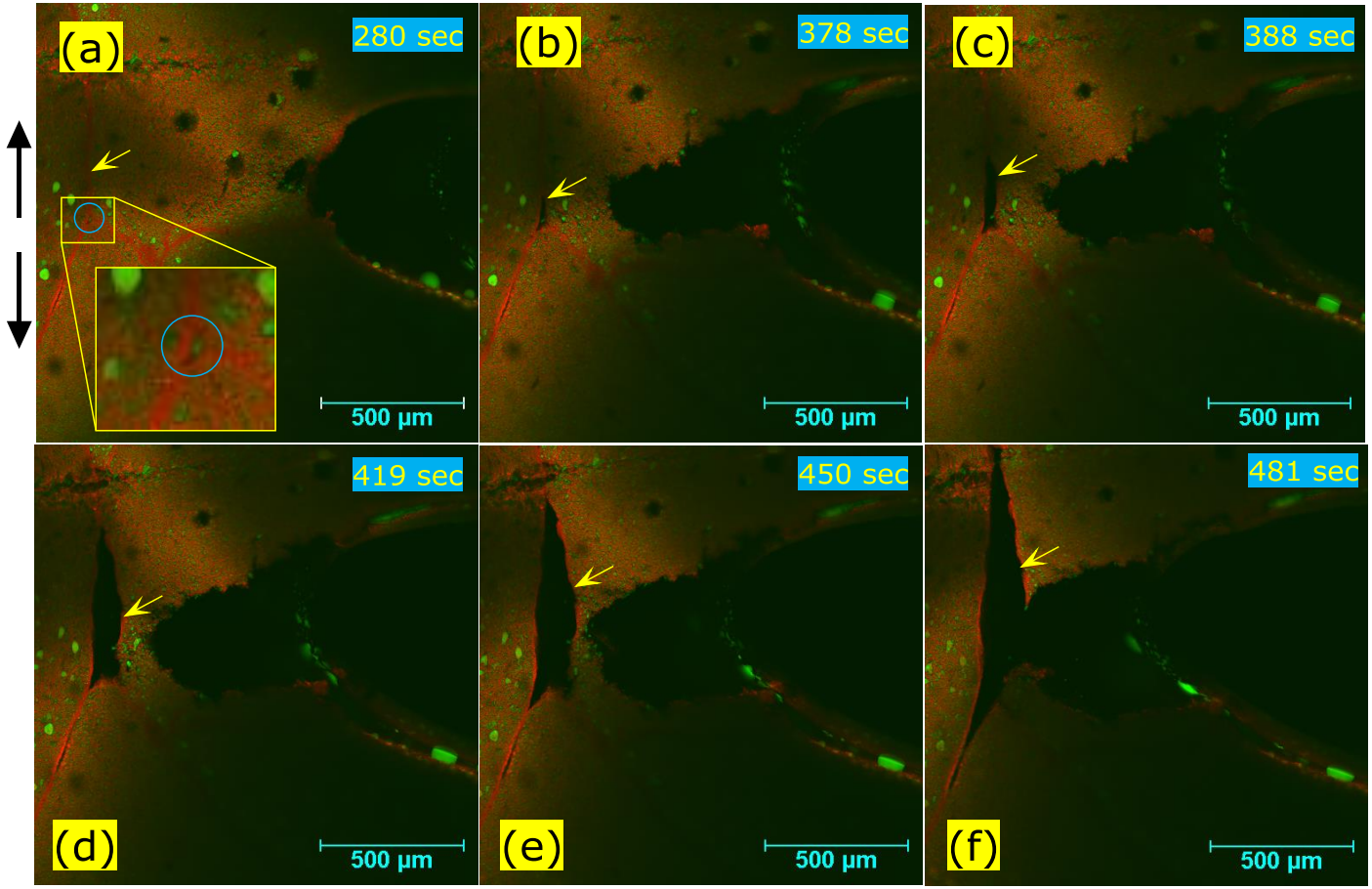


Figure 5.7. Sequence of confocal scanning laser microscopy (CSLM) images of cheese taken during tensile deformation of a notched cheese sample after 90 d of ripening, illustrating the fracture behaviour in the presence of an inherent defect at a curd granule junction. The circle, in the micrograph and in the inset (a), shows the inherent defect (~32 µm) at a curd granule junction and the arrow shows the curd granule junction. Both the inherent defect and the notch (b-e) grew gradually and (f) merged during tensile deformation. The protein phase appears red while the fat phase appears green. Arrows beside the micrographs indicate the direction of tensile deformation.

5.5 Conclusions

This study successfully achieved dynamic visualization of semi-hard cheese microstructure under large-strain tensile deformation. On deformation, the fat globules/pools were detached from the protein network and subsequently released, confirming that the fat globules within semi-hard cheese matrix are not bonded to the protein matrix but rather weakly held/entrapped within the proteins matrix. Moreover, the cheese matrix fractured (at least in part) along curd granule junctions, suggesting the presence of localized weak spots along the curd granule junctions within the cheese matrix. Inherent micro-defects were observed within the cheese matrix, and these defects were most likely to be present at the curd granule junction. It was proposed that these micro-defects could be a key underlying factor in the formation of undesirable slits or cracks. During fracturing, stress profiles of experimental cheeses varied with the levels of maturation, ripening temperature, coagulant type or residual chymosin activity, suggesting that the fracture behaviour of semi-hard cheese can be modulated by changing maturation levels, ripening temperature, using a different coagulant type, or inhibiting the residual chymosin activity. Overall, this study demonstrated the applicability of dynamic *in situ* imaging of cheese microstructure to better understand fat-protein interactions as well as predicting weak zones within a cheese matrix. Such structural information is particularly relevant for understanding texture and flavour perception, as well as mechanisms of formation of undesirable slits and cracks within the cheese matrix. Furthermore, the approach used in this study could be applied to establish a greater understanding of structure-fracture relationships in cheese, as well as other food products.

5.6 Supplementary materials

Supplementary Table 1. General overview of the treatments and ripening regimens used in the study[1]

Treatment	Cheese type[2]			
	Control	noWR	CC	PepA
Rennet type	FPBC	FPBC	FPCC	FPBC
Chymosin inhibitor	Not added	Not added	Not added	Added
Ripening regimen	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 8 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d

[1]FPBC = fermentation-produced bovine chymosin; FPCC = fermentation-produced camel chymosin

[2]noWR = cheese without warm room ripening; CC = cheese made using fermentation- produced camel chymosin as a coagulant; PepA = cheese containing chymosin inhibitor i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

Supplementary Table 2. Compositional parameters at 20 d and pH at 1 d of ripening in semi-hard cheeses[1]

Compositional factors	Cheese types[2]				*P*-value
	Control	noWR	CC	PepA	
Moisture (%, w/w)	40.83 ± 1.61[a]	40.89 ± 2.47[a]	40.95 ± 3.36[a]	41.84 ± 2.51[a]	0.96
MNFS (%, w/w)	56.24 ± 1.54[a]	56.11 ± 2.20[a]	56.26 ± 3.11[a]	57.05 ± 2.28[a]	0.96
Protein (%, w/w)	25.32 ± 0.90[a]	25.57 ± 1.47[a]	25.44 ± 1.56[a]	25.39 ± 1.48[a]	0.98
Fat (%, w/w)	27.42 ± 1.06[a]	27.16 ± 1.77[a]	27.29 ± 1.98[a]	26.70 ± 1.65[a]	0.95
FDM (%, w/w)	46.33 ± 0.91[a]	45.92 ± 1.46[a]	46.18 ± 0.85[a]	45.88 ± 1.28[a]	0.96
Salt (%, w/w)	1.34 ± 0.12[a]	1.38 ± 0.14[a]	1.38 ± 0.09[a]	1.36 ± 0.08[a]	0.96
S/M (%, w/w)	3.28 ± 0.31[a]	3.39 ± 0.51[a]	3.38 ± 0.41[a]	3.25 ± 0.08[a]	0.95
Total calcium (mg/100 g cheese)	867 ± 30[a]	861 ± 30[a]	837 ± 34[a]	842 ± 27[a]	0.60
pH (1 d)	5.18 ± 0.03[a]	5.21 ± 0.03[a]	5.19 ± 0.03[a]	5.23 ± 0.03[a]	0.28

[1]MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio; Control, control cheeses; noWR, cheeses without warm room ripening; CC, cheeses made using fermentation-produced camel chymosin; PepA, cheeses containing chymosin inhibitor i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

[2]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean ± standard deviation of data from three replicate trials.

5.7 References

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Chapter 6: Solubility of carbon dioxide in renneted casein matrices: Effect of pH, salt, temperature, partial pressure, and moisture-to-protein ratio

6.1 Abstract

The contribution of individual components of cheese matrices on CO_2 solubility, especially the protein or moisture phases, remains unclear. Therefore, this study investigated the solubility behaviour of CO_2 in model casein matrices, representing the moisture-protein phase of cheese. Renneted casein matrices were prepared from micellar casein concentrate (MCC) and the salt and pH levels of the gels were modulated by adding salt and glucono delta-lactone to the MCC solutions prior to renneting. Different moisture-to-protein levels were achieved by freeze-drying, incubation of samples at different relative humidity, or by applying varying pressures during gel manufacture. The CO_2 solubility of samples decreased ($P < 0.05$) linearly with both increasing temperature (between 5 °C and 25 °C) and salt-in-moisture content (between 0.06 and 3.2%), whereas solubility of CO_2 increased ($P < 0.05$) with increasing pH (between 5.4 and 6.2). A complex relationship was observed between CO_2 solubility and the moisture-to-protein ratio of experimental samples; the CO_2 solubility first decreased with increasing moisture-to-protein ratio (M/P) from ~0.03 to ~1.7, followed by a gradual increase with increasing M/P from ~1.7 to 2.5. Overall, the solubility or absorption capacity of protein matrices were significantly influenced by varying levels of moisture-to-protein ratio, salt content, pH, temperature and partial pressure. This knowledge may be applied to improve the quality and consistency of eye-type cheese, and in particular to avoid development of undesirable splits and cracks.

6.2 Introduction

In some eye-type cheeses, such as Emmental and Maasdam, propionic acid bacteria produce a high level of carbon dioxide, especially during warm-room ripening. The rate and extent of gas production and its behaviour in the cheese matrix (e.g., solubility and diffusivity) are considered important factors for the development of eyes, but are also implicated in the undesired development of slits or cracks within those cheese types (Daly, McSweeney, & Sheehan, 2010b). It is believed that CO_2 produced within the cheese matrix first solubilizes/dissolves within the components of the cheese matrix. Once the cheese body becomes saturated with gas, it then diffuses to the nuclei for eye-development or diffuses outward through the cheese rind. It is reported that the ~50% of the total CO_2 gas produced is dissolved in the cheese body (Walstra, Wouters, & Geurts, 2005).

Studies have suggested that the solubility capacity of the cheese matrix largely depends on factors such as cheese composition, temperature, and partial pressure. Carbon dioxide solubilises in both the aqueous and fat phases of cheese; however, the solubility capacity of each phase is temperature-dependent (Jakobsen, Jensen, & Risbo, 2009). CO_2 solubility in the aqueous phase of cheese has been reported to decrease with increasing temperature whereas the CO_2 solubility in the fat phase has been reported to increase with increasing temperature (Jakobsen et al., 2009). Acerbi, Guillard, Guillaume, and Gontard (2016) studied the effect of temperature, partial pressure, salt and moisture content on the solubility behaviour of CO_2 in semi-hard cheese. Those authors observed a decrease in CO_2 solubility with increasing temperature and salt level. However, a complex relationship was observed with moisture level, which has been attributed to concomitant changes in protein

content with changing moisture levels. Those authors recommended conducting further research to clarify the influence of nitrogen content on CO_2 solubility in cheese.

Although several studies have investigated CO_2 solubility behaviour in food matrices (Acerbi et al., 2016) or in pure fat (Jakobsen et al., 2009; Truong, Palmer, Bansal, & Bhandari, 2017), solubility behaviour of CO_2 in dairy protein matrices is not yet fully understood. In fact, studies have neglected the effect of protein content on the CO_2 solubility (Jakobsen et al., 2009; Acerbi et al., 2016). However, it is difficult to investigate the effect of each individual component on solubility behaviour in a multi-component food system, as changing of one compositional parameter results in consequential changes to other compositional parameters. Therefore, studies using model systems may be better suited to understand the effect of each component individually on solubility behaviour of CO_2.

The primary aim of this study was to investigate the effect of moisture-to-protein ratio on solubility of CO_2 in renneted-casein gel matrices rather than simply in a protein-only matrix, as it is not possible to vary protein content without changing the moisture level. In the majority of food matrices, protein is mostly present in a hydrated state, and its level of hydration is dependent on product type; for example, there are low levels of protein hydration in dairy powders as compared to cheese. An additional aim of this study was to elucidate the effect of varying levels of salt, pH, temperature and partial pressure on the solubility of CO_2 in model renneted-casein gel matrices.

6.3 Materials and methods

6.3.1 Preparation of renneted casein matrix

Liquid micellar casein concentrate (MCC; protein content = 14.55%, w/w; total solids: 18.34%, w/w) was produced as reported by Xia et al. (unpublished data) and stored at -18 °C. Prior to use in experiments, the MCC was thawed in a water bath at 50 °C and an aliquot (400 g) was placed in a 500 mL beaker with 0.03 % (w/w) sodium azide (BDH Chemicals, Poole, England) as a preservative. The desired salt concentration and pH levels of the final gels, were achieved by mixing varying levels of NaCl (0, 1.5, or 2.5%, w/w) and glucono-δ-lactone (GDL; 0.5, 1.2, or 2%, w/w; Sigma-Aldrich) into the MCC with a magnetic stirrer. Three minutes after salt and GDL addition, fermentation-produced bovine chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 0.82 mL/kg MCC. Rennet addition was based on MCC protein content. All renneted milk concentrates were incubated at 32 °C for 30 min to induce gel formation and stored overnight at 4 °C for completion of GDL hydrolysis.

On the following day, all gels were incubated in a water bath at 40 °C for 1 h to promote expulsion of whey/moisture. Each gel was then collected into a mould and pressed vertically under increasing pressure (up to 195 kPa) for 3 h to obtain the desired final moisture content. All gels were then vacuum-packed (Falcon 52, Original Henkelman vacuum system, 's-Hertogenbosch, the Netherlands), and stored at 4 °C.

6.3.1.1 Preparation of a model system to investigate the effect of partial pressure and temperature

To investigate the effect of partial pressure and temperature on CO_2 solubility, three identical casein matrices were prepared of moisture and salt content ~60% (w/w) and ~2% (w/w), respectively, and with a pH of ~6.8.

6.3.1.2 Preparation of a model system to investigate the effect of salt

To investigate the effect of salt on the CO_2 solubility, three casein matrices were prepared in triplicate, by adding three different salt levels, i.e., 0, 1.5, and 2.5%, w/w, to the MCC. Other parameters, including levels of protein and moisture, and pH, were all kept constant.

6.3.1.3 Preparation of a model system to investigate the effect of pH

To investigate the effect of pH on the CO_2 solubility, three casein matrices, of three different pHs, were prepared in triplicate, by adding varying levels of GDL (0.5, 1.2, or 2%, w/w) to the MCC. Other parameters, including levels of salt, protein and moisture, were all kept constant. A series of preliminary experiments were conducted to determine the levels of GDL necessary to achieve the desired pH value, ranging between 5.4 and 6.2.

6.3.1.4 Preparation of a model system to investigate the effect of moisture to protein ratio

Approaches, such as application of variable pressure during manufacture, freeze-drying, or incubation of samples in various relative humidity environments, were applied to achieve desired hydration levels of the casein matrices. Increasing pressing pressure up to 98 kPa for 2 h was applied to achieve casein matrices with a moisture content of ~67% (w/w), whereas increasing pressure up to 197 kPa for 3 h

was applied to achieve casein matrices with a moisture content of ~59% (w/w). Casein matrices with a moisture content of ~47% (w/w) or ~34% (w/w) were prepared by incubating small slices of casein gel (each of ~2 g, initial moisture content of ~59%) for 1 to 2 weeks, at 4 °C, in desiccators containing a saturated solution of LiCl. Casein matrices of very low moisture content (~2%, w/w) were prepared by freeze drying. Some freeze-dried samples were rehydrated to achieve a moisture content of 15% (w/w) or 19% (w/w) by incubating (for 2 weeks at 25 °C) in a hermetically sealed container maintaining a relative humidity of 97% (using saturated potassium sulphate) or 100% (using pure water), respectively.

6.3.2 Composition analysis

Moisture, protein and salt contents were determined as described by Lamichhane, Kelly, and Sheehan (2018a). The fat content of samples was determined using the Röse-Gottlieb method (IDF, 1996). The pH of gel samples was determined by directly inserting a penetrating pH probe (HQ11d, Hach) into gel samples.

6.3.3 Determination of freezable and non-freezable moisture

Levels of freezable and bound moisture were determined using a differential scanning calorimeter (DSC; Q200, TA Instruments, New Castle, DE, USA) as described by McMahon, Fife, and Oberg (1999). Freezable moisture is defined as water freezeable at −40 °C, whereas non-freezable moisture was defined as the water that did not freeze at −40 °C (McMahon et al., 1999).

6.3.4 Modified atmosphere packaging

In triplicate, experimental samples of 1.5 to 2 g, were each individually packaged into pouches (length = 25 cm, width = 18.5 cm; Amcor Flexibles,

Denmark) of high gas impermeability using a modified atmosphere packaging machine equipped with a two-gas mixture (A 300; Multivac, Germany). To investigate the effect of partial pressure and temperature on CO_2 solubility, samples were packed with a CO_2:N_2 gas mixture of 0:100; 30:70; 60:40 and 100:0 and stored at 5 °C, 12 °C and 25 °C for 2 d. To investigate the effect of salt, pH and casein hydration, samples were packed under 100% CO_2 and stored at 5 °C for 2 days.

6.3.5 Determination of the concentration of CO_2 in the headspace of modified atmosphere packages

The concentration of CO_2 in the headspace of modified atmosphere packages was determined using a headspace gas analyser (CheckMate 9900, PBI-Dansensor A/S, Ringsted, Denmark). To prevent gas leakage from the packaging material during measurement, a septum (diameter = 15 mm, MACON Europe A/S, Denmark) was attached to the top of the packaging materials, which was pierced by the needle of the headspace gas analyser.

6.3.6 Determination of the concentration of CO_2 in renneted-casein matrices

The concentration of carbon dioxide in the renneted-casein matrix was determined using a titration method as described in previous studies (Gill, 1988; Jakobsen et al., 2009; Truong et al., 2017). Briefly, a pair of side-armed conical flasks (100 mL; Pyrex), one containing 10 mL of 0.5 M H_2SO_4 and another containing 3 mL of 0.1 N $Ba(OH)_2$, connected by a reinforced PVC tube, were used for extraction and subsequent scavenging of CO_2 from experimental samples. Experimental samples (~1.5 g) held under modified atmosphere packaging were transferred immediately to the flask containing 0.5 M H_2SO_4, which was sealed using a neoprene stopper. Therein, the CO_2 evolved from the experimental sample reacted with $Ba(OH)_2$ (present in the other flask) and produced a $BaCO_3$ precipitate.

After at least 24 h, the residual $Ba(OH)_2$ was titrated against a standard HCl solution (0.1 M) using phenolphthalein as an indicator.

6.3.7 Statistical analysis

Statistical analyses of the data were performed using SigmaPlot version 14 (Systat Software, Inc., San Jose, California, USA). The effect of treatment on CO_2 solubility of casein matrices was determined performing one way ANOVA followed by *posthoc* Student-Newman-Keuls tests. Before ANOVA evaluation, data were checked for homoschedasticity and normality by performing Brown-Forsythe and Shapiro–Wilk tests, respectively. The level of significance was set at $P \leq 0.05$. Regression analyses of the data were performed using SigmaPlot version 14 (Systat Software, Inc., San Jose, California, USA).

6.4 Results and discussions

6.4.1 Effects of partial pressure and temperature on solubility of CO_2

A linear relationship was observed between the concentrations of CO_2 in the experimental samples and the CO_2 partial pressure of the headspace of corresponding samples at all three temperatures investigated (Figure 6.1a). These results are in agreement with previous studies on cheese (Jakobsen et al., 2009; Acerbi et al., 2016) and on anhydrous milk fat (Truong et al., 2017). The linear regression equations obtained had very high coefficients of determination (R^2 = 0.98-0.99), thus validating Henry's law for the casein matrix studied. However, a small deviation from the origin was observed at zero CO_2 partial pressure. This deviation in CO_2 solubility of samples ranged between 0 and 2.68 mmol/kg. Similar deviations from the origin have previously been observed in cheese (Jakobsen et al., 2009; Acerbi et al., 2016) and in anhydrous milk fat (Truong et al., 2017) and were

attributed to the inherent presence of carbamate or carbonate species within the sample (Jakobsen et al., 2009; Acerbi et al., 2016). We believe that such a small offset plays a negligible role in the overall determination of the relationship between the physicochemical parameters of the matrix and CO_2 solubility.

The influence of temperature on CO_2 solubility in cheese (containing fat, protein and moisture) (Jakobsen et al., 2009; Acerbi et al., 2016) and in pure fat systems (Truong et al., 2017) has been previously studied, with a decrease in CO_2 solubility being observed with increasing temperature in semi-hard cheeses.

Based on CO_2 solubility behaviour in pure water and pure butterfat, Jakobsen et al. (2009) concluded that the solubility of CO_2 in the aqueous phase of cheese decreased with increasing temperature, whereas CO_2 solubility in the fat phase increased with increasing temperature. However, those authors did not take into account the influence of protein content, which was present in a considerable amount (30.6%, w/w) within the semi-hard cheese matrices studied. Moreover, the aqueous phase of cheese is more complex than pure water. Therefore, we investigated the role of temperature on solubility behaviour of CO_2 in casein matrices. The casein matrices had a moisture-to-protein ratio of ~1.8, similar to those found in some semi-hard cheese types such as Maasdam (Lamichhane et al., 2018a).

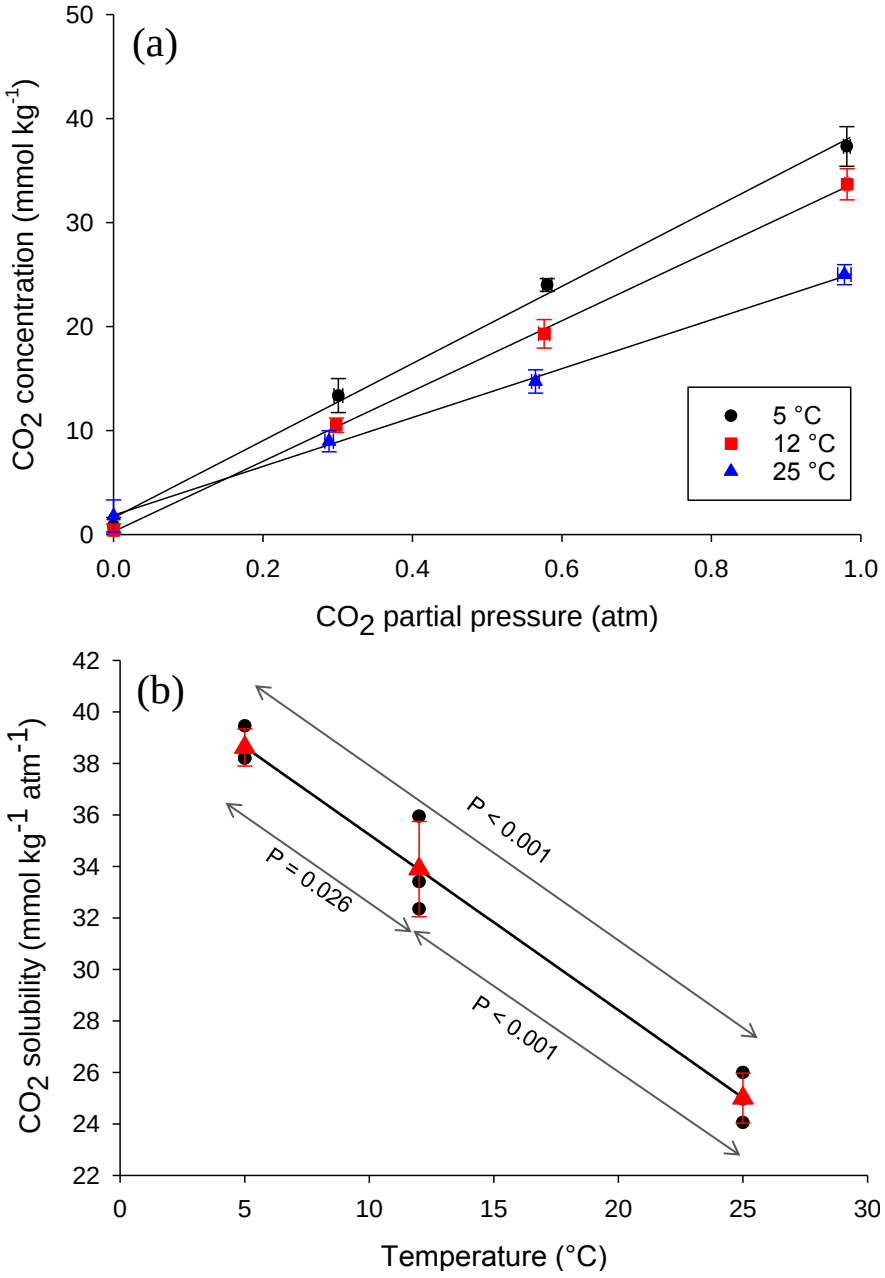


Figure 6.1. (a) Carbon dioxide concentration in casein matrices as a function of carbon dioxide partial pressure in the headspace of modified atmosphere packages and temperature. (b) Carbon dioxide concentration in the casein matrices as a function of temperature; (▲), mean of data from three replicate experiments; (●), individual data points; P represents P-value. Error bars represent standard deviations of means (n = 3). Average composition (± standard deviation) of samples: moisture content = 61.43±1.32 (%, w/w), protein content = 31.20±0.05 (%, w/w), fat content: 0.39±0.10 (%, w/w), salt content = 1.90±0.05 (%, w/w), salt-in-moisture content = 3.09±0.05 (%, w/w), and pH = 5.84±0.02.

It was observed in this study that the solubility of CO_2 in a casein matrix decreased ($P < 0.05$) linearly ($R^2 = 0.96$) as the temperature increased, with ~35% lower CO_2 solubility observed at 25 °C than at 5 °C (Figure 6.1b). It is proposed that the random molecular motion of the CO_2 gas molecules increases with increasing temperature (Cofie-Agblor, Muir, Sinicio, Cenkowski, & Jayas, 1995), thereby reducing the forces of attraction between CO_2 gas and the casein matrix, with subsequent release of CO_2 from the casein matrix.

Slits and cracks are usually observed during cold room storage and therefore we propose that the changes in CO_2 solubility with changing ripening temperature may contribute to the occurrence of such defects. Cheeses, such as, Maasdam and Emmental are pre-ripened for 1 to 2 weeks at 8-10 °C before warm room ripening (~23 °C) for 4-6 weeks for the development of eyes, and are finally stored at 2-4 °C. Propionic acid bacteria produce a high level of CO_2, especially during warm-room ripening, and a proportion of CO_2 produced dissolves in the cheese body, while the remainder diffuses to nuclei for eye-formation or diffuses outward through the cheese rind. Diffusion of CO_2 to nuclei causes expansion of cheese; it is reported that the Emmental cheese volume increases by ~25% due to production of CO_2 gas (Walstra et al., 2005). However, during cold storage (2-4 °C), it is expected that a proportion of carbon dioxide present in nuclei may solubilize in the cheese body as the solubility capacity of the cheese increases with decreasing temperature, which may result in a contraction of cheese. Moreover, the temperature of commercial cold rooms may fluctuate due to a range of circumstances, such as, increased external ambient temperature, or the arrival of new batches of cheese (from warm rooms) into the cold rooms. Such fluctuations in storage temperature may lead to contraction and expansion of the cheese body, resulting weakening of cheese structure, and thereby

contributing to the formation of cracks and slits. Further research is recommended to validate this hypothesis.

6.4.2 Effect of salt content on solubility of CO_2

The effect of three different salt concentrations on CO_2 solubility within the casein matrices was studied (Figure 6.2). Carbon dioxide solubility in the casein matrices decreased ($P = 0.004$) by ~22% with increasing average salt content from 0.04% (salt-in-moisture content: 0.06%, w/w) to 1.89% (salt-in-moisture content: 3.13%, w/w). This decrease in solubility may be attributed to a salting-out effect on the solubility of CO_2 in the aqueous phase of the casein matrix by NaCl in accordance with previous studies (Liu, Hou, Yang, & Han, 2011; Carvalho, Pereira, Gonçalves, Queimada, & Coutinho, 2015; Acerbi et al., 2016).

Although the effect of salt on CO_2 solubility behaviour in water and in aqueous solutions is well known, the role of NaCl on CO_2 solubility behaviour in solid food matrices is less documented. Acerbi et al. (2016) studied the impact of salt content on CO_2 solubility behaviour in semi-hard cheese matrices and, in agreement with our results, observed a significant decrease in CO_2 solubility (by ~25%) on increasing salt levels from 0.0 to 2.7 % (w/w).

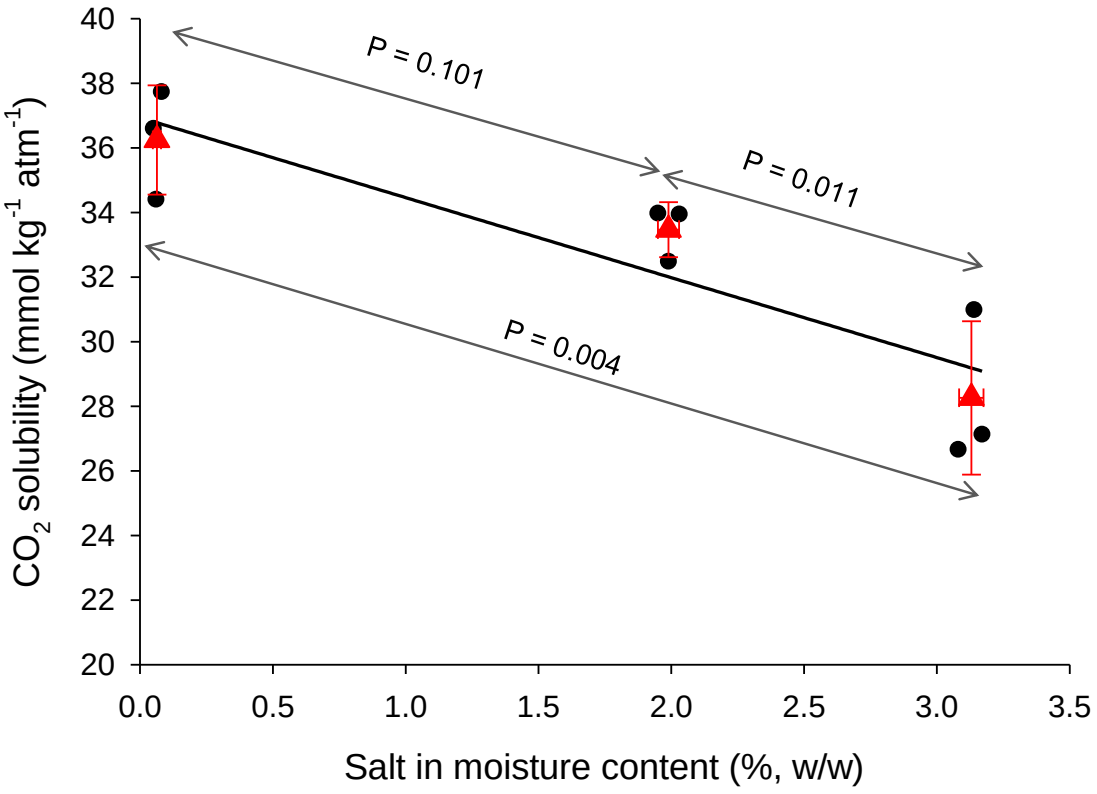


Figure 6.2. Effect of salt-in-moisture content on the solubility of carbon dioxide in casein matrices; (▲), mean of data from three replicate experiments; (●), individual data points; P represent P-value. Error bars represent standard deviations of means (n = 3). Average composition (± standard deviation) of samples: moisture content = 61.38±1.30 (%, w/w), protein content = 31.11±1.78 (%, w/w), fat content = 0.39±0.08 (%, w/w), and pH = 5.37±0.03.

The salt (or salt-in-moisture) concentration range selected was similar to those found in eye-type cheeses and those involving propionic acid bacteria (PAB) fermentation cheeses (Guinee, 2004; Lamichhane, Sharma, Kennedy, Kelly, & Sheehan, 2019). In brine-salted cheese types, salt diffuses inward from the rind to the centre of the cheese matrices, resulting in a decreasing salt gradient from the rind to the centre (Guinee, 2004). Time for attainment of equilibrium in salt-in-moisture content within the cheese matrix depends on composition, size and shape of the cheese and ripening conditions, among other factors. It has been reported that Gouda (10 kg wheel) and Emmental (60-130 kg wheel) cheese takes 7-9 weeks and > 4 months, respectively, for attainment of equilibrium in salt-in-moisture content within the cheese matrix (Guinee, 2004; Daly, McSweeney, & Sheehan, 2010a). Salt content in cheese can vary from batch to batch due to manufacture derived variables (e.g., temperature of brine and brining time), or over season due to variation in the composition of milk. Daly et al. (2010a) observed a significantly higher salt content in the interior area of Emmental cheese blocks produced in late in the season of manufacture than those produced at early in the season. Such intra-cheese and inter-cheese variation in salt content will likely result in heterogeneity in the local concentration of CO_2 within, or between, cheese blocks, leading to variable internal pressure within or between cheeses. This may result in some areas of the cheese matrices or some batches of cheeses, which may be more prone to development of slits or cracks. Although no specific locations for development of splits and cracks within the cheese matrices has been reported, a lower number of eyes have been found near the rind of Emmental cheese, where salt content is higher, than in the centre of the cheese (Guggisberg et al., 2015; Bisig et al., 2019).

6.4.3 Effect of pH on solubility of CO_2

Solubility CO_2 within the casein matrices increased ($P < 0.001$) by ~41% on increasing pH from 5.4 to 6.15 (Figure 6.3) with a quadratic relationship ($R^2 = 0.93$) providing a better fit than a linear relationship ($R^2 = 0.84$). Interestingly, the CO_2 solubility increased ($P = 0.026$) by ~14% on increasing pH from 5.4 to 5.8, whereas the CO_2 solubility increased ($P = 0.001$) by ~24% on increasing pH from 5.4 to 6.2. Such an increase in solubility is attributed to dissociation of an increasing fraction of the dissolved CO_2 as HCO_3^- with increasing pH (Gill, 1988). In solution, CO_2 can exist as dissolved CO_2, carbonic acid, bicarbonate or carbonate ions, and the fraction of carbonic acid, bicarbonate or carbonate ions in solution depends on the pH of that solution (Equation 6.1). With increasing pH from 5.4 to 6.15, increasing quantities of carbonic acid will dissociate as bicarbonate ions (HCO_3^-) and hydrogen ions (H^+). As a result, higher quantities of CO_2 will dissolve in the aqueous phase of the casein matrix (Jakobsen & Bertelsen, 2002). At pH values below 8, the carbonate ions (CO_3^-) in solution are present in negligible amounts (Dixon & Kell, 1989).

$$H_2O + CO_2 \longleftrightarrow HCO_3^- + H^+ \longleftrightarrow CO_3^- + 2H^+ \quad (6.1)$$

Very little is known regarding the effect of pH on CO_2 solubility in solid food systems and no consensus have been found among the studies. Gill (1988) on investigating the effect of pH of the muscle tissue of beef, pork and lamb observed a linear increase in the solubility of CO_2 with increasing pH from 5.4 to 6.9. Similarly, Jakobsen and Bertelsen (2006) reported a slightly higher solubility in meat tissue with higher pH (5.83) than lower pH (5.66). However, a difference of 0.5 pH units in the two meat types or fish types did not influence the solubility of CO_2 (Sivertsvik, Rosnes, & Jeksrud, 2004; Sivertsvik & Jensen, 2005). This discrepancy may be

attributed to a comparison of data between different samples with different compositions and possibly of different buffering capacity.

Dissolution of CO_2 in the aqueous phase of food matrices can decrease pH because of formation of carbonic acid (Singh, Wani, Karim, & Langowski, 2012). There was a concern that addition of CO_2 to the protein matrices would have resulted in reduction in pH in the samples, thus confounding our results relating to the effect of pH on CO_2 solubility. Therefore, the pH of the samples was measured before and after packaging in a modified atmosphere. It was observed that storage of small pieces (~10 g) of the casein matrices (3.1 % salt-in-moisture, 60% moisture content, and 32% protein content) under a 100% CO_2 environment for 2 d reduced the pH by ~0.1 unit (data not shown). However, for water samples of similar initial pH and salt-in-moisture content, pH decreased by ~2.3 units when stored under 100% CO_2 environment for 2 d (data not shown). The comparatively small decrease in pH of the casein matrices as compared to water may be attributed to its high buffering capacity, as food matrices of higher buffering capacity are expected to exhibit a greater resistance to pH change. Buffering capacity of food matrices largely depends on their composition. Proteins, inorganic phosphate and organic acids are the main constituents contributing to its buffering capacity of cheese (Salaün, Mietton, & Gaucheron, 2005).

During ripening, pH within the eye-type cheese matrices increases, due to the proteolytic liberation of basic compounds and metabolism of lactic acid by propionic acid bacteria, among other factors (Sheehan, Fenelon, Wilkinson, & McSweeney, 2007; Lamichhane et al., 2018a). Studies have reported an increase in pH from 5.2-5.3 at 7-11 d (before warm-room ripening) to ~5.5 at 35-41 d (after warm-room

ripening) and 6.0-6.1 at 270 d of ripening in Swiss, Dutch and related eye-type cheeses (Govindasamy-Lucey, Jaeggi, Martinelli, Johnson, & Lucey, 2011; Lamichhane et al., 2018a). Therefore, an increase in pH, especially above pH 5.8, during ripening of cheese may contribute to an increase in solubility of CO_2 within the cheese matrices. Moreover, natural cheese matrices can have both macroscopic and microscopic pH gradients (Burdikova et al., 2015), which may lead to heterogeneity in the concentration of CO_2 within the cheese matrices.

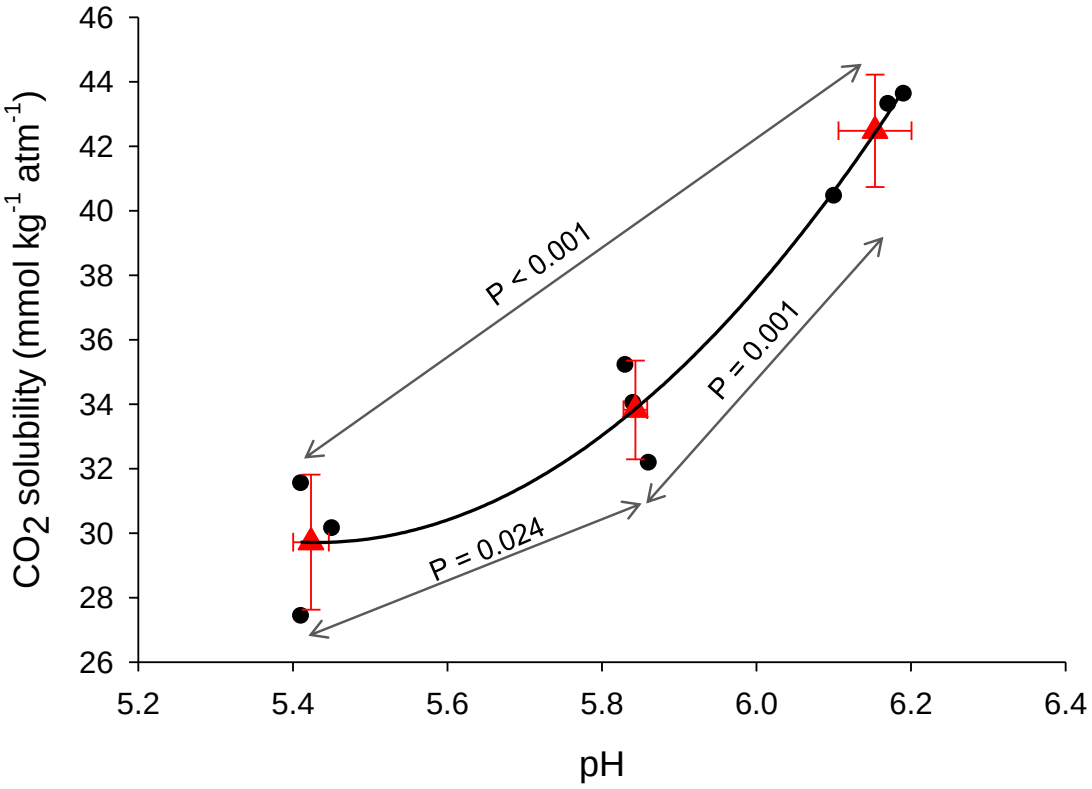


Figure 6.3. Effect of pH on carbon dioxide solubility in the casein matrices; (▲), mean of data from three replicate experiments; (●), individual data points; P represent P-value. Error bars represent standard deviation of mean (n = 3). Average composition (± standard deviation) of samples: moisture content = 61.05±0.9 (%, w/w), protein content = 31.81±0.96 (%, w/w), fat content = 0.39±0.08 (%, w/w), and salt content = 1.92±0.03 (%, w/w), salt-in-moisture content = 3.14±0.08 (%, w/w).

6.4.4 Effect of moisture-to-protein ratio on CO_2 solubility

To investigate the effect of moisture-to-protein ratio on CO_2 solubility, casein matrices having different moisture and protein contents were prepared.

Casein matrices with an average moisture-to-protein ratio of 0.027±0.009 [corresponding to an average moisture and protein content of 2.10±0.67% (w/w) and 78.72±1.26% (w/w) respectively] retained a considerable amount of CO_2, i.e., 161.7±24.68 mmol kg^{-1} atm^{-1} at 5 °C (Figure 6.4), in agreement with the results of Mitsuda, Kawa, Yamamoto, and Nakajima (1975). Those authors observed that dried casein and gelatin powders retained a considerable amount of CO_2 when stored under high CO_2 partial pressure. Although the exact reasons are not known, CO_2 adsorption by reactive sites in protein is considered as an important factor (Mitsuda et al., 1977; Cundari et al., 2009). Mitsuda et al. (1977) investigated the reactivity of certain particular functional groups involved in CO_2 gas adsorption by protein. The authors concluded that the α-amino, ε-amino and guanidinium groups are the preferred sites for CO_2 adsorption by protein in the gas-solid phase system. Cundari et al. (2009) analysed the binding of CO_2 to protein utilizing a combination of bioinformatics, molecular modelling, and first-principles quantum mechanics, and concluded that the hydrogen bonds between the functional groups of the amino acids and the oxygen sites on the carbon dioxide were involved in the CO_2 adsorption process.

The relationship between moisture-to-protein ratio and CO_2 solubility was non-linear (Figure 6.4), and can be divided into three distinct regions: (1) a rapid decrease in CO_2 solubility on increasing the moisture-to-protein ratio from ~0.03 to ~0.5; (2) a relatively slower decrease in CO_2 solubility with increasing moisture-to-protein ratio from ~0.5 to ~1.7; and (3) a small but significant increase in CO_2

solubility on increasing moisture-to-protein ratio from ~1.7 to ~2.5. Around a 4-fold decrease in CO_2 solubility was observed when the average moisture-to-protein ratio of casein matrices increased from ~0.03 to 0.5. Mitsuda et al. (1975) also reported similar solubility behaviour of CO_2 in dried casein and gelatine powder as a function of moisture content. The solubility of CO_2 in casein or gelatine powder decreased by >90% when their moisture content increased from ~5 or 10% (w/w) to 20 or 40% (w/w). The authors also observed a rapid decrease in CO_2 solubility of casein or gelatine powder prior to a gradual decrease in their CO_2 solubility on increasing moisture levels. Pre-adsorbed water may interact with the reactive sites of casein matrixes making those reactive sites unavailable for interaction with CO_2, thus decreasing the CO_2 adsorption/solubilisation capacity of hydrated casein matrices. A similar moisture-dependent CO_2 adsorption behaviour has also been observed in other non-food materials. For example, Ozdemir and Schroeder (2009) observed a lower CO_2 adsorption capacity of wet coals to that of dried coals. Those authors speculated that the adsorbed water occupies the pore space or the active sites for the adsorption of CO_2.

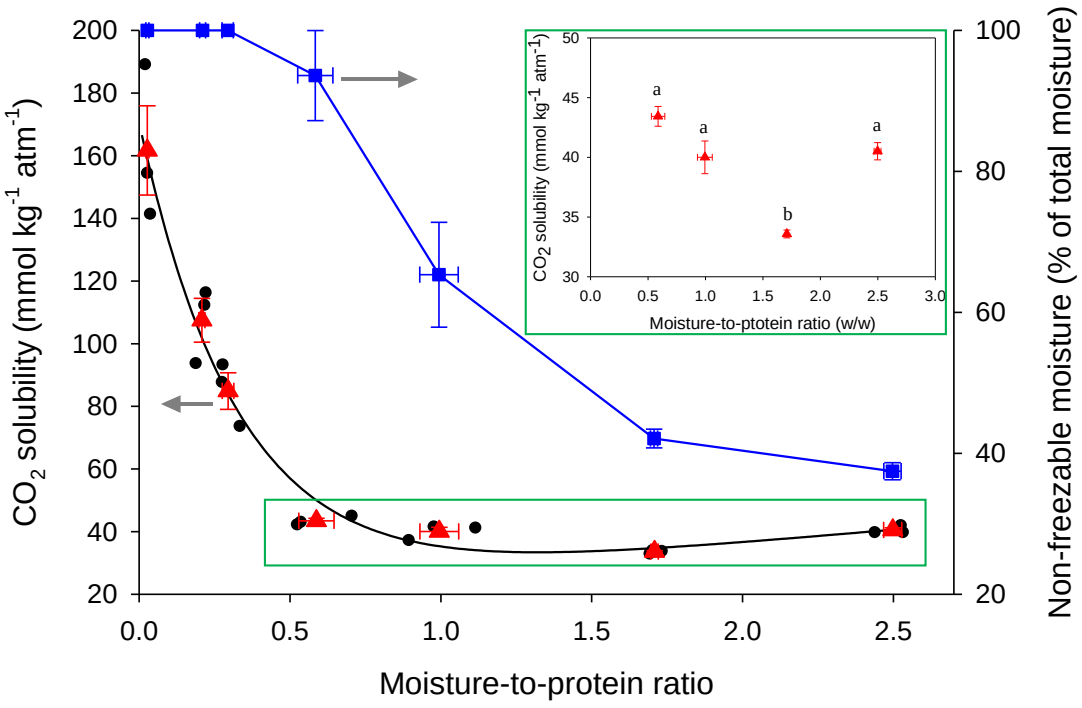


Figure 6.4. Relationships between moisture-to-protein ratio and (▲) CO_2 solubility or (■) non-freezable moisture (% of the total moisture) in renneted-casein matrices; (●) individual data points. Inset: magnification of CO_2 solubility data for casein matrices of moisture-to-protein ratio between 0.5 and 2.5; means with different letters differ ($P < 0.05$). Error bars represent standard deviations of means ($n = 3$).

Water in casein matrices is either present as a bulk (freezable at -40 °C) or bound form (non-freezable at -40 °C) (McMahon et al., 1999; Lamichhane, Kelly, & Sheehan, 2018b), and the latter is typically considered to be so-called primary hydration water and primarily related to the solvation of polar and charged residues (Huppertz et al., 2017). In casein matrices of moisture-to-protein ratio up to 0.5, almost all moisture (>98% of total moisture) was found to be in non-freezable form (Figure 6.4). This result further supports the hypothesis that the pre-adsorbed water may interact with the reactive sites (e.g., polar and charged residues) of casein matrices and making those reactive sites unavailable for CO_2 interaction.

The CO_2 solubility in casein matrices first decreased ($P < 0.05$) by ~23% with increasing moisture-to-protein ratio from ~0.5 to ~1.7 and then increased ($P < 0.05$) by ~21% with increasing moisture-to-protein ratio from ~1.7 to ~2.5 (Figure 6.4, inset). Such complex relationships observed between CO_2 solubility and moisture-to-protein ratio may be attributed to interactive effects of moisture and protein content on CO_2 solubility. This suggests that both water and protein components of casein matrices have an important role on CO_2 solubility.

In eye-type cheeses, the moisture-to-protein ratio is between 1.2 and 2.0, such as ~1.8 in Maasdam (Lamichhane et al., 2018a), ~1.7 in Edam (Guinee, 2016), and ~1.25 in Emmental (Deegan et al., 2013). Therefore, the CO_2 solubility studied in casein matrices with a protein-to-moisture ratio between 1.0 and 2.0 are particularly important for hard and semi-hard eye-type cheeses. Moisture-to-protein ratios in cheese may also vary on a batch-to-batch basis, due to seasonal variations in the composition of milk and thus the resultant cheeses. Therefore, these results could form the basis for development of a robust model for prediction of CO_2 solubility in

a wide variety of cheeses of different compositions, as models reported in previous studies were limited to the cheese type under study (Jakobsen et al., 2009; Acerbi et al., 2016).

Although the CO_2 solubility behaviour studied in casein matrices with a protein-to-moisture ratio below 1.0 is not relevant to natural cheese matrices, such knowledge may be useful when designing modified atmosphere packaging for cheese powders, where the protein to moisture ratio in spray-dried cheese powders was reported to vary between 0.05 and 0.12 (Felix da Silva, Larsen, Hougaard, & Ipsen, 2017).

6.5 Conclusions

This study investigated the solubility behaviour of CO_2 in casein matrices, representing varying conditions of the protein-water phase of semi-hard cheese matrices. Both compositional (i.e, moisture-to-protein ratio and salt-in-moisture content) and ripening-related (i.e., pH and temperature) parameters had a significant influence on CO_2 solubility of casein matrices.

It is proposed that fluctuation in the ripening and storage temperature may lead to contraction and expansion of the cheese body, resulting in weakening of cheese structure, and thereby contributing for the formation of cracks and slits. Further research is recommended to validate this hypothesis. Another possibility is that the cheese matrix becomes oversaturated with CO_2 gas, it can no longer contain all the solubilized gas due to changes in ripening related parameters, such as, temperature and pH, and rather than creating eyes the gas accumulates at the weak points in the matrix, e.g., at micro-defects and this leads to the formation of a slit or crack.

Variation in the cheese composition from batch to batch due to differences in milk composition or manufacture derived variables, such as time of the day of manufacture, plant temperature, temperature of brine and brining times, and rennet-to-casein ratio, may result in certain batches being more at risk for development of slits and cracks.

Overall, the result obtained from this study could form the basis for development of a robust model for CO_2 solubility in a wide variety of cheese types or where the composition of cheese may vary within a commercial cheese production plant. Such knowledge may help to improve the quality and consistency of eye-type cheese by minimizing or avoiding development of splits and cracks.

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Chapter 7: General discussion and recommendations for future research

7.1 General discussion

Ireland produces over 200,000 tonnes cheese per annum, over 90% of which is exported, to a value of €815 million. The UK is the largest export market, accounting for €407 million in exports in 2018 (Central Statistics Office, 2019), followed by Germany, the Netherlands, Algeria and France. In 2018, Cheddar accounted for ~67% of total cheese exports, predominantly to the UK (Central Statistics Office, 2019). However, there has also been growth in the export of continental and eye-type cheeses and this is projected to grow further, resulting from the development of a new 20,000 tonne per annum Jarlsberg cheese plant and a 45,000 tonne per annum Gouda type cheese plant in Ireland.

Abolition of milk quotas in 2015 has significantly increased Irish milk production and cheese has been targeted as a vital end-product for this increased milk pool due to continued increases in global cheese consumption, its high end-use versatility, its potential for significant added value, and as a profitable outlet for surplus milk fat (Sheehan, 2013).

In response to the increased milk production, increased market growth and consumer demand for other non-Cheddar cheese types in emerging markets, the Irish cheese industry is substantially investing to diversify from a heavy dependence on the production of Cheddar cheese into other cheese varieties. Similarly, changes to or creation of new trade agreements and barriers, such as through Brexit, have created a further trigger for Irish cheese industries to diversify their product portfolio and markets.

Recently, a public-private partnership between Teagasc and Ornua focused on developing a continental eye-type cheese for a key European export market. However, technological challenges exist to converting a highly seasonal Irish milk

supply of varying composition into Continental-type cheeses of consistent physico-chemical composition, with consistent mechanical and structural properties, ripening patterns and ultimately sensory quality from a textural, aesthetic and flavour perspective. Moreover, very little research has been published on the physicochemical, microstructural, textural and rheological properties, and ripening characteristics of mesophilic Continental semi-hard type cheeses with a propionic acid fermentation, such as Maasdam.

It is noteworthy that consistency in quality for Continental cheese types can be much more demanding to achieve than for Cheddar, not least in the development of eyes or of absence of undesired slits and cracks. Such defects result in poor aesthetic quality (a key retail requirement) and poor performance under high speed slicing for global food service markets, with consequent economic loss. Resolving this issue for Irish cheese producers would offer a very significant competitive advantage in key international markets.

Development of textural defects, such as, slits and cracks within the cheese matrix is an international problem in the manufacture and ripening of Swiss-, Dutch- and related eye-type cheeses, leading to downgrading of the product. The root cause is often linked to four specific areas (Daly, McSweeney, & Sheehan, 2010b): (1) initial physicochemical and textural defects arising due to variations in the cheese manufacture processes; (2) weakening during ripening of the structural integrity of the cheese matrix linked to calcium solubilisation and/or protein hydrolysis; (3) microbial activity resulting in late-gas formation; or (4) variable levels of carbon dioxide solubility within the individual moisture, fat and protein phases of the cheese matrix linked to conditions of changing temperature during ripening. Therefore, the

aim of this PhD study was to explore these parameters individually to understand and control the underlying causes.

It is now well recognized that many of the desirable properties and functionalities of cheese are largely determined by its structure; thus, Chapter 1 reviewed the most recent literature to provide a comprehensive review on structure-function relationships in cheese. In particular it provides an overview of how functional properties of cheese are influenced by the structural organization of cheese components and their interactions, as well as by environmental factors (e.g., pH and temperature).

The first research chapters investigated the effects of milk centrifugation, at a centrifugal force of ~9,000 × g, and incorporation of high heat-treated centrifugate on Maasdam cheese characteristics (Chapter 2 & 3). This was to determine the influence of the commercial process of centrifugation of milk to remove clostridia spores in particular, and other materials from milk. As expected, Maasdam cheeses made from centrifuged milk has significantly lower butyric acid levels (Chapter 3). However, no further significant influence of centrifugation was observed on the composition, texture, primary and secondary proteolysis (Chapter 2), and volatile organic compounds (except butyric acids; Chapter 3) of Maasdam cheese in comparison to control cheeses. This suggests that centrifugation of milk before cheese-making is a suitable method for controlling undesirable butyric acid fermentation without significantly altering the texture and other ripening characteristics of Maasdam cheese. However, some studies have reported a drastic reduction in the number of eyes in cheeses made from centrifuged or microfiltered milk (Thierry et al., 2010; Guggisberg et al., 2015) and this has been attributed to removal of micro-particles from the cheese-milk; these microparticles are known to

act as nuclei for development of eyes within the cheese matrices. Moreover, a reduction in the number of eyes within the cheese matrix may result in higher CO_2 overpressure, which can increase in the development of slits or cracks (Guggisberg et al., 2015). No definitive trends for eye and cracks or slits characteristics were observed in this study and further research is recommended, possibly including analysis of a large number of commercial samples over the course of a manufacturing season.

Reincorporation of the high heat-treated centrifugate into cheese-milk, as practised commercially to retain cheese yield, resulted in increased moisture in non-fat substance levels and decreased hardness levels in Maasdam cheeses in the current study. This has the potential to influence the structural properties of the cheese matrix and thus its ability to retain eye-quality, so care is required when incorporating high heat-treated centrifugate into the cheese matrix. However, given that the protein content of high heat-treated centrifugate is ~7% (w/w), not reincorporating this stream into cheese will lead to economic loss to the manufacturer (Kosikowski & Mistry, 1990); therefore, we propose two potential solutions to this problem: (1) slight modifications to the cheese-making procedure to counteract any increase in cheese moisture content, such as cutting curds to a smaller size or extending the vat residence time (i.e., time from cut to pitch); or (2) using this stream for manufacture of other dairy products (if possible), such as, yoghurt and high-heat milk powder.

Cheese fracture properties, especially shortness or brittleness have previously been associated with development of undesirable slits or cracks (Grappin, Lefier, Dasen, & Pochet, 1993; Rehn et al., 2011). Proteolysis and solubilisation of colloidal

calcium are two important age-related parameters determining the textural and rheological properties of cheeses (O'Mahony, Lucey, & McSweeney, 2005). However, no previous studies have been conducted to elucidate the effects of specific hydrolysis of α_{S1}- and β-caseins as well as different levels of insoluble calcium on the fracture behaviour of washed-curd brine-salted semi-hard cheeses in a single study. Therefore, an experiment was designed to investigate the individual contribution of primary proteolysis and solubilisation of colloidal calcium on fracture properties of washed-curd brine-salted semi-hard cheeses (Chapter 4). Specific hydrolysis of caseins in the semi-hard cheeses was achieved during ripening through the following approaches: (1) addition of a chymosin inhibitor, i.e., pepstatin A, to the curd/whey mixture during cheese manufacture; (2) substitution of fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC); and (3) modulation of ripening temperature. The study showed that cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein, were found to be more rigid, indicating that modulation of hydrolysis of α_{S1}-casein was an effective means for maintaining the strength of the cheese matrix during ripening. However, cheeses with low levels of intact β-casein or insoluble calcium content were more likely to be shorter in texture, suggesting that these parameters are potentially significant causes of development of slits and cracks in eye-type cheeses.

For the first time, a novel dynamic *in situ* imaging technique was applied to understand the microstructural changes occurring in semi-hard eye-type cheeses under large-strain tensile deformation (Chapter 5). Tensile deformation was used to mimic the effects of eye formation which results in some curd granules being moved apart. We observed micro-cracks within the semi-hard cheese matrices, especially at

curd granule junctions, and these developed into larger cracks which fractured along the curd granule junctions under tensile deformation. Based on these results, we propose that the presence of micro-cracks within the cheese matrix could be one possible factor for development of splits and cracks defects within the semi-hard cheese matrices. Therefore, it is suggested that research needs to be directed towards removing or reducing those defects within the cheese matrix, for example, by avoiding the entrapment of whey pockets, air bubbles or free fat between curd granules during cheese manufacture. Furthermore, this approach could be applied to establish a greater understanding of structure-fracture relationships in cheese, as well as other food products.

Among other factors, the behaviour of CO_2 gas within the cheese matrix, including solubility, is considered a critical factor in the development of eyes and splits or cracks within the cheese matrices (Acerbi, Guillard, Guillaume, & Gontard, 2016; Lamichhane, Kelly, & Sheehan, 2018). However, very little is known in relation to the solubility of CO_2 in various components of cheese matrices, especially the protein-moisture phase. Therefore, we investigated the solubility behaviour of CO_2 in a range of casein matrices, representing varying conditions of the protein-moisture phase of semi-hard cheese matrices (Chapter 6). The CO_2 solubility of casein matrices significantly depends on the protein-to-moisture ratio as well as on environmental factors, such as, pH, salt, and temperature. The moisture-to-protein ratio, pH and salt level in cheese have been reported to vary from batch to batch due to manufacture-derived variables (e.g., time of day of manufacture) or over season due to variation in the composition of milk (White, Broadbent, Oberg, & McMahon, 2003; Daly, McSweeney, & Sheehan, 2010a). Moreover, moisture, salt and pH gradients within brine-salted cheeses have been reported in previous studies (Guinee

& Fox, 2004; Burdikova et al., 2015). Such intra-cheese and inter-cheese variation in cheese composition will likely result in heterogeneity in the concentration of CO_2 within or between cheese blocks, leading to variable internal pressure within or between cheeses. This may result in some areas in cheese matrices or some batches of cheeses being more prone to development of slits or cracks. In addition, changing ripening temperature or fluctuations in storage temperature may lead to contraction and expansion of the cheese body due to the exchange of CO_2 between cheese eyes and body, resulting in weakening of the cheese structure, and thereby contributing to the formation of cracks and slits.

In conclusion, research undertaken in this thesis on the underlying factors associated with development of undesirable splits and cracks within semi-hard eye-type cheeses has greatly expanded knowledge in the area. Its key findings are:

- Incorporating high heat-treated centrifugate (also called bactofugate) into cheese-milk increased the level of moisture in non-fat substances and decreased the hardness of resultant cheeses, which will have the potential to influence subsequent eye characteristics, and split or crack development;
- Cheeses with low levels of intact β-casein or insoluble calcium were found to be shorter in texture, suggesting that these parameters are potentially significant causes of development of slits and cracks in eye-type cheeses;
- When the curds are stretched in a manner mimicking eye formation, micro-cracks transformed into slits and cracks. Thus micro-cracks are proposed to be a key basis for the formation of undesirable slits or cracks within the cheese;
- Carbon dioxide solubility within a casein matrix depends significantly on its composition (i.e., protein-to-moisture ratio and salt-in-moisture content) and

ripening-related parameters (i.e., pH and temperature). It is proposed that such variations will alter CO_2 gas concentration in the cheese body and subsequently in the eyes, leading to contraction (when CO_2 gas present in eyes is solubilised in the cheese body) or expansion (when CO_2 gas diffuses from the cheese body to eyes). Such expansion and contraction may result in mechanical fatigue or weakening of the cheese structure, and thereby contribute to the formation of cracks and slits.

Overall, incorporation of high heat-treated centrifugate into cheesemilk, specific hydrolysis of caseins and solubilisation of colloidal calcium during ripening, and the presence of inherent micro-cracks within cheese matrices have all been shown to contribute to weakening of cheese structure. Furthermore, changes in CO_2 solubility relating to variable cheese composition, or with changing ripening parameters, are proposed to produce mechanical strain (contraction or expansion) in cheese matrices due to the exchange of CO_2 between the eyes and body of the cheese, resulting in mechanical fatigue or weakening of the cheese structure. Another possibility is that the cheese matrix becomes oversaturated with CO_2 gas, it can no longer contain all the solubilized gas due to changes in ripening related parameters, such as, temperature and pH, and rather than creating eyes the gas accumulates at the weak points in the matrix, e.g., at micro-defects and this leads to the formation of a slit or crack. It is expected that the combined effect of all parameters may lead to an overall weakening of cheese structure and thereby contribute to the development of undesirable slits and cracks (Figure 7.1).

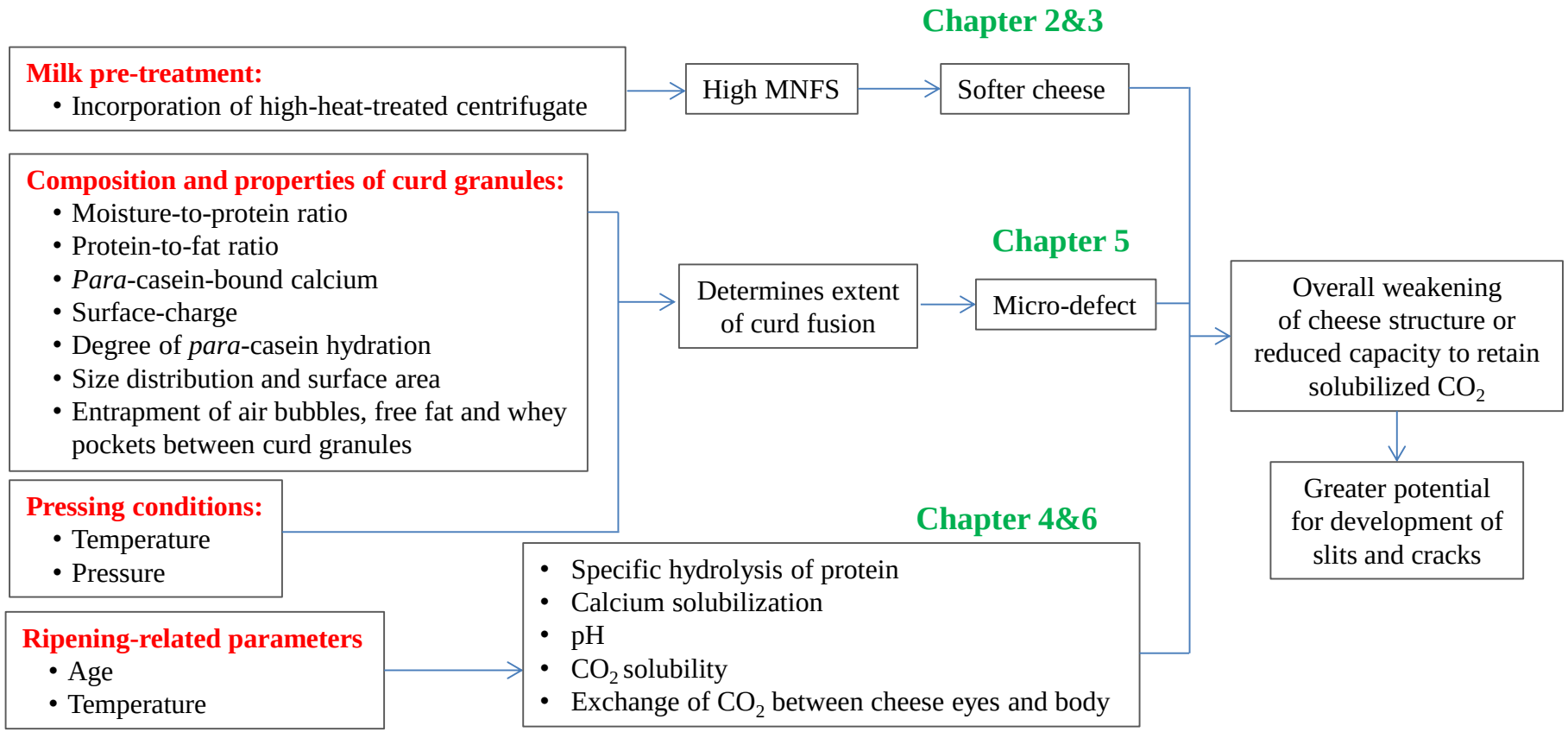


Figure 7.1 Schematic overview of key findings of this PhD thesis; MNFS, moisture in non-fat substance.

7.2 Recommendations for future research

Similar to centrifugation (at a centrifugal force of ~9,000 × g), microfiltration (pore size = 1.4 µm) is also widely used by cheese industries for removal of *Clostridium* spores from cheese-milk prior to continental cheese manufacture (Beuvier et al., 1997). However, very little is published on investigating the effect of microfiltration on the microbiological, physico-chemical and ripening characteristics as well as on eye-development characteristics of semi-hard continental cheese types. Such knowledge could be beneficial to cheese industries that use microfiltration as a milk-pretreatment method for removal of *Clostridium* spores. Sensory analysis of Maasdam cheese made from centrifuged milk, as well as cheese made from centrifuged milk containing high heat-treated (HHT) centrifugate may also provide corroborating support to the volatile results obtained by gas-chromatography mass-spectrometry analysis in the current study.

Shortness or brittleness of cheese has previously been associated with undesirable slits or cracks (Grappin et al., 1993; Rehn et al., 2011). Thus, strategies for maintaining higher levels of intact β-casein or insoluble calcium in the cheese curd during ripening could be applied to reduce the shortness of cheese texture, which in turn may help to reduce or avoid the incidence of undesirable slits or cracks within the cheese matrix. High levels of β-casein may be achieved by greater retention of inhibitors of plasmin or plasminogen activators; studies have also reported inhibition of plasmin activity by whey proteins (Bastian, Hansen, & Brown, 1993; Politis, Zavizion, Barbano, & Gorewit, 1993). Milk plasmin content is reported to be lower in early lactation and higher in late lactation (Bastian, Brown, & Ernstrom, 1991; Sheehan, 2013). Similarly, approaches to retain higher levels of insoluble calcium, perhaps through fortification could also be the subject of further

research. Addition of calcium chloride to the curd/whey mixture during cheese manufacture may increase the serum calcium, which may prevent solubilisation of colloidal calcium from the casein micelles. However, addition of calcium chloride to the curd-whey mixture may alter other cheese-making parameters, which need to be taken into consideration.

It is recommended that further investigations should focus on the causes of micro-defects within the cheese matrix, which in turn may allow the development of strategies to avoid formation of those defects in cheese matrices. Such investigations could focus on:

- The properties of curd granules, such as, composition, surface charge, degree of *para*-casein hydration (g water/ g protein), protein-to-fat ratio, solid-to-liquid fat ratio, which determines their potential to deform and flow into, and fuse with, other curd particles when subjected to moulding and pressing (Guinee, 2016);
- Size distribution and surface area of curd granules, which affects the packing arrangement of curd granules within cheese matrices (Guinee, 2016);
- Pressing conditions, such as, temperature and pressure, which are known to affect the solid-to-liquid fat ratio, and molecular interactions between the networks of curd granules (Guinee, 2016).

Application of microscopy and spectroscopy techniques, such as, atomic force microscopy (AFM) and Raman spectroscopy, may provide a more detailed insight into the composition of, and molecular interactions between, the networks of curd granules.

The application of advanced non-invasive techniques, such as, X-ray computed tomography (CT) and magnetic resonance imaging (MRI) (Guggisberg et

al., 2015; O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016) could be applied to investigate the distribution and growth kinetics of splits or cracks during ripening of semi-hard eye-type cheeses.

Further research should be applied to investigate the effect of primary and secondary proteolysis, lipolysis and fatty acid composition on CO_2 solubility in semi-hard cheeses, which would achieve a greater understanding of the influence of age-related changes on CO_2 solubility within cheese matrices.

Overall, the research undertaken in this thesis provides a greater understanding of underlying issues leading to the development of undesirable split or crack defects and also recommends areas for further research. The knowledge gained through this thesis will help to develop strategies to minimize or remove split and crack defects within semi-hard cheese matrices made from a seasonal milk supply.

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Appendix: Reprints of published articles

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Symposium review: Structure-function relationships in cheese[1]

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ABSTRACT

The quality and commercial value of cheese are primarily determined by its physico-chemical properties (e.g., melt, stretch, flow, and color), specific sensory attributes (e.g., flavor, texture, and mouthfeel), usage characteristics (e.g., convenience), and nutritional properties (e.g., nutrient profile, bioavailability, and digestibility). Many of these functionalities are determined by cheese structure, requiring an appropriate understanding of the relationships between structure and functionality to design bespoke functionalities. This review provides an overview of a broad range of functional properties of cheese and how they are influenced by the structural organization of cheese components and their interactions, as well as how they are influenced by environmental factors (e.g., pH and temperature).
Key words: cheese, structure, function, interaction

INTRODUCTION

Overall, the global consumption of cheese is increasing continuously and is projected to increase by ~13.5% between 2016 and 2025 (OECD/FAO, 2016). Simultaneously, consumers/end-users have increasingly been demanding enhanced physico-chemical properties, sensory and nutritional quality, and optimal usage characteristics of cheese, all at a reasonable cost. This is primarily driven by factors such as growing consumer awareness of the role of diet in health and well-being, the potential to use structure to influence flavor release and sensory experience, and the extensive use of cheese as an ingredient in food retail applications. Such expanding consumer demands have triggered the focus of food researchers and cheese producers toward the improvement in the quality of existing products or the design of new innovative products.

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It is now well recognized that many of the desirable properties of cheese are largely determined by its structure. For example, structure plays an important role in determining the mechanical, rheological, and cooking properties of heated and unheated cheese (Lucey et al., 2003; Guinee, 2016), eye formation in several types of hard (e.g., Swiss type or Emmental) and semi-hard (e.g., Maasdam type) cheese (Daly et al., 2010), and texture perception (Rogers et al., 2009). More recently, it has also been reported that food structure plays a key role in flavor release (Taylor, 2002) and in the digestion and the absorption of nutrients (Parada and Aguilera, 2007; Singh et al., 2015). Apart from containing basic nutrients, the nutritional value of food can also be enhanced by introducing health-promoting and bioactive compounds, such as polyphenols and peptides. In this context, the cheese matrix can potentially be used as a delivery vehicle for bioactives and probiotics (Sharp et al., 2008; Rashidinejad et al., 2016). Thus, a better understanding of the complex interrelationship between structure and functionality (i.e., the so-called structure-function relationship) is necessary to design of cheese types with specific functionalities. However, the full extent of the relationships between structure and functionality of cheese is not fully understood. The aim of this review is to provide an appropriate knowledge of how cheese structure may be manipulated to control and predict the functional properties of cheese.

CHEESE COMPONENTS AND STRUCTURE

Caseins, the main structural component of cheese, are present in the form of a network in the cheese matrix in which fat globules, water, minerals, bacteria, and dissolved solutes such as lactose, lactic acid, soluble salts, and peptides are all interspersed. The spatial arrangements of these components and their interactions determines the structure of cheese, which is influenced by relative volume fractions of each component and their properties (e.g., residual charge on the casein, composition of membrane materials of fat globules, and state of minerals, water, and fat), cheese manufacturing procedures, maturation conditions, and environmental

conditions (e.g., pH, temperature, and solvent quality/ionic strength), among other factors.

Like other food types, cheese encompasses a hierarchical structure, with scales that span from the molecular to the macroscale (Figure 1). At a macroscopic level, cheese is the assembly of curd particles (resulting from cutting of the gel in the case of brine-salted cheeses), or curd chips or pieces (resulting from milling of curds and dry salting, such as in Cheddar and Stilton cheese manufacture; Guinee, 2016). Eyes, slits/cracks, visible crystals, and mechanical openness are also macrostructural features of cheese. At the microscopic level, cheese is composed of microstructural components, such as the casein network, fat globules, and water droplets. At further higher levels of magnification (nano or molecular scale), microstructural components of cheese are formed from molecules and atoms. Structures at the macro, micro, nano, and molecular levels of organization all have an important role in various properties of cheese. Various techniques to study cheese structure, such as microscopy, rheology, magnetic resonance, and dynamic light scattering, have been reviewed extensively (e.g., Everett and Auty, 2008; El-Bakry and Sheehan, 2014).

From a materials science perspective, cheese can be viewed as a 2-phase composite material (also called "filled gels" or "gelled emulsions") containing fat globules as a filler in a protein gel matrix (Barden et al., 2015). Several researchers used this approach to study the role of milk fat and protein network on the mechanical and rheological properties of cheese (Rogers et al., 2010; Barden et al., 2015; Thionnet et al., 2017).

MOLECULAR INTERACTIONS WITHIN THE CHEESE MATRIX

Various molecular forces and interactions that act between the cheese components are considered important as they can influence the functionality of cheese. For example, it is suggested that the localized balance of the attractive and repulsive forces between casein controls the melting of heated cheese (Lucey et al., 2003). Moreover, the nature and extent of interactions of flavor compounds and nutrients with the food matrix can influence their release patterns in the mouth during mastication and in the gut during digestion, and this can in turn affect the sensorial and nutritional properties of food (Parada and Aguilera, 2007; Gierczynski et al., 2011). For such reasons, knowledge of molecular interactions and forces that act between cheese components is vital.

Some studies have characterized the interactive forces in milk gels and cheese curd using different dissociating agents such as urea, SDS, and EDTA (Lefebvre-Cases et al., 1998; Gagnaire et al., 2002; Zamora et al., 2012). These dissociating agents are known to disrupt specific types of bond or interaction; for example, hydrophobic interactions and hydrogen bonds can be disrupted by SDS or urea, respectively, whereas ionic bonds involving calcium salts are broken by the chelating effects of EDTA (Zamora et al., 2012). Lefebvre-Cases et al. (1998) characterized the interactive forces in rennet- and acid-induced milk gels using different dissociating agents, and the results of their study suggested that hydrophobic interactions and calcium bonds were the most important forces for the stabilization of the structure of rennet milk gels. The contribution of hydrogen bonds seemed comparatively less important for the stability of rennet gel structure than the aforementioned forces. In acid-induced milk gels, hydrophobic and electrostatic interactions and hydrogen bonds have been shown to be important forces, whereas the contribution of calcium bonds have been found to be less important, most probably due to solubilization of colloidal calcium at low pH (Lefebvre-Cases et al., 1998). Calcium bonding, electrostatic interactions, and hydrogen bonds (to a lesser degree) contribute to the formation and stability of the *para*-casein matrix (after pressing) in Emmental cheese (Gagnaire et al., 2002). The major interaction

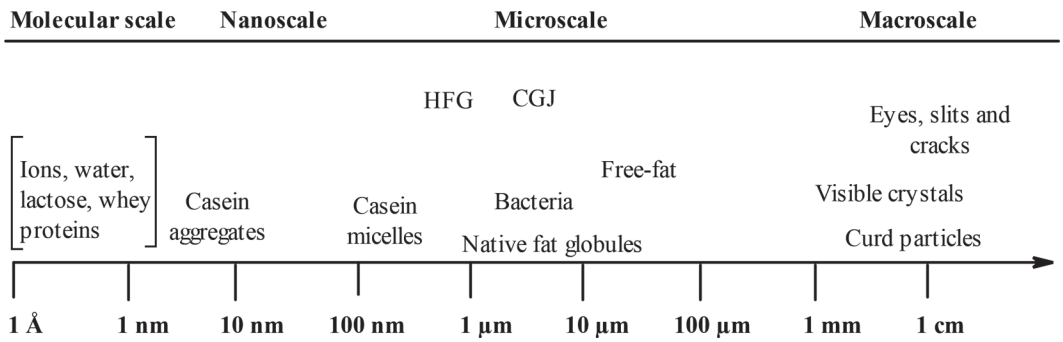


Figure 1. Characteristic length scales in cheese. HFG = homogenized fat globules, CGJ = curd granule junction.

forces responsible for the structural organization of cheese components are defined as follows.

Electrostatic Interactions

Electrostatic interactions are important for food components that have a permanent electrical charge, such as dipoles or ions (McClements et al., 2009). Cheese is a complex system, and many components present in the cheese matrix are known to have electrical charge. For example, casein contains several AA residues with ionizable groups along their polypeptide chains, including phosphoseryl residues (Horne, 1998).

Electrostatic interactions between charged species are sensitive to the surrounding environment, particularly pH and ionic strength. The electrical charge of ionizable groups of food components depends on their pKa values relative to the pH of the surrounding aqueous solution (McClements et al., 2009). The aqueous phase of the cheese matrix contains several monovalent (e.g., Na^+) and multivalent ions (e.g., Ca^{2+}), and their level determines the ionic strength of the aqueous phase of the cheese matrix. The magnitude and range of the electrostatic repulsion between the casein in the cheese matrix may decrease with increasing ionic strength of the surrounding aqueous solution due to electrostatic screening effects (McClements et al., 2009).

Calcium-sensitive casein can cross-link with free calcium ions via calcium bridging, which is a type of electrostatic interaction (Dalgleish, 1983). Such interactions are considered important for the aggregation of renneted casein micelles during coagulation of milk (Dalgleish and Corredig, 2012). The binding of calcium by casein is suggested to decrease with increasing ionic strength and with decreasing temperature (<40°C; Horne and Lucey, 2014).

Hydrophobic Interactions

Hydrophobic interactions are strong attractive forces between hydrophobic side groups of molecules in aqueous solution. The molecular origin of hydrophobic interactions is the fact that water molecules can form relatively strong hydrogen bonds with other water molecules, but not with nonpolar groups (McClements et al., 2009). Caseins have a significant fraction of nonpolar regions along their polypeptide chain (Horne, 1998). Thus, it is expected that the hydrophobic interactions may play an important role in determining the casein interactions in the cheese matrix (Lucey et al., 2003). The strength of hydrophobic interaction tends to increase with increasing temperature (McClements et al., 2009). Thus, it is believed that these interactions may make a significant contribution to the functionality of heated cheese (Lucey et al., 2003).

Hydrogen Bonding

Hydrogen bonds are simply an interaction between an electronegative atom (e.g., O, N, F, and Cl) and a hydrogen atom covalently bound to similar electronegative atoms. This is the one type of electrostatic force that tends to decrease in strength as the temperature increases (McClements et al., 2009). Hydrogen bonding is known to play a major role in hydration of proteins (Petukhov et al., 2004). Protein, including casein, contains several groups, such as carbonyl, amine, amide, and hydroxyl, which are able to interact with water through hydrogen bonding. Hydration of casein is considered important for the development of desirable texture and cooking properties of some cheese types, such as Mozzarella (Guo et al., 1997). Moreover, several studies have also suggested that the texture and cooking properties of low-fat cheeses can partially be improved by increasing the water-binding capacity of the protein matrix through approaches, such as modulation of pH, and varying the level of colloidal calcium phosphate (**CCP**) and sodium chloride (NaCl) of the cheese matrix (Paulson et al., 1998; Sheehan and Guinee, 2004; McMahon et al., 2005; Johnson et al., 2009).

Disulfide Bonding

Disulfide bonds are a covalent bond formed between 2 thiol groups. This bond is considered important for cheese made from high heat-treated milk (e.g., queso blanco). High heat treatment of cheese milk unfolds heat sensitive whey proteins, exposing thiol groups that can form disulfide links with other reactive thiol groups of whey protein and casein through classical thiol-disulfide exchange reactions (Kethireddipalli and Hill, 2015). This type of reaction is influenced by redox potential (Poole, 2015), although little is published on this with regard to cheese matrices.

FUNCTIONAL PROPERTIES OF CHEESE

Key Properties for Use as an Ingredient

Cheese is extensively used as an ingredient in many foods. Two main functional requirements of cheese when used as an ingredient are (1) machinability (the ability of cheese to be shredded/diced/cut/sliced), and (2) specific cooking and melting properties (Lucey, 2008). Functional requirements of cheese largely depend on end-use application. For example, when cheese

is used a topping on pizzas and lasagna, it needs to melt and stretch in a specific manner, whereas melting is undesirable when visual identity and shape of cheese on cooking is required, such as for queso blanco and paneer, although a certain degree of softening is desirable (Guinee, 2016). The various functional properties required when cheese is used as ingredients have been discussed extensively (Lucey, 2008; Guinee, 2016).

Texture Perception

Texture is an important factor determining the quality and identity of food, including cheese (Lawrence et al., 1987; Foegeding and Drake, 2007). For cheese, the main evaluation of texture occurs in the mouth (during mastication). However, in some eye-forming cheese types, such as Emmental and Maasdam, visual properties are also important. Proper eye development, encompassing number, size, shape, luster, and distribution, is an important textural property in those cheese types (Lucey et al., 2003; Daly et al., 2010).

During consumption, food is subjected to a complex series of oral manipulations, including ingestion, size reduction, and mixing with saliva, to form a bolus for safe swallowing, collectively termed as oral processing (Foegeding et al., 2010). Behavior of food during oral processing, such as breakdown patterns and extent of interaction (coating) with the oral surfaces, is thought to play an important role in the texture perception, and the structure and chemical composition of food can influence their behavior during oral manipulation (van Vliet et al., 2009; Foegeding et al., 2010). For example, the desirable texture of full-fat Cheddar cheeses compared with low-fat cheeses is considered to be partly due to role of fat in the desirable breakdown patterns of cheese during oral manipulation (Rogers et al., 2009; Foegeding et al., 2010). Similarly, the desirable texture of aged cheese is attributed to the age-related structural changes in the protein matrix, resulting in specific breakdown pattern during chewing (Rogers et al., 2009). Moreover, several studies of emulsion gels have reported that the properties of emulsion droplets, extent of droplet-matrix interactions, distribution of emulsion droplets, and characteristics of the gel matrix can all influence texture perception (Sala et al., 2007; Liu et al., 2015; Oliver et al., 2015). Thus, a fundamental knowledge of how the structure of cheese influences a specific textural response could be useful for designing cheese with desired texture profiles.

Flavor Release and Perception

Like texture, flavor (comprising taste and aroma) is also an important attribute of cheese. The heterogeneous mixture of several hundred volatile and nonvolatile flavor compounds in cheese are the result of complex biochemical reactions during maturation, such as proteolysis, lipolysis, and glycolysis (McSweeney, 2004). During consumption, flavor compounds are released from the food matrix and diluted with saliva, which need to be transported to the flavor receptors in the mouth and nose for flavor perception to occur (Taylor, 2002). The correct balance and concentration of a wide range of flavor compounds, their release profile during oral processing, and the concentration and the rate at which those flavor compounds reach the receptors can all influence overall flavor perception (Taylor, 2002).

Food structure appears to play a key role on release of flavor compounds. Several studies on pure gels (e.g., protein gels, carrageenan gels), mixed gels (e.g., whey protein-polysaccharides), emulsion-filled gels (where emulsion droplets are embedded within a gel matrix), or solid lipoproteic colloid foods have shown that the gel structure affects volatiles and tastant release profile (Stieger and van de Velde, 2013; Kuo and Lee, 2014); in general, weaker gel textures and more porous structures gave higher flavor compound release during mastication. For example, Kuo and Lee (2014) reported that the rate of sodium release increased with increasing porosity and pore size of solid lipoproteic colloid foods. Proteolysis weakens the structure of protein network due to breakdown of the protein network; thus, it may be assumed that proteolysis facilitates the release of sapid compounds during mastication (Sousa et al., 2001).

Other factors, such as properties of the flavor compounds (e.g., volatility, solubility, and affinity toward the food matrix), and the subject's oral processing behavior (e.g., chewing, saliva flow rate, and air flow rate through the mouth and nose), can also influence the release of flavor compounds from the food matrix during oral manipulation (Taylor, 2002; Gierczynski et al., 2011). In addition, it is now well accepted that overall flavor perception can also be influenced by perceptual interactions between various sensory modalities (e.g., aroma, taste, and texture). For example, Visschers et al. (2006) reported that the intensity of aroma perceived by subjects decreased with increasing firmness of the food. In another study, the odor of Comté cheese enhanced the perception of saltiness in model cheeses (Lawrence et al., 2011).

Based on the above considerations, it is clear that the flavor profile of food/cheese can potentially be enhanced by modifying food structure. This approach could be manipulated to facilitate reduction of sodium and saturated fat without significantly altering flavor attributes or to ameliorate flavor defects in reduced-fat and reduced-salt cheeses. Such approaches merit further research.

Nutritional Properties

Cheese, a nutrient-dense dairy product, is a good sources of proteins, vitamins, and minerals, particularly calcium, and phosphorus. However, the traditional method for evaluation of the nutritional quality of food (i.e., based on its composition) has recently been criticized because the method often neglects the effect of food matrix (structure) on nutrient release and absorption (Parada and Aguilera, 2007; Singh et al., 2015). For example, in an in vivo study using 6 mini-pigs, Barbé et al. (2013) reported that a renneted-gel matrix slowed down the rate of digestion of protein and absorption of AA as compared with liquid milk, probably due to lesser accessibility for digestive enzymes. In another study, Lamothe et al. (2012) studied the digestion pattern of Cheddar and Mozzarella cheese using an in vitro stomach model. The results of their study suggested that the degradation of protein and the kinetics of fatty acid release are closely associated with the physical characteristics of the cheese matrix; cheeses that exhibited greater cohesiveness and elasticity were more slowly degraded during digestion and gave slower rates of fatty acid release. More recently, some studies have reported that calcium in cheese can influence the free fatty acid bioaccessibility by producing insoluble calcium soaps with long-chain fatty acids at intestinal pH conditions (Ayala-Bribiesca et al., 2017). A better understanding of the role of cheese structure on digestion and absorption of nutrients within the gastrointestinal environment is key to designing cheese with enhanced nutritional quality. This area was reviewed in detail recently by Singh et al. (2015).

Delivery of Bioactives and Probiotics

Several studies have reported the potential for using the cheese matrix as a delivery vehicle for bioactives, such as vitamins (Madziva et al., 2006), minerals, and polyphenols (Rashidinejad et al., 2016). For example, Rashidinejad et al. (2016) successfully used full-fat hard cheese as a delivery vehicle for liposomal nanoencapsulated green tea catechins. Moreover, the cheese matrix can also serve as a vehicle for probiotic delivery (Sharp et al., 2008). High buffering capacity and the dense protein network of cheese are thought to protect probiotic bacteria against the harsh acid environment in the stomach (Gomes da Cruz et al., 2009), making cheese a potentially suitable carrier for probiotics.

However, it should be noted that many bioactives have an undesirable taste and odor, such as metallic taste of mineral salts, bitter taste of peptides, and fishy taste and odor of marine oils rich in n-3 fatty acids (Augustin and Sanguansri, 2008), which can alter the sensory properties of cheese. Moreover, the metabolites from high numbers of viable and metabolically active bacterial cells can also alter the sensory attributes of cheese. Therefore, these details need consideration when using cheese as a delivery vehicle for bioactive compounds and probiotics.

ROLE OF STRUCTURAL ELEMENTS AND THEIR INTERACTIONS ON FUNCTIONAL PROPERTIES OF CHEESE

The composition and the structural organization of cheese determine its functionality. In this section, we therefore focus on how the properties and the structural organization of the different phases of cheese, and the interactions between them influence cheese functionality. For the sake of simplicity, we have divided cheese structure into 4 phases: (1) protein phase, (2) fat phase, (3) aqueous phase, and (4) gas phase, particularly carbon dioxide (CO_2).

Protein Phase

Formation and Rearrangement of Protein Network. Formation of a protein network is a crucial step in cheese manufacture. The destabilization of casein micelles is one of the first steps in the manufacture of cheese. The mechanisms of destabilization of casein micelles by different means have been discussed extensively elsewhere (Dalgleish and Corredig, 2012). The destabilized casein micelles aggregate into chain and clusters, leading to formation of a 3-dimensional gel.

Several studies have reported that the factors, such as concentration of casein (Karlsson et al., 2007), properties of casein micelle (e.g., casein micelle size; Logan et al., 2014), and coagulation conditions (e.g., pH, temperature, and rennet concentration; Wium et al., 2003; Ong et al., 2011a, 2012), can all influence the coagulation process. This may influence the arrangement of casein into protein matrix and also the microstructure and the quality of the final cheese. For example, milk renneted at lower pH (pH 6.1) gave gels with more compact protein network than in gels renneted at higher pH (pH >6.3; Ong et al., 2012). Moreover, the texture of resulted Cheddar cheese was different; that is, cheese made using milk renneted at lower pH (pH 6.1) had lower chewiness, gumminess, cohesiveness, and springiness than cheese made using milk renneted at higher pH (pH 6.7 or 6.5). Increased solubilization of CCP, accelerated rennet activity, and reduced charge repulsion between micelles at a lower milk pH are most likely to alter the rate and extent of aggregation, possibly leading to different microstructures of gels and cheese curds (Ong et al., 2012). In

other studies, the coarseness of the protein network of the gel or cheese increased with increasing coagulation temperature (Wium et al., 2003; Ong et al., 2011a). This is probably due to enhancement of the protein network rearrangement and increasing strength of hydrophobic interactions at higher coagulation temperatures. Moreover, the calcium-binding by *para*-casein is suggested to increase with increasing temperature within the normal milk-coagulation temperature regimen, which may influence the aggregation kinetics of fully renneted casein micelles (Dalgleish, 1983; Horne and Lucey, 2014). This suggests that the functionality of the final cheese can be modified by optimization or modulation of initial cheese-making conditions. Thus, the influence of initial cheese-making conditions on the final properties of cheese should not be underestimated.

Rennet-induced gels are inherently unstable and likely to undergo intraparticle, interparticle, and interstrand rearrangements (Mellema et al., 2002). The ultimate result of such rearrangements is syneresis (expulsion of whey; Mellema et al., 2002). The rate and extent of syneresis is promoted by various cheese-making processes, such as cutting, stirring, scalding, and pressing (Dejmek and Walstra, 2004). Syneresis is considered as an essential step during cheese manufacture because it affects the composition and texture of final cheese, as reviewed extensively by Dejmek and Walstra (2004). However, in some cheese types, such as Quark (also called Quarg) and cottage cheese, syneresis can also occur in the finished product during storage, termed wheying-off, which is generally considered as undesirable (Guinee, 2016).

To date, there is significant knowledge on how milk composition and renneting conditions affect the gel structure. However, the link between gel structure and macroscopic behavior of the gel, such as syneresis and water-holding, is not yet fully understood. Structural parameters, such as dimensions of protein strands, network pore size, and volume fraction of the pores and protein network, can be characterized at different structural levels by using different microscopic techniques (Langton and Hermansson, 1996; Ong et al., 2011b). Such structural information is relevant in understanding the effects of milk composition and gelation conditions on the macroscopic behavior of gels, such as syneresis and water-holding properties of gel.

Casein-Mineral Interactions. In cheese, significant levels of minerals are associated with the protein network (Lucey and Fox, 1993). Calcium and phosphate (PO_4) are the 2 most important minerals found in cheese, and are present in both soluble and colloidal form. However, it is well recognized that the calcium and phosphate associated with the casein are an important structural unit in cheese. The level of calcium associated with casein (micellar calcium) varies widely between cheese types, ranging from less than 5 mg/g of protein in feta and cottage cheese to ~24 mg/g of protein in Gouda and Emmental cheese (Remillard and Britten, 2011).

Modulation of levels of colloidal calcium in the cheese matrix can alter the texture and cooking properties of cheese. For example, decreased levels of colloidal calcium is associated with the softening of cheese texture [at least in Cheddar; O'Mahony et al. (2005)] and increased melt and flow properties (O'Mahony et al., 2006; Choi et al., 2008), attributed to the reduction in calcium-induced casein-casein interactions (Lucey et al., 2003). This mechanism is supported by the studies of Pastorino et al. (2003a) and McMahon et al. (2005), who observed a more homogeneous microstructure in cheeses with low levels of calcium than those with high levels of calcium, when observed using scanning electron microscopy; this indicates the proteins in the former are less aggregated than in the latter cheeses.

Micellar calcium levels in cheese are considered important in conferring an elastic texture to cheese (Lucey and Fox, 1993), which is important in the case of eye-forming cheese types, such as Emmental and Gouda, to accommodate gas produced during warm-room ripening for smooth eye formation (Daly et al., 2010). Moreover, an elastic texture is also important for sliceability of cheese without fracturing or crumbling or sticking to cutting implements (Guinee, 2016).

It has also been reported that increased hardness due to increase in micellar calcium levels slowed down the disintegration during in vitro digestion, which in turn can affect nutrient bioaccessibility (Ayala-Bribiesca et al., 2016).

Age-Related Changes in the Protein Matrix. During maturation, the structure of the protein network alters due to complex physical and biochemical changes in the cheese matrix, such as proteolysis by various proteolytic agents, demineralization of casein, and hydration of the casein networks (at least in Mozzarella), as reviewed by Guinee (2016).

Recently, fermentation-produced camel chymosin has received attention because of its much higher ratio of milk clotting to general proteolytic activity than bovine chymosin (Kappeler et al., 2006). Cheddar cheeses made using recombinant camel chymosin were generally found to be harder with less bitter and brothy flavors, and with lower levels of proteolysis than cheeses made from bovine chymosin or microbial rennet (Hannilase; Bansal et al., 2009; Soodam et al., 2015). Moynihan et al. (2014) suggested the use of recombinant camel chymosin to extend the shelf-life performance of low-moisture partly skim Mozzarella because its baking properties, such as blister quantity, strand thickness,

hardness, and chewiness, on baked pizzas were maintained for a longer time in storage than in cheeses made with bovine calf chymosin. Apart from residual coagulants, indigenous milk enzymes and enzymes produced by starter and nonstarter bacteria also contribute to the proteolysis of cheese, particularly in high-cooked cheese varieties in which the residual chymosin activity is very low, most probably due to heat denaturation of chymosin (Sousa et al., 2001; Sheehan et al., 2007). Plasmin is considered the most important indigenous milk proteolytic enzyme, and its activity in high-cook cheese varieties (e.g., Emmental and grana-type cheeses) is comparatively higher than those in low-cook cheese varieties, (e.g., Cheddar), most probably due to thermal inactivation of inhibitors of both plasminogen activators and plasmin (Sheehan, 2013). Plasmin has an optimum pH of ~7.5 and thus makes a major contribution to the ripening of cheese types with high pH (~7), such as mold-ripened (e.g., Camembert) and smear-ripened (e.g., Tilsit) cheese varieties (McSweeney, 2004; Sheehan, 2013). The role of plasmin and other indigenous milk enzymes in casein hydrolysis and their contribution to the quality of cheese has been reviewed extensively (Sousa et al., 2001; Kelly and McSweeney, 2003; McSweeney, 2004; Kelly et al., 2006).

Varying degree of hydrolysis of casein in different cheese types have been reported (Table 1). The rate and extent of casein hydrolysis is influenced by factors, such as cheese type, ripening temperature, level and types of coagulant, and cheese compositions (e.g., moisture in nonfat substance, Table 1). α_{S1}-Casein has been considered to be the principal structural element in several cheese varieties, such as Cheddar and Emmental (Lawrence et al., 1987; Gagnaire et al., 2002), with the hydrolysis of α_{S1}-CN thus being associated with a weakening of the protein network (Creamer and Olson, 1982). However, more recent studies have shown that softening of cheese in the early stages of ripening is primarily due to solubilization of CCP (O'Mahony et al., 2005).

Some studies indicated that the specific hydrolysis patterns of casein and the resulting peptide profiles can influence the melting and stretching properties of cheese. For example, Bogenrief and Olson (1995) observe a degree of melt of Cheddar cheese is more closely related to the extent of β-CN hydrolysis than the hydrolysis of α_{S1}-CN. In another study, Emmental cheeses made with *Lactobacillus helveticus* as a starter culture exhibited greater stretchability (2.5 times higher) than those with *Lactobacillus delbrueckii* (Richoux et al., 2009). Moreover, the stretchability of cheese was strongly correlated with the proportion of hydrophobic peptides in the pH 4.6-soluble nitrogen fraction. This finding is further supported by the study of Sadat-Mekmene et al. (2013), who also observed high stretchability in Swiss-type cheese made with 2 different strains of *Lactobacillus helveticus* (i.e., ITGLH77 and ITGLH1). Moreover, the stretchability was correlated with hydrophobic peptides, regardless of casein origin (i.e., whether α_{S1}-CN, α_{S2}-CN, or β-CN), and with a lower degree of proteolysis. These hydrophobic peptides may interact with the protein matrix or with other large peptides via hydrophobic forces, possibly forming fibers in the cheese matrix (Richoux et al., 2009).

Fat Phase

During cheese manufacture, milk fat globules are entrapped within the protein gel network, and processes such as scalding, cheddaring, hot water stretching, and pressing, can cause aggregation, coalescence, and disruption of the fat globules. In the cheese matrix, fat globules can exist as intact (spherical fat globules covered with native membrane materials), aggregated (clumps of circular fat globules), coalesced (spherical but larger than typical milk fat globules), elongated (especially in pasta-filata cheese-types), or even non-globular forms (Michalski et al., 2007; Rogers et al., 2010; Ong et al., 2011b) (Figure 2). The microstructure of fat globules can influence the physical properties of cheese. For example, although Everett and Olson (2003) did not find a correlation between fat-globule-circularity and free-oil formation in Cheddar cheese, fat globule size (Feret's diameter) in Mozzarella cheese has been positively correlated with meltability and free oil in a study by Ma et al. (2013).

Several factors, such as fatty acid compositions, native milk fat globule (**NMFG**) size, level of fat, and properties of fat globule membrane materials, can influence various properties of cheese.

Fatty Acid Composition. The fatty acid composition of milk fat [which is influenced by factors such as stage of lactation, breed of cow, genetics, and diet composition, Månsson (2008)] can alter the rheological and textural properties of cheese. Palmitic acid (C16:0) and oleic acid (C18:1) are the major saturated and unsaturated fatty acids in milk that have high and low melting points, respectively (Coppa et al., 2011); a higher ratio of C18:1 to C16:0 is known to produce more creamy and less firm cheese (Coppa et al., 2011; Bocquel et al., 2016). Bocquel et al. (2016) observed ~30% decrease in hardness of Raclette cheese when the ratio of C18:1 to C16:0 in cheese milk increased from 0.8 to 1.0. In the case of Raclette cheese, increased hardness increases the risk of cracks forming, which significantly affects the quality of cheese (Bocquel et al., 2016). The effect of fatty acid composition on the mouthfeel of cheese is not yet fully understood. However, it may be assumed

Table 1. Extent of hydrolysis of casein at different stages of ripening in different cheese varieties[1]

Cheese type	Level of casein hydrolysis	Age and temperature	Coagulant type	% MNFS	% S/M	Method	Reference
Cheddar	48.8% of α_{S1}-CN[2] 16.8% of β-CN[2]	45 d at 7°C	Single-strength calf rennet extract (0.15 mL/kg of milk)	55	3.75	Urea-PAGE	Bogenrief and Olson (1995)
Cheddar	44.7% of α_{S1}-CN[2] 59.8% of β-CN[2]	45 d at 7°C	*Cryphonectria parasitica* (0.051 mL/kg of milk)	55.7	3.69	Urea-PAGE	Bogenrief and Olson (1995)
Cheddar	~80–85% of α_{S1}-CN[2] ~10% of β-CN[2]	90 d at 8°C	Chymosin (0.3 mL/L of milk)	57	2.85	Urea-PAGE	O'Mahony et al. (2005)
Cheddar	~78% of α_{S1}-CN[2]	180 d at 8°C	Fermentation-produced calf chymosin (0.3 mL/L of milk)	56	3.3	Urea-PAGE	Bansal et al. (2009)
Cheddar	~50% of α_{S1}-CN[2]	180 d at 8°C	Fermentation-produced camel chymosin (0.035 mL/L of milk)	56	3.3	Urea-PAGE	Bansal et al. (2009)
Part-skim Mozzarella	~50–80% of α-CN[2]	28 d at 4°C	Single-strength calf rennet extract (0.42 mL/L of milk)	64–66	ND	SDS-PAGE	Fife et al. (1996)
Mozzarella (direct acidification)	~55% of α_{S1}-CN[3] ~15% of β-CN[4]	15 d at 5°C	Double-strength rennet, chymosin (0.025 mL/kg of milk)	66	2.5	Capillary electrophoresis	Dave et al. (2003)
Mozzarella (direct acidification)	~90% of α_{S1}-CN[3] ~50% of β-CN[4]	15 d at 5°C	Double-strength rennet, chymosin (0.1 mL/kg of milk)	66	2.5	Capillary electrophoresis	Dave et al. (2003)
Swiss cheese	~75–80% of α_{S1}-CN ~75–80% of β-CN	60 d (7 d at 4°C, 21 d at 21°C, and 32 d at 4°C)	*C. parasitica* (0.05 mL/kg of milk)	55	ND	Capillary electrophoresis	White et al. (2003)
Emmental	~50% of α_{S1}-CN ~40% of β-CN	40 d (20 d at 12°C, and 20 d at 24°C)	Calf rennet (0.3 mL/kg of milk)	54	ND	Urea-PAGE	Sadat-Mekmene et al. (2013)

[1]ND = not determined; MNFS = moisture in nonfat substance; S/M = salt-to-moisture ratio.

[2]Expressed as a percent of the level at 1 d of ripening.

[3]Initial (100%) intact α_{S1}-CN level is based on the peak area of d-1 α_{S1}-CN plus the peak area of d-1 α_{S1}-CN (f24–199) times 1.1314 (to account for the loss of 23 of the 198 peptide bond in α_{S1}-CN).

[4]Initial (100%) intact β-CN level is based on the peak area of β-CN at d 1.

that the complex crystallization behavior due to fatty acid composition can alter the in-mouth coalescence of fat globules during oral processing, which can in turn affect the fat-related sensory perception. For example, high solid fat content in emulsion droplets enhances the coalescence of emulsion droplets and reduces friction during oral processing of emulsion-filled gels (Liu et al., 2015).

Native Milk Fat Globule Size. The size of NMFG ranges from <0.2 to >15 μm, with an average diameter of ~4 μm (Huppertz and Kelly, 2006). Studies have shown that the cheese manufactured from milk with different fat globule size differ compositionally and texturally. For example, Camembert and Emmental cheese produced from milk with small fat globules (**SFG**, ~3 μm), separated using microfiltration, had higher moisture content, softer texture, and underwent greater proteolysis during ripening than cheese made from milk with large fat globules (**LFG**, ~6 μm; Michalski et al., 2003, 2004). More recently, Logan et al. (2017) reported that the Cheddar cheese made from milk with SFG (~2.7 μm) was less firm at the early stages of ripening, and was less cohesive, less chewy, and less springy throughout maturation than cheese made from milk with LFG (~5 μm). However, the exact effect of NMFG size on cheese properties was not determined in these studies because the effect of NMFG size is confounded with the cheese moisture level.

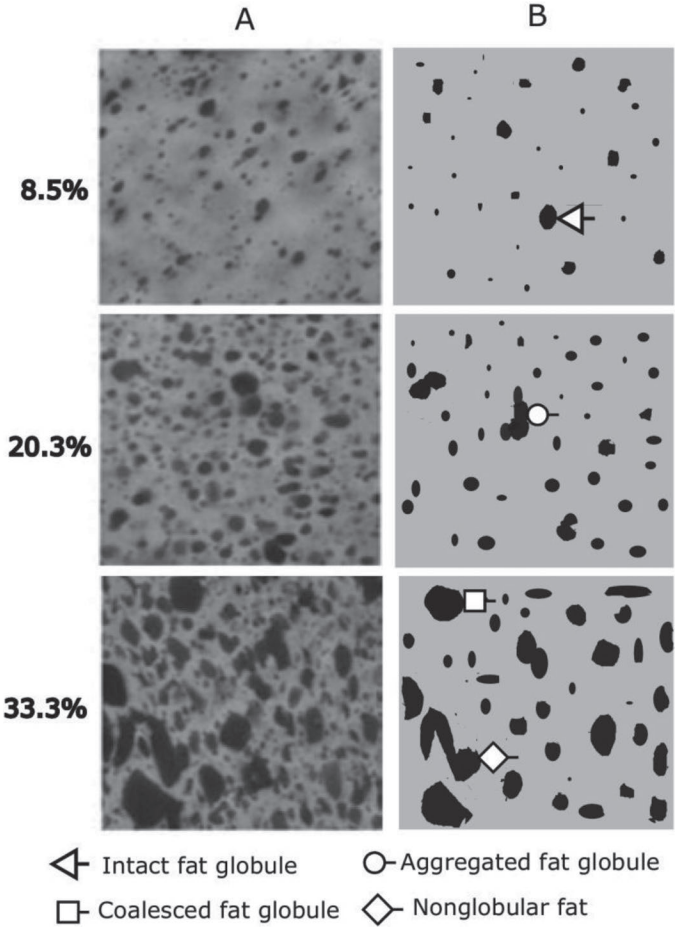


Figure 2. Column A shows the confocal laser scanning microscopy images (100 μm × 100 μm) of Cheddar cheese (age: 12 wk) at 3 different fat levels (8.5, 20.3, and 33.3%). Column B is the schematic representation of Cheddar cheese microstructure, with intact fat globules, aggregated fat globules, coalesced fat globules, and nonglobular fats. The dark and gray areas represent fat globules and protein network within the cheese matrix, respectively. Adapted from Rogers et al. (2010) with permission. Copyright (2010) American Dairy Science Association.

In another study, Michalski et al. (2007) made Emmental cheeses from milk with SFG and control milk, and adapted the process to obtain similar moisture content. The authors found that Emmental cheeses with SFG exhibited higher stretchability and elasticity, and improved sensory characteristics compared with control cheeses, despite the moisture content being similar for both cheeses. This may be attributed to the effect of NMFG size on microstructure of cheese. Emmental (Michalski et al., 2007) and Cheddar (Logan et al., 2017) cheeses made from SFG appeared less aggregated and less coalesced than control cheeses or cheeses made from LFG, when observed using confocal laser scanning microscopy. Moreover, more intact fat globules covered with phospholipids were observed in Cheddar cheeses from SFG than those from LFG when observed using confocal laser scanning microscopy (Logan et al., 2017). A recent study of an emulsion-filled gel system has reported that the mechanical properties of emulsion gels are influence by the magnitude of droplet clustering or aggregation. Droplet clustering or aggregation enhanced the stiffness of emulsion-filled gels (Oliver et al., 2015).

Moreover, the fat globule size can alter the casein strand formation during rennet-induced coagulation of milk; this could be another reason for observed differences in the properties between the cheeses made from milk with small and large fat globules. Depending on the size of NMFG and the pore size of the protein network, the NMFG can act as an "inert-filler," "structure-breaker" (Michalski et al., 2002), or has even been suggested as being held weakly within the protein matrix (Everett and Olson, 2000; Logan et al., 2015).

Level of Fat. A reduction in levels of fat without a proportionate increase in the levels of moisture will increase the concentration of casein in the protein matrix, leading to a compact protein matrix and a lower degree of fat coalescence (at least in Cheddar; Guinee et al., 2000; Rogers et al., 2010; Figure 2). Such changes on the cheese structure may have consequences for texture, opacity, and rheological and cooking properties of cheese (Guinee et al., 2000; Johnson et al., 2009; Rogers

et al., 2009; Rogers et al., 2010). The effect of fat reduction on texture, flavor, cooking properties, and color of cheese has been reviewed by Johnson et al. (2009).

To better understand the role of milk fat content on the mechanical and rheological properties of cheese, and to simplify the complex cheese system, model filler particles, such as Sephadex beads (Barden et al., 2015) or glass beads (Thionnet et al., 2017), have been used instead of milk fat in some studies. These studies suggested that the mechanical properties of cheese depend on the rheological properties of both the gel matrix and filler particle and on the volume within the cheese occupied by the filler particles (Barden et al., 2015; Thionnet et al., 2017). This knowledge may be useful in developing the replacement of milk fat in low-fat cheeses with other fat-like components, such as hydrocolloids (Thionnet et al., 2017).

Interactions Between Fat Globules and Protein Matrix. The interactions between milk fat globules and the protein matrix in cheese largely depend on the composition of fat globule membrane materials. Although a subject of debate (Everett and Auty, 2008), it is generally accepted that the NMFG membrane, which is composed mainly of specific proteins and phospholipids, does not chemically interact with the surrounding protein matrix (Michalski et al., 2002).

The nature and extent of interactions between fat globules and protein network can be controlled by modifying the surface properties of fat globules. Such modulation of fat-protein interactions can alter the cheese structure, which in turn can affect the mechanical, rheological, and sensorial properties of cheese. For example, Everett and Olson (2003) compared the microstructure and rheological properties of Cheddar cheese manufactured from recombined milk containing fat globules coated with casein or whey proteins. The microstructure of fat globules appeared elongated and clustered in cheeses made from milk with fat globules coated with α_{S2}-CN (a relatively poor emulsifier) compared with other experimental cheeses in which fat globules were coated with other proteins. Moreover, the former cheeses fractured at a lower strain and with a lower stress than the other experimental cheeses.

The effect of interactions between fat globules and protein matrix on texture perception of cheese is not yet fully understood. However, several studies on emulsion-filled gel systems reported a significant effect of filler-matrix interactions on the texture perception. Emulsion-filled gels are prepared by embedding emulsion droplets into a gel matrix, and are a representative model food system for a broad variety of food products, including cheese (Sala et al., 2007). Depending on the properties of the emulsifiers on the surface of emulsion droplets, emulsion droplets can either be bound to the gel matrix (called bound filler) or not (called unbound filler; Sala et al., 2007). Unbound fat has been found to be related to the enhancement of fat-related sensory perception rather than bound fat droplets in emulsion-filled gels (Sala et al., 2007; Liu et al., 2015). The unbound droplets underwent more coalescence than bound droplets during oral manipulation, leading to lower friction and enhancement of fat-related sensory perceptions (Liu et al., 2015).

Aqueous/Serum Phase

Water. Water in cheese can be broadly classified as bound or bulk water. Bound water is strongly associated with protein and other components of the cheese matrix, and this water is not available as a solvent, whereas bulk water is loosely associated within the protein matrix and retains a large solvent capacity and is freezable at −40°C (McMahon et al., 1999). Bulk water may be either present within the channels surrounding the fat (free water) or entrapped within the protein matrix (entrapped water). The distribution and state of water in cheese depends on factors such as cheese-type and age. In most cheese varieties, most of the water is present within the protein matrix. However, in young Mozzarella cheese, a significant amount of water is present in the fat-serum channel. During aging, this water is gradually absorbed into the protein matrix, which has been confirmed by studies undertaken using nuclear magnetic resonance technique (Kuo et al., 2001; Smith et al., 2017). Moreover, during maturation, hydrolysis of each peptide bond releases 2 new charged groups (NH_3^+/COO^-) that can bind the available free water and thus can alter the state of water in cheese (Creamer and Olson, 1982). This might be a possible reason for a slight decrease in water activity (a_w) in Cheddar cheese during ripening, from a mean of ~0.965 at 1 d to ~0.956 at 270 d (Hickey et al., 2013).

Cheese generally becomes softer as the levels of moisture increase. Two main reasons have been reported for the texture softening effect of moisture: (1) water in the cheese matrix plays the role of a plasticizer (low-viscosity lubricant; Marshall, 1990), and (2) increasing the levels of moisture results in a corresponding decrease in the levels of casein, which is the principal structuring component (McMahon et al., 2005). However, the effect of water on melt properties of cheese is rather complex. Increasing total moisture content of cheese does not necessarily increase the meltability of cheese (Pastorino et al., 2003c; McMahon et al., 2005). Instead, melt properties of cheese are reported to be more related to casein-water interactions (which are largely influenced by pH, ionic strength, and the levels of CCP) than total moisture content (McMahon et al., 1999).

Components of the Aqueous/Serum Phase. The components present in the aqueous phase, such as nitrogen fractions (water-soluble protein, enzymes, peptides, or free AA), minerals, carbohydrates (lactose, galactose, and glucose) and organic acids, and their level influence the environment, mainly pH, a_w, and ionic strength, of the cheese matrix (Salaün et al., 2005; Hickey et al., 2013). This can in turn affect the structure of the protein phase in cheese and thus on the texture, rheological, and cooking properties. Moreover, the correct balance and concentration of components of the serum phase can influence the flavor profile of cheese. Components, such as hydrophobic peptides, lactose, lactate, and free AA, have been found to be positively associated with bitter, sweet, sour, and umami flavor intensities, respectively (Møller et al., 2013).

The level of ions and their valance determines the ionic strength of serum phase of the cheese matrix, which can alter protein interactions. Salt (NaCl) has a major contribution to the ionic strength of the cheese matrix, because a varying amount of salt, ranging from ~0.5% (wt/wt) to 6% (wt/wt), is added in cheese, mainly for flavor and preservation (Guinee, 2004). Addition of salt up to certain concentrations can promote protein-water interactions, probably due to a "salting-in" effect, leading to hydration and swelling of the casein matrix (Guinee, 2004). Several studies have elucidated the role of salt in hydration and solubilization of casein. Guo et al. (1997) observed a higher levels of intact casein in the serum phase obtained from centrifugation (~12,500 × g for 75 min at 25°C) of brine-salted Mozzarella cheese than in unsalted cheese. Pastorino et al. (2003b) observed a more homogeneous protein matrix with less serum pockets in Munster cheese injected with salt than cheese without salt injection, as observed by scanning electron microscopy. Everett et al. (2014) reported an increase in NaCl-soluble proteins with increasing concentration of salt solution up to 6% (wt/wt) when unsalted Cheddar curd was immersed in varying brine concentrations (0–25%, wt/wt). However, very high salt concentrations can promote protein-protein interactions, probably due to a "salting-out" effect, leading to protein aggregation and contraction of the cheese matrix.

Salt in the cheese matrix not only contributes to the saltiness of cheese, but can also enhance the flavor intensity of sapid compounds; moreover, salt can suppress the unwanted flavor, e.g., bitterness (Møller et al., 2013). Thus, reduction in salt content is sometimes associated with flavor defects. For example, the flavor profile of Cheddar cheese deteriorated when the level of salt was reduced by 50% (Møller et al., 2013). In another study, consumer liking for low-salt cheeses was low and they were able distinguish even a 30% salt reduction (Ganesan et al., 2014).

The effect of salt in structure, texture, rheological, and cooking properties of cheese has been discussed extensively (Guinee, 2004; Everett et al., 2014). A general overview of the role of salt in cheese is depicted in Figure 3.

Gas Phase (Particularly Carbon Dioxide)

Formation of smooth eyes in eye-forming cheese types, such as Emmental and Maasdam, is considered an important quality parameter. Cheese matrix structure, the rate and extent of gas production and its behavior in the cheese matrix (e.g., solubility and diffusivity), and the presence of nuclei are known to play an important role in desirable eye formation (Daly et al., 2010). In this section of review, we focus on the role of CO_2 in the eye formation.

An appropriate understanding of CO_2 production, and its solubility and diffusivity in the cheese matrix, is necessary to obtain desirable quality of eyes without splits and cracks. Carbon dioxide in the cheese matrix is mainly produced due to lactate fermentation by propionic acid bacteria during the warm room ripening. The rate and extent of CO_2 production is influenced by factors, such as strains of propionic acid bacteria, ripening temperature, and cheese composition (Daly et al., 2010). Acerbi et al. (2016a) determined the rate of production of CO_2 in semi-hard cheese to be ~10 to 15 mmol/kg·d at constant temperature (25°C) and salt-to-moisture ratio (2%, wt/wt). Carbon dioxide solubilizes in the fat and aqueous phases of cheese. However, its solubility is largely temperature dependent [i.e., the solubility of CO_2 in the fat phase is lower at low temperature (e.g., 4°C) than at high temperature (at least up to 20°C)]; the opposite holds true for the solubility of CO_2 in water (Jakobsen et al., 2009). The solubility of CO_2 in semi-hard cheese was determined as ~37 mmol/kg·atm at 2°C and ~30 mmol/kg·atm at 25°C (Acerbi et al., 2016b). Thus, any changes in the ripening conditions or cheese composition or both can alter the solubility of CO_2 within the cheese matrix, which in turn can affect the internal pressure of cheese. It is necessary to control the internal pressure of cheese, because overpressure can lead to slits or cracks, which are unappealing to consumers. Moreover, these cheeses can produce a lot of fines or broken portions during size reduction operations, such as slicing and dicing, resulting in lost revenue to manufacturers (Martley and Crow, 1996). On the other hand, small or no eyes ("blind" cheese) will be formed if the gas pressure is inadequate.

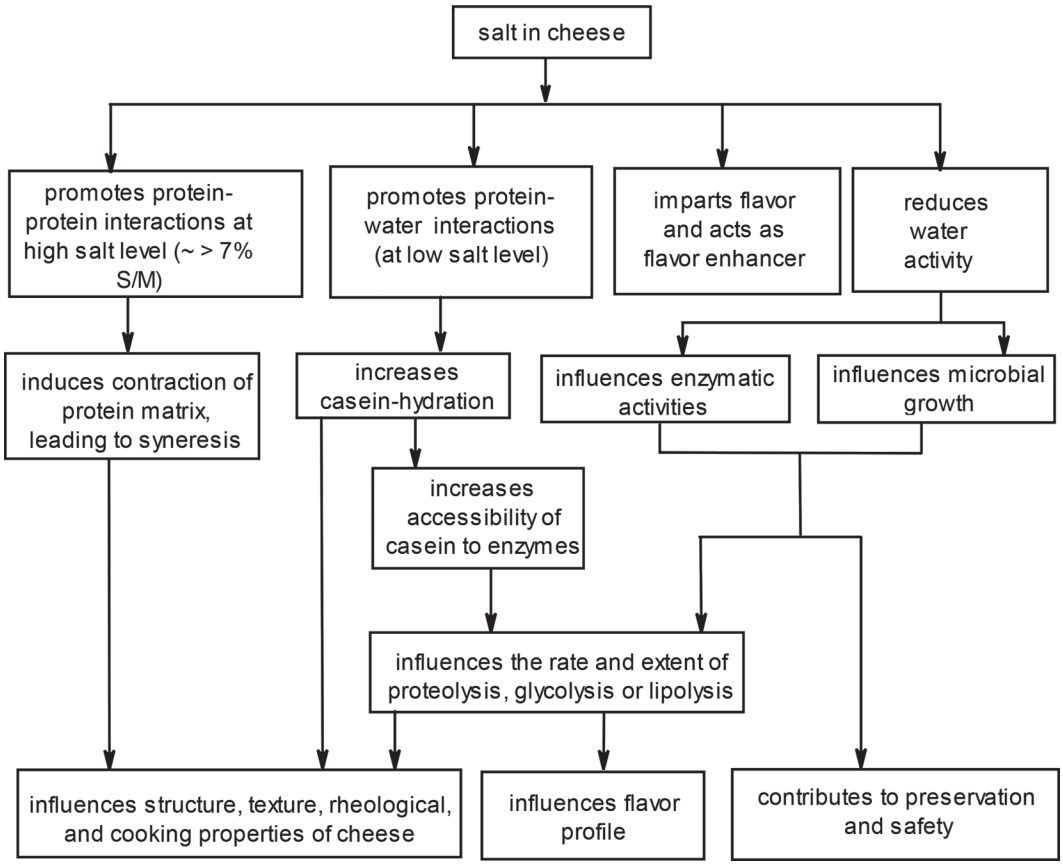


Figure 3. Role of salt in cheese. S/M = salt-to-moisture ratio.

Carbon dioxide produced in the cheese matrix diffuses within the cheese matrix or escapes from the cheese. The diffusion of CO_2 within the cheese is thought to obey the second law of Fick [Equation [1]; where D is the effective diffusivity coefficient (m^2/s)], which describes the change in concentration (c) with time (t) at any place (x) as a function of the local concentration gradient for a mono-directional diffusion (Acerbi et al., 2016c).

$$\frac{dc}{dt} = D\left(\frac{d^2c}{dx^2}\right). \quad [1]$$

Diffusion of CO_2 to nuclei generates pressure at nuclei which are the primary site for eye formation. Diffusivity of CO_2 within the cheese matrix is one of the most important factors affecting eye growth in eye-forming cheese types, and it is influenced by cheese composition and structure, and ripening conditions. For example, a higher level of diffusivity of CO_2 has been observed in more aged semi-hard cheeses compared with young cheeses, and this has been attributed to age-related changes in the cheese matrix, such as proteolysis and demineralization of casein (Acerbi et al., 2016c).

EFFECT OF ENVIRONMENTAL FACTORS ON STRUCTURAL AND FUNCTIONAL PROPERTIES OF CHEESE

pH

It is widely recognized that pH has a strong influence on the texture, rheological, and cooking properties of cheese, mainly via altering the casein-casein, mineral-casein, and casein-water interactions, through its effect on casein charge and calcium solubility. At higher pH values (~5.4), the proportion of calcium associated with casein (micellar calcium) is relatively higher than at low pH [unless colloidal calcium is solubilized by other means, such as addition of calcium chelators; Choi et al. (2008); McAuliffe et al. (2016)]. Higher levels of micellar calcium promote casein-casein interactions within the cheese matrix. Such strong casein-casein interactions are known to increase the structural rigidity of the cheese matrix; as a consequence, cheese tends

to be more firm, elastic, and less meltable (McMahon et al., 2005). At intermediate pH (~5.1), casein-casein interactions decrease as the negatively charged regions of the casein (e.g., phosphoserine residues) are exposed due to partial solubilization of micellar calcium, and the resultant cheese tends to be softer and more meltable (Lucey et al., 2003). However, lowering the pH toward 4.7 increases the strong casein-casein interactions as the casein approach their isoelectric point, and adversely affects the melt, flow, and stretch properties of cheese (Lucey et al., 2003; Pastorino et al., 2003a).

It seems that that pH may have an indirect effect related to its influence on the distribution of calcium (soluble or casein-associated) at pH above 5 (at least in Cheddar and direct-acidified nonfat Mozzarella cheese; Pastorino et al., 2003a; McMahon et al., 2005). Below 5, pH seems to have a direct effect (i.e., charge neutralization). If the cheese curd has very low levels of calcium, then increasing pH may simply increase protein-water interactions, probably due to increases in electrostatic repulsion forces between the charged groups of protein, which is illustrated by the work of Monteiro et al. (2009). Those authors investigated the effect of pH on the microstructure and functionality of hot-pack cream cheese, in which calcium level is very low, by using exposure to acetic acid or ammonia vapor to modulate pH after manufacture. The microstructure of cheese appeared more continuous or swollen with increasing pH (Figure 4), indicating that the casein is increasingly hydrated with increasing pH; moreover, cheese firmness decreased, whereas cheese meltability increased, with increasing pH.

The pH of cheese can alter the size of the protein aggregates and their arrangement in the protein matrix (Hall and Creamer, 1972; Lawrence et al., 1987; Pastorino et al., 2003a). Pastorino et al. (2003a) developed a model for the protein matrix of cheese at different pH, and reported that the diameter of protein aggregates were relatively higher at pH 5.3 (10 to 12 nm) than at pH 4.7 (2 to 4 nm) in their model. Moreover, the protein aggregates at pH 5.3 have relatively more well-defined structure than those at pH 4.7. The proposed model is in agreement with the study of Hall and Creamer (1972), who observed bigger protein aggregates (10 to 15 nm) in Gouda cheese (pH, ~5.3) than in Cheshire cheese (3 to 4 nm), with relatively low pH (~4.6), when examined using scanning electron microscopy; moreover, the protein in latter cheese is less well organized. Taneya et al. (1992) also reported a less well-defined protein network structure of curd at pH 5.0 than at pH 5.4 when observed with transmission electron microscopy, and the authors concluded that curd having pH 5.4 is suitable for stretching during manufacture of string cheese. Pastorino et al. (2003a) speculated that the size of the protein aggregates and their arrangement in the cheese matrix may alter the texture, rheological, and cooking properties of cheese because the strength of material is known to be influenced by factors such as the extent of cross-linking, and the orientation or the structural regularity of the constituents of the material (Pastorino et al., 2003a).

It is well known that mold-ripened cheeses (e.g., Camembert and Brie), have a macroscopic pH gradient between the surface and interior of cheese (McSweeney, 2004). However, it has recently been found that cheese can have a microscopic pH gradient. Burdikova et al. (2015) observed pH micro-heterogeneity in natural cheese matrices using fluorescence lifetime imaging microscopy (Figure 5). The local variation of pH in cheese matrix may influence the molecular interactions at local level, which may lead to local heterogeneity in the microstructure of cheese. Nevertheless, more research is needed to gain greater understanding in this area.

Temperature

Temperature influences the structure of cheese through its effect on the components of cheese and their interactions, including changes in the physical state of fat and the molecular interactions between the casein. These changes in the structure of cheese are important to the textural, rheological, and cooking properties of heated or unheated cheese. At low temperatures (below

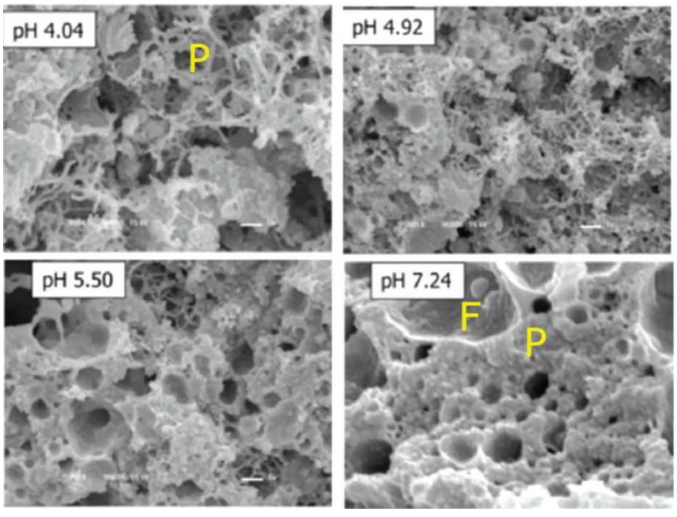


Figure 4. Scanning electron micrographs (9,000×) of hot pack cream cheese with different pH values. Cheese sample were exposed to ammonia vapor to increase the pH or acetic acid vapor to decrease the pH. The pH of the untreated control cheese was 4.92. P = protein matrix, F = spherical imprints in the protein matrix left by fat globules that were extracted during sample preparation. Scale bar represents 1 μm. Adapted from Monteiro et al. (2009) with permission. Copyright (2009) Institute of Food Technologists. Color version available online.

20°C), a significant proportion of milk fat is in a solid state (Lopez et al., 2006). Lopez et al. (2006) observed more than half (~54% of total fat content) of the milk fat present in Emmental cheese is in crystallized form at 4°C. Solid milk fat in cheese is known to act as reinforcing fillers, contributing to elastic properties of unheated cheese (Rogers et al., 2010; Shima and Tanimoto, 2016). Moreover, it has been suggested that the contact area between the casein increases with decreasing temperature as they expand at low temperature, probably due to weakening of hydrophobic interactions (Lucey et al., 2003). Thus, the firmer texture of cheese at low temperature (<20°C) is considered due to the combined effect of higher proportion of solid fat and increased contact area between casein. The firm texture of cheese at low temperature is generally suitable for size reduction operation because it is easier to cut cheese cleanly (Lucey, 2008).

During heating of cheese, the proportion of liquid fat increases dramatically; at ~40°C, almost all fat in cheese is in a liquid state (Lopez et al., 2006). Liquid fat acts as a plasticizer between casein strands, making cheese more soft and flexible (Shima and Tanimoto, 2016). Although fat has an important role in the initial softening of cheese during heating, it is now well accepted that the casein interactions have a major role on the melting properties of cheese. Lucey et al. (2003) proposed a mechanism for melting of cheese during heating based on the dual-binding model proposed by Horne (1998). The authors speculated that the localized balance of the attractive and repulsive forces between casein controls the cheese melting and the behavior of cheese at elevated temperature. During heating, casein networks in cheese are thought to contract, probably due to strengthening of hydrophobic interactions. Magnetic resonance studies have also indicated that contraction of the protein network occurs in cheese with increasing temperature, since levels of free water in cheese increased as it was heated from 20 to 60°C (Vogt et al., 2015; Smith et al., 2017). Contraction of the casein network is suggested to reduce the size of the contact area between casein, leading to weakening of the cheese matrix (Lucey et al., 2003). Weakening of the cheese matrix during heating is indicated by the changes in the rheological parameters, that is, decreases in dynamic moduli (G′ and G″) and an increase of loss tangent with increasing temperature. More recently, mid-infrared and synchronous fluorescence spectroscopies, coupled with chemometrics, have been suggested as valuable tools for understanding the role of temperature on the melt behavior of cheese (Boubellouta and Dufour, 2012).

Kim et al. (2011) investigated the effect of baking temperature (180°C for 25 min) on the properties of reduced-fat and full-fat Cheddar cheeses by reacting heated cheeses with different dissociating agents, such as SDS, EDTA, and urea; the results indicated that the skin formation in reduced-fat cheeses is the result of a high degree of protein-protein interactions, which involve disulfide bonds and hydrophobic interactions and, to some extent, ionic bonds with calcium.

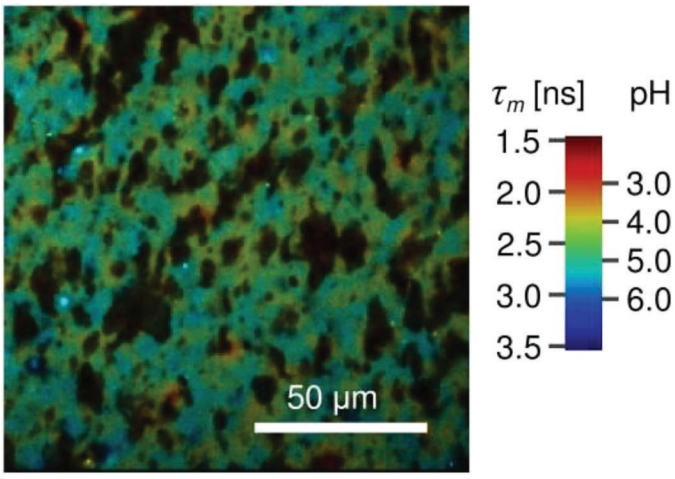


Figure 5. Fluorescence lifetime imaging microscopy (FLIM) image of natural cheese sample stained with Oregon Green 488. Shown are apparent local variations of fluorescence lifetime and thus pH. Localized spots with pH as low as 4.0 are observed. The dark areas most likely represent fat within the cheese matrix. The pseudocolor scale of the FLIM images is calibrated both in lifetime (τ_m) and pH values. Reprinted with permission from Burdikova et al. (2015). Copyright (2015) Lausanne: Frontiers Research Foundation. Color version available online.

CONCLUSIONS

This review documents the current understanding on structure-function relationships in cheese. A fundamental knowledge of how the structure of cheese influences various functionalities is necessary to design cheeses with enhanced physico-chemical properties, and of optimal sensory and nutritional quality. Such knowledge is particularly important for the improvement of the quality of cheeses such as those with low fat content because fat reduction is often associated with undesirable changes in texture, flavor, and cooking properties. Similarly, reduction in sodium has also been linked particularly with flavor defects. Cheese structures have been shown to play an important role in texture perception and in release of flavor compounds during mastication. Structuring of the cheese matrix for controlled release of nutrients, and delivery of bioactives and probiotics is an area of key importance in the development of functional cheese with beneficial properties beyond basic nutrition. A detailed knowledge of molecular

interactions and forces that act between cheese components is vital as they can influence the functionality of cheese, such as physico-chemical properties of heated and unheated cheese, and also the release patterns of flavor compounds or nutrients in mouth during mastication or in the gut during digestion. This also creates a need to further develop analytical methods for determination of molecular forces or interactions within the cheese matrix. Similarly, advanced microscopy techniques allied with image analysis, mathematical modeling, and computer simulations will also help to establish a greater knowledge of the link between structure and functionality of cheese. In addition, the growing number of studies using model food systems, such as emulsion-filled gels and solid lipoproteic colloid foods, offers potential, and such approaches need to be applied to research and innovation in natural cheeses. Overall, an appropriate knowledge of structure-function relationship is key to the design of future cheese types with specific functionalities.

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Effect of milk centrifugation and incorporation of high-heat-treated centrifugate on the composition, texture, and ripening characteristics of Maasdam cheese

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ABSTRACT

This study investigated the effect of centrifugation (9,000 × *g*, 50°C, flow rate = 1,000 L/h), as well as the incorporation of high-heat-treated (HHT) centrifugate into cheese milk on the composition, texture, and ripening characteristics of Maasdam cheese. Neither centrifugation nor incorporation of HHT centrifugate into cheese milk had a pronounced effect on the compositional parameters of any experimental cheeses, except for moisture and moisture in nonfat substance (MNFS) levels. Incorporation of HHT centrifugate at a rate of 6 to 10% of the total milk weight into centrifuged milk increased the level of denatured whey protein in the cheese milk and also increased the level of MNFS in the resultant cheese compared with cheeses made from centrifuged milk and control cheeses; moreover, cheese made from centrifuged milk had ∼3% higher moisture content on average than control cheeses. Centrifugation of cheese milk reduced the somatic cell count by ∼95% relative to the somatic cell count in raw milk. Neither centrifugation nor incorporation of HHT centrifugate into cheese milk had a significant effect on age-related changes in pH, lactate content, and levels of primary and secondary proteolysis. However, the value for hardness was significantly lower for cheeses made from milk containing HHT centrifugate than for other experimental cheese types. Overall, centrifugation appeared to have little effect on composition, texture, and ripening characteristics of Maasdam cheese. However, care should be taken when incorporating HHT centrifugate into cheese milk, because such practices can influence the level of moisture, MNFS, and texture (particularly hardness) of resultant cheeses. Such differences may have the potential to influence subsequent eye development characteristic, although no definitive trends were observed in the present study and further research on this is recommended.

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Key words: centrifugation, heat-treatment, Maasdam cheese, texture, ripening characteristic

INTRODUCTION

Various milk pretreatment methods have been applied before cheesemaking to enhance quality, consistency, and functionality of different cheese varieties (Kelly et al., 2008; Johnson, 2017). Centrifugation of milk using a special centrifuge (also called Bactofuge, Alfa Laval, Richmond, VA) at a centrifugal force of ∼9,000 × *g* (at 50°C) is a pretreatment method widely used by the cheese industry for removal of *Clostridium* spores before cheesemaking. After centrifugation, milk is divided into 2 streams, namely (1) centrifuged milk containing low bacterial cells and spores count, which account for ∼97% of the feed volume, and (2) centrifugate containing high bacterial cells and spores count, which account for ∼3% of the feed volume (Kosikowski and Mistry, 1990).

Some cheese producers apply high heat treatment to the centrifugate to inactivate bacterial cells and spores and recycle the stream back into centrifuged milk before cheesemaking to minimize protein losses, as it contains ∼7% protein (Kosikowski and Mistry, 1990). High heat treatment of milk results in denaturation of whey proteins (Rynne et al., 2004), which can form complexes with whey proteins (in the serum phase) and casein micelles (Donato and Guyomarc'h, 2009). Such complexes are believed to hinder the aggregation of destabilized casein micelles during rennet-induced coagulation of milk (Vasbinder et al., 2003), and thus reduce the ability of the gels to undergo syneresis, leading to cheese curd with higher levels of moisture and moisture in nonfat substance (**MNFS**). Moisture in the cheese matrix acts as a plasticizer between the protein strands and softens the cheese texture (Lamichhane et al., 2018a). Moreover, the higher moisture and MNFS content within the cheese matrix can enhance the microbial and enzymatic activities (Beresford et al., 2001), which can alter the ripening characteristics of cheese (Rynne et al., 2004, 2007).

Some *Clostridium* spp. have been reported to be associated with late blowing defect of cheese, which is manifest as production of gas (e.g., CO_2 and H_2) and formation of high levels of butyric acid, resulting in downgraded cheeses (Klijn et al., 1995; Le Bourhis et al., 2007; Garde et al., 2011). Although the effect of centrifugation on efficacy of removal of *Clostridium* spores from milk and late blowing defect of cheese have been a research focus for several studies (Langeveld, 1971; Su and Ingham, 2000), its effect on composition, texture, and ripening characteristics of cheese has to date received little attention. As well as removal of *Clostridium* spores from milk, centrifugation also removes indigenous milk bacterial cells and somatic cells from milk by ~87 and 75 to 95% of the total count, respectively (Te Giffel and Van Der Horst, 2004; Wieking, 2004).

Maasdam is a brine-salted, large-eye forming, semihard cheese combining the traits of both Swiss and Dutch-type cheeses. Both lactic and citric acid fermentation occur during the first 24 h of manufacture and propionic acid fermentation occurs during warm-room ripening. Very little research has been published on the physicochemical properties and ripening characteristics of Maasdam and similar cheese types, such as Jarlsberg.

The aim of our study was to evaluate the effect of (1) centrifugation and (2) the incorporation of the high-heat-treated (**HHT**) centrifugate into cheese milk on the composition, pH, primary and secondary proteolysis, lactic acids levels, and texture of Maasdam cheese during ripening. In our study, centrifugation refers to the separation of bacteria and spores at a centrifugal force of ~9,000 × *g* (at 50°C with a flow rate of 1,000 L/h), whereas centrifugal separation refers to separation of milk into cream and skim milk. A parallel study was conducted investigating the effect of milk centrifugation and incorporation of HHT centrifugate on microbial composition and the levels of volatile organic compounds of Maasdam cheese (Lamichhane et al., 2018b).

MATERIALS AND METHODS

Milk Supply and Treatments

Raw whole milk was obtained from a local dairy company. From raw milk, 3 different cheese milk streams were prepared (Figure 1). Part of the raw milk was separated at 55°C (centrifuge disc separator, GEA Westfalia, Oelde, Germany) to give skim milk and cream. Control cheese milk (**CT**) was prepared by adding a portion of the resultant cream to skim to achieve a protein-to-fat ratio of 1.13:1. The remaining whole milk was centrifuged (Bactofuge disc separator, type: D3187M, Alfa Laval, Richmond, VA) at a centrifugal force of ~9,000 × *g* (at 50°C with a flow rate of 1,000 L/h) to provide centrifuged whole milk and centrifugate (also called sludge or bactofugate), which accounts for approximately 3 to 6% of the total milk feed. Centrifuged whole milk was then separated to give skim and cream. Centrifuged cheese milk (**CF**) was prepared by adding portions of the cream into the skim milk to achieve a protein-to-fat ratio of 1.13:1. High heat treatment (120°C for 26 s, plate heat exchanger, APV Schweig AG, Worb, Switzerland) was applied to centrifugate to inactivate spores and bacteria, and this centrifugate was combined with a portion of centrifuged cream and skim milk to produce the third cheese milk; that is, centrifuged milk containing HHT centrifugate (**CFHHT**). As the protein content of centrifugate after high heat treatment varied between 3.76 and 6.36% (wt/wt) between trials, HHT centrifugate was added to centrifuged milk on a protein basis rather than weight basis [i.e., approximately 12% (wt/wt) of the total protein was from HHT centrifugate in CFHHT milk; on weight basis, HHT centrifugate was added at a level of 6.6 to 10.3% (wt/wt), depending on the protein content of HHT centrifugate]. All cheese milk types (CT, CF, and CFHHT) were standardized to a protein-to-fat ratio of ~1.13:1 and pasteurized (72°C for 15 s) before cheese manufacture.

Cheese Manufacture

Three experimental Maasdam cheese types [i.e., cheese made from control milk (CT cheese), centrifuged milk (CF cheese), and centrifuged milk containing HHT centrifugate (CFHHT cheese)] were each manufactured on 3 different occasions in replicate cheesemaking trials over a 3-mo period. Standardized and pasteurized cheese milks were pumped into cylindrical, jacketed cheese vats. Each vat contained automated variable speed cutting and stirring equipment (APV Schweig AG). All cheese milks (380 kg/vat) were inoculated at 31°C with frozen direct vat inoculate cultures (Chr. Hansen Ltd., Cork, Ireland), including (1) mixed strains of mesophilic bacteria (C950, 18 mg/kg of milk), consisting of *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Leuconostoc*; (2) *Lactobacillus helveticus* (LH-B01, 4.8 mg/kg of milk); and (3) propionic acid bacteria (**PAB**; PS-60, 7 mg/kg of milk). Calcium chloride (34%, wt/vol) was added at a level of 0.3 mL/kg of milk to each vat. Rennet (Chy-Max Plus, ~200 IMCU/mL; Chr. Hansen Ltd.), diluted ~1:10 with deionized water, was added at a level of 0.2 mL/kg of milk after a 40-min ripening period at 31°C. All gels were cut at a constant firmness (storage or elastic modulus, **G′**) value of 35 Pa (as measured using

a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments, New Castle, DE), and the resultant curd particle/whey mixture was allowed to heal for 7 min before being stirred continuously for another 7 min. Stirring was then stopped and a portion of whey (34 kg/100 kg of cheese milk) was removed. After whey removal, reverse osmosis water at ~50°C (23 kg/100 kg of cheese milk) was used to cook the curd to 37°C at a rate of 0.2°C/min with continuous stirring. After the curd washing and cooking steps, whey and curd were drained into a prepress vat and curds were vertically prepressed under warm whey for 25 min, with increasing pressure from 3 to 5 kPa. Whey was then drained from the prepress vat and the prepressed curd was subsequently cut into 10-kg wheels (3 wheels from each vat), placed into 10-kg molds, and pressed vertically under increasing pressure from 3.3 to 14 kPa for ~3.5 h. When the pH of the cheese curds reached 5.49 to 5.51, cheese wheels were transferred to a saturated brine solution (23% wt/wt NaCl, 0.56% $CaCl_2$, pH 5.2, and 18°C) for 24 h. After brining, cheese wheels were vacuum-packed in CO_2-permeable bags and transferred to the ripening room. The cheeses were ripened at 8°C for 10 d (preripening), at 23°C for 30 d (warm-room ripening), and finally stored at 4°C for 140 d.

Rennet Coagulation Properties

In all 3 replicate cheesemaking trials, 2 min after rennet addition and stirring, a representative sample of milk was removed from the vat and placed into a cell of a small amplitude oscillatory rheometer (AR 2000ex, TA Instruments). A concentric-cylinder measuring geometry, consisting of a cylindrical bob and cup, was used. The dynamic changes in rheology during the coagulation process were monitored using a dynamic time sweep analysis with an angular frequency of 1.0 Hz, and a strain of 0.01 at 31°C, within the linear viscoelastic region (strain <0.03) reported for rennet milk gels (Mateo et al., 2010). Total time to reach a G′ value of gels of 35 Pa (at which cutting of the gel in the cheese vat was initiated) after rennet addition was calculated.

Milk and Cheese Composition Analysis

The composition of raw milk, centrifugate, and pasteurized cheese milks were analyzed using a Fourier transform infrared spectrophotometer (MilkoScan FT 120, Foss Electric, Hillerød, Denmark). Raw milk and centrifuged milk samples were analyzed for SCC (cells/mL) with a fluoro-opto-electronic counter (Fossomatic FC, Foss). Casein number, NPN, and levels of whey protein denaturation as percentage of total whey protein of HHT centrifugate and pasteurized cheese milk samples of 1 representative trial were determined as described by Rynne et al. (2004). Grated cheese samples were analyzed at 11 d at least in duplicate for moisture, fat, protein, salt, calcium, and pH, as described by Sheehan et al. (2007a).

For determination of lactose and galactose content, cheese samples were extracted as described by Zeppa et al. (2001). Grated cheese samples (10 g) were mixed

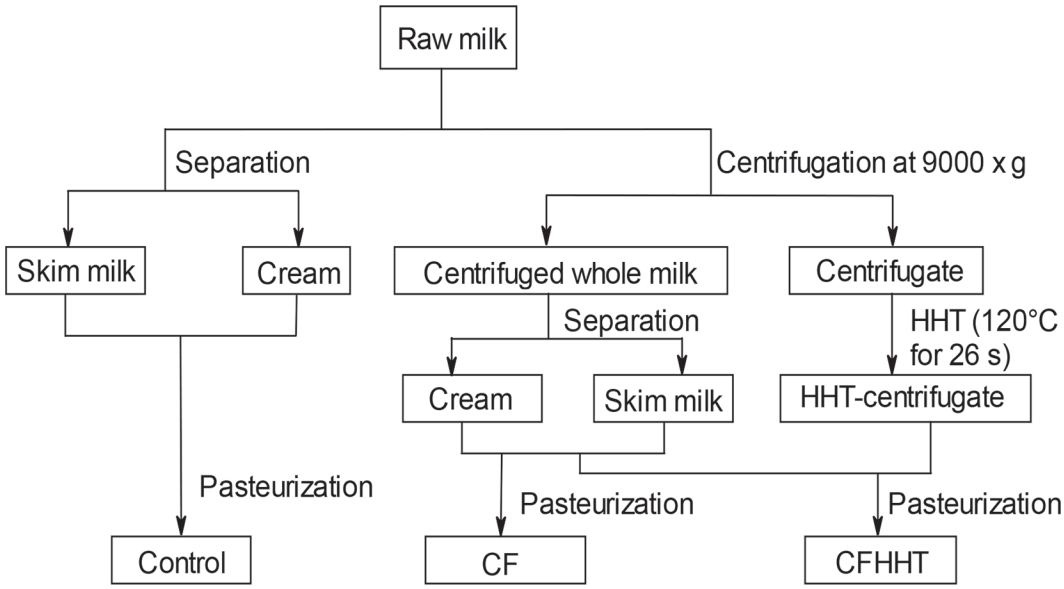


Figure 1. Flowcharts of the preparation of cheese milks [i.e., control, centrifuged (CF), and centrifuged milk containing high-heat-treated centrifugate (CFHHT)]. HHT = high heat treatment; HHT-centrifugate = high-heat-treated centrifugate.

with 50 mL of 0.013 N H_2SO_4 and stomached for 10 min using a stomacher (Iul Instruments, Barcelona, Spain) before centrifugation at 7000 × g for 5 min. The supernatant was then filtered using a 0.2-μm nylon filter. The extracted samples were then analyzed by HPLC (Waters Alliance 2695 separation module, Waters, Milford, MA) with an Aminex HPX-87C Carbohydrate column 300 × 7.8 mm (Bio-Rad, Hertfordshire, UK) under the following working conditions: injection volume of, 50 μL; mobile phase of 0.009 N H_2SO_4; flow rate of 0.5 mL/min; column temperature of 60°C; and refractive index detection (Waters 2414 RI detector). Quantification of lactose and galactose was based on the external standard method as described by Hou et al. (2014).

SDS-PAGE Analysis

The individual proteins in the raw milk, centrifugate before and after HHT, and cheese milks were identified by SDS-PAGE. All milk samples were diluted, using Milli-Q water (Millipore, Billerica, MA), to a protein concentration of ~6 μg/μL. A portion of the diluted samples were further diluted with SDS sample buffer [NuPAGE LDS Sample Buffer (4×; Thermo Fisher Scientific, Waltham, MA), composed of lithium salt, glycerine, sulfuric acid, and monododecyl ester]. For reducing SDS-PAGE, samples were treated with dithiothreitol [NuPAGE Sample Reducing Agent (10×); concentration = 500 mmol/L] at a level of 10% (vol/vol) of the total sample volume mixture. All samples were heated at 70°C for 10 min, cooled, and loaded on SDS-PAGE gels (NuPAGE 12% Bis-Tris mini gels) at a rate of 10 μg/well before running in SDS running buffer [NuPAGE MOPS SDS Running Buffer (1×)] at constant voltage of 200 V for 50 min using Mini Gel Tank (XCell SureLock Mini, Thermo Fisher Scientific). After electrophoresis, the gels were stained as described by McCarthy et al. (2012). The SDS-PAGE gels were scanned using an Epson V700 film scanner (Epson, Suwa, Nagano, Japan). The identities of principal protein bands in the milk samples were determined using prestained protein molecular weight marker (PageRuler Prestained Protein Ladder, 10 to 180 kDa, Thermo Fisher Scientific).

pH and L- and D-Lactate Analysis

The pH of cheese samples were measured on cheese slurry prepared by mixing 20 g of grated cheese and 12 g of deionized water at different time points throughout ripening (Sheehan et al., 2007a). The sample extraction method, as outlined for lactose and galactose content, was used for D- and L-lactic acid content determination. The extracted samples were analyzed for D- and L-lactic acid content using HPLC (Waters Alliance 2695 separation module) equipped with chiral column [Chirex 3126 (D)-penicillamine, column 150 × 4.6 mm, Phenomenex, Cheshire, UK], as described by Hou et al. (2014).

pH 4.6-Soluble Nitrogen and Free Amino Acids

The levels of pH 4.6-soluble N (% of total N) and free amino acids (**FAA**) of the cheeses were measured after 1, 11, 41, 65, 97, 140, and 180 d as described by Fenelon and Guinee (2000) and Sheehan et al. (2007a), respectively.

Texture Profile Analysis of Cheese

Texture properties were analyzed by TAHDi texture profile analyzer (Stable Micro Systems, Goldalming, Surrey, UK), equipped with a 70-mm (diameter) compression plate and a 100-kg load cell. Cheese was cut into 6 cube-shaped samples (25 mm^3) using a Cheese Blocker (Bos Kaasgreedschap, Bodengraven, the Netherlands), wrapped in tin foil, and stored overnight at 4°C. Cheese samples (~4°C) were compressed to 40% of their original height in 2 consecutive bites at a rate of 60 mm/min (Henneberry et al., 2015). Texture profile analyzer parameters were calculated as previously described by Chevanan et al. (2006).

Statistical Analysis

Three experimental cheese types were each manufactured on 3 different occasions in replicate cheesemaking trials. An ANOVA, using IBM SPSS software version 24 (IBM Corp., 2016), was applied to determine the effect of treatment on milk and cheese composition. A split-plot design was used to determine the effects of treatments (centrifugation or addition of HHT centrifugate into cheese milk), ripening time, and their interactions on pH, lactic acid-to-protein ratio, lactic acid, proteolysis, and texture. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple-comparison test was used for paired comparison of treatment means at a 5% level of significance. IBM SPSS software version 24 (IBM Corp., 2016) was used to perform Pearson correlation between lactate-to-protein ratio and pH.

Table 1. Composition of centrifugate and pasteurized cheese milks[1]

Compositional parameter	Centrifugate[2]		Cheese milk[2]		
	Before HHT	After HHT	CT	CF	CFHHT
Protein (%, wt/wt)	6.10[a]	5.11[a]	3.38[b]	3.29[b]	3.32[b]
Fat (%, wt/wt)	0.23[a]	0.22[a]	2.98[b]	2.90[b]	2.92[b]
Lactose (%, wt/wt)	4.52[a]	4.05[a]	4.74[b]	4.66[b]	4.64[b]
Protein/fat	—	—	1.134[b]	1.135[b]	1.137[b]
Casein number	79.22	89.72	79.56	79.16	80.69
NPN (%, wt/wt)	5.91	5.57	6.33	6.57	6.47
Native whey protein (% of total)	14.86	4.71	14.11	14.27	12.85
WPD[3] (% of total whey protein)	—	68.30	5.81	4.77	14.24

[a,b]Values within a row not sharing common superscripts differ ($P < 0.05$) in the case of protein, fat, lactose and protein/fat.

[1]HHT = high heat treatment; CT = control milk; CF = centrifuged milk; CFHHT = centrifuged milk containing high-heat-treated centrifugate.

[2]Data presented are the mean of data from 3 replicate trials for protein, fat, lactose, and protein/fat; for other parameters, data are from 1 representative trial. For parameters without superscripts, statistical analysis was not carried out because the data are from only 1 representative trial.

[3]WPD = whey protein denaturation.

RESULTS AND DISCUSSION

Raw Milk, Centrifugate, and Cheese Milk Composition

The average fat, protein, and lactose contents of raw milk used for the 3 replicate cheesemaking trials were 4.01, 3.41, and 4.73% (wt/wt), respectively. Somatic cell counts of the raw milk between trials ranged between 1.2×10^5 and 2.4×10^5 cells/mL. The SCC of milk depends on factors such as breed and parity, stage of lactation, udder health, and also individual and environmental factors, as well as management practices (Li et al., 2017; Panthi et al., 2017). In general, the SCC of milk from healthy cows is less than 2×10^5 (Li et al., 2014). The efficacy of centrifugation process ($9{,}000 \times g$ at 50°C with a flow rate of 1,000 L/h) for removal of SCC was ~95% relative to the SCC in raw milk, in close agreement with the study of Wieking (2004), who reported the efficacy of centrifugation as ~95%. It is generally accepted that the SCC of cheese milk negatively influences the cheese making and final cheese quality (Panthi et al., 2017). However, in contrast, a recent study (Li et al., 2017) suggested that the somatic cells have minimal effect per se on the cheesemaking and final cheese quality. This suggests that more research is needed to better understand the role of somatic cells in cheese quality.

The composition of centrifugate before and after HHT and cheese milks is shown in Table 1. The level of whey protein denaturation (**WPD**; as a percentage of total whey protein) in centrifugate after HHT was 68.30%. It is widely recognized that HHT denatures whey proteins; for example, Rynne et al. (2004) observed that 34% of total whey protein was denatured when milk was heated at 87°C for 26 s. The protein and lactose content of centrifugate decreased, although not significantly, after HHT. This is probably due to slight dilution with process flush water when utilizing a low volume of centrifugate (~40–45 kg) in pilot-scale processing. Although fat, protein, and lactose contents of cheese milks were not statistically different, the level of WPD was ~2.5- to 3-fold higher in CFHHT milk (14.2%) than in CT (5.8%) and CF (4.8%) milks. The low level of WPD in CT and CF milk is attributed to pasteurization (72°C for 15 s) of milk, whereas the level of WPD in CFHHT milk is attributed to both pasteurization and incorporation of HHT centrifugate. The level of WPD in pasteurized milk is in close agreement with that reported by Rynne et al. (2004).

SDS-PAGE Analysis

The individual proteins in raw milk, centrifugate before and after HHT, and cheese milks were analyzed using SDS-PAGE (Figure 2). The SDS-PAGE patterns of centrifugate before HHT were different compared with centrifugate after HHT, with a lower intensity of the bands corresponding to α-LA, β-LG, and other minor proteins, such as lactoferrin and BSA, in HHT centrifugate. Moreover, some large protein aggregates were observed at the entry of stacking gel in the HHT centrifugate, as denoted by X in Figure 2A. Similar to our result, Patel et al. (2006) also observed heat-induced large aggregates in HHT milk samples. Heat-denatured whey proteins can form complexes with themselves and with caseins, particularly κ-CN, through disulfide interchange reactions and hydrophobic forces (Jean et al., 2006; Patel et al., 2006; Kethireddipalli and Hill,

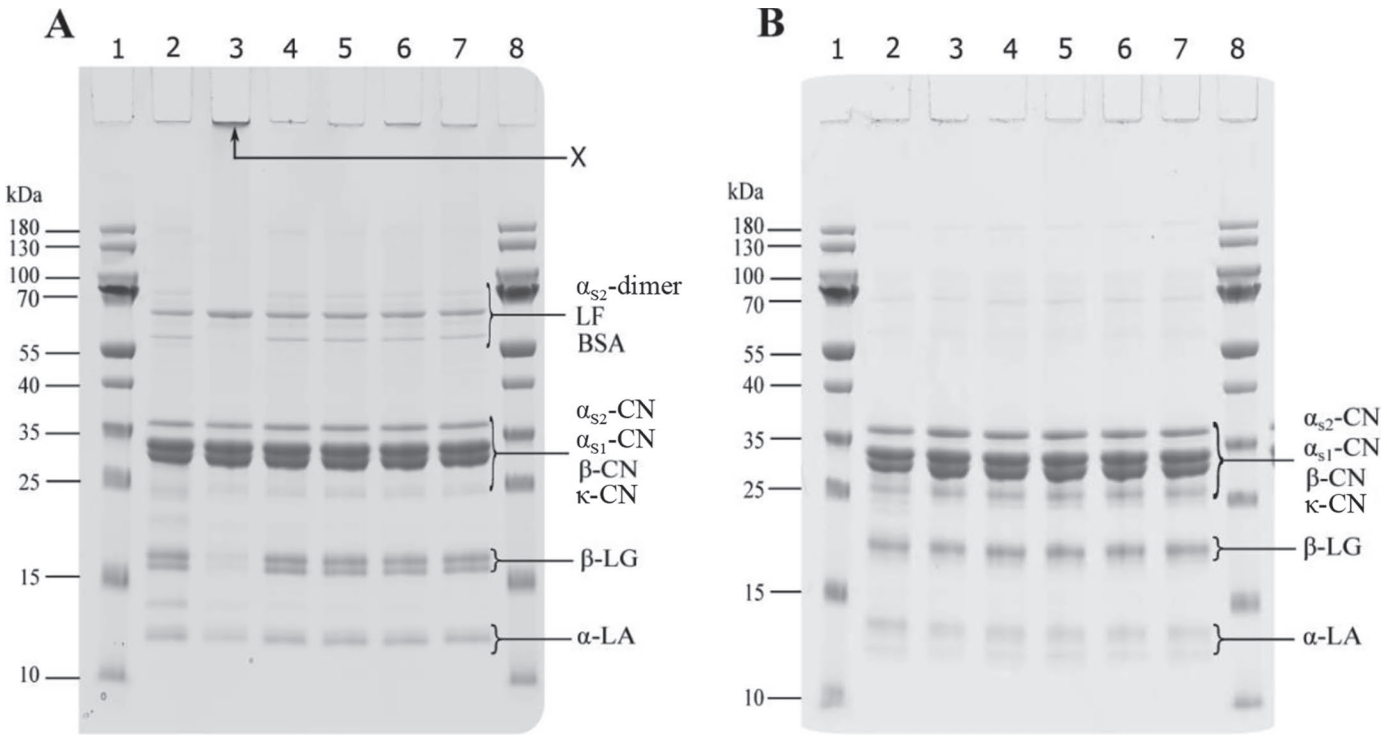


Figure 2. The SDS-PAGE patterns of milk samples under (A) nonreducing and (B) reducing conditions. Lane 1 and 8 = prestained protein molecular weight marker; lane 2 = centrifugate before high heat treatment; lane 3 = centrifugate after high heat treatment; lane 4 = raw milk; lane 5 = pasteurized (72°C for 15 s) control cheese milk; lane 6 = pasteurized centrifuged milk containing high-heat-treated (HHT) centrifugate; and lane 7 = pasteurized centrifuged milk. Protein aggregates in HHT centrifugate are denoted by X. LF = lactoferrin. Samples shown are from 1 representative trial.

2015). In reducing SDS-PAGE, the large heat-induced aggregates in HHT centrifugate disappeared when the samples were reduced with dithiothreitol; moreover, the SDS-PAGE patterns in reduced gels appeared similar (Figure 2B). This suggests that the protein aggregates in HHT centrifugate may be bonded by various molecular forces, such as hydrophobic forces and disulfide bonding (Donato and Guyomarc'h, 2009).

The SDS-PAGE patterns of raw milk and cheese milks appeared similar, although the level of denatured whey protein was higher in cheese milks, especially CFHHT, than in raw milk. This suggests that the SDS-PAGE analysis is not very sensitive for differentiating between low levels of whey protein denaturation.

Rennet Coagulation Characteristics

The average time to reach an elastic shear modulus (G′) value of 35 Pa for CFHHT, CT, and CF milks after rennet addition was 45.1, 38.7, and 40 min, respectively. Several factors can influence the rennet-induced coagulation of milk, such as milk composition and renneting conditions (e.g., pH, temperature, and ionic strength; Guinee et al., 2006; Ong et al., 2011, 2012). Some studies have reported that the heat-induced complexes formed between whey protein and κ-CN on the surface of the casein micelles inhibit the primary phase of rennet coagulation (i.e., decrease the accessibility of enzyme to κ-CN; Van Hooydonk et al., 1987). However, more recent studies have suggested that WPD has minimal effect on the primary phase of rennet coagulation; instead, WPD has a more clear effect on the secondary phase of rennet coagulation [i.e., aggregation (fusion) of destabilized casein micelles; Vasbinder et al., 2003]. The casein-whey protein and whey protein aggregates (in serum phase) can sterically hinder the aggregation of destabilized casein micelles (Waungana et al., 1996; Vasbinder et al., 2003). In the current study, although the level of denatured whey protein of CFHHT was ~2.5- to 3-fold higher than that of CT and CF milks, the rennet coagulation time of CFHHT milk did not differ statistically significantly to the others. The levels of WPD (as a percent of total whey protein) for all experimental cheese milks were below 15%, and it seems that such levels of WPD had no pronounced effect on the rennet-induced coagulation of milk. More pronounced effects were reported previously for severely heat-treated milk (Rynne et al., 2004).

Table 2. Compositional parameters and pH at 11 d of ripening in Maasdam cheeses[1]

Compositional parameter[2]	Cheese type			SEM	*P*-value
	CT	CF	CFHHT		
Moisture (%, wt/wt)	44.83[a]	44.15[a]	47.83[a]	0.72	0.057
MNFS (%, wt/wt)	58.9[a]	58.14[a]	61.78[b]	0.63	0.013
Protein (%, wt/wt)	24.04[a]	24.57[a]	23.44[a]	0.30	0.348
Fat (%, wt/wt)	23.9[a]	24.07[a]	22.61[a]	0.44	0.376
FDM (%, wt/wt)	43.31[a]	43.07[a]	43.29[a]	0.35	0.966
Salt (%, wt/wt)	1.53[a]	1.50[a]	1.73[a]	0.05	0.183
S/M (%, wt/wt)	3.41[a]	3.40[a]	3.61[a]	0.07	0.450
Total calcium (mg/100 g)	821[a]	800[a]	837[a]	12.19	0.514
pH (11 d)	5.28[a]	5.31[a]	5.27[a]	0.01	0.548
Lactose (mg/100 g)					
1 d	54.39[a]	41.82[a]	66.72[a]	16.26	0.861
11 d	20.42[a]	43.72[a]	62.63[a]	15.07	0.585
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	—
Galactose (mg/100 g)					
1 d	28.09[a]	29.57[a]	35.34[a]	4.11	0.798
11 d	27.61[a]	27.26[a]	30.82[a]	4.22	0.947
41 d	0.00[a]	0.00[a]	0.00[a]	0.00	—

[a,b]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean of data from 3 replicate trials.

[1]CT = control cheese; CF = cheese made from centrifuged milk; CFHHT = cheese made from centrifuged milk containing high-heat-treated centrifugate.

[2]MNFS = moisture in nonfat substance; FDM = fat in DM; S/M = salt-to-moisture ratio.

Cheese Composition

The composition of the experimental cheeses is shown in Table 2. Centrifugation of milk had no significant effect on mean levels of moisture, MNFS, protein, fat, salt, total calcium, lactose, and galactose of final cheeses. However, incorporation of HHT centrifugate into the centrifuged milk increased ($P < 0.05$) the mean levels of MNFS of the resultant cheeses. The average moisture content of CFHHT cheese was ~3% higher than control and CF cheeses, which was expected and sizeable in magnitude for cheese moisture; however, the difference was not statistically significant ($P = 0.057$). This is explained by a degree of variation in the compositional data between trials, which influenced the statistical analysis of the data. The coefficients of variation of average moisture content of the experimental cheeses between 3 trials were below 5%, which is considered acceptable (Thomsson et al., 2014). Higher moisture and MNFS levels in CFHHT cheeses are partly attributed to the negative effect of HHT centrifugate on syneresis (expulsion of whey) of rennet-induced milk gels. Denatured whey protein present in HHT centrifugate can sterically hinder the aggregation (fusion) of destabilized casein micelles, as described before, and thus hinders syneresis (Pearse et al., 1985; Walstra et al., 1985; Vasbinder et al., 2003). Moreover, the high water-binding capacity of the denatured whey proteins may increase the level of MNFS in the CFHHT cheeses (Donato and Guyomarc'h, 2009). These results are in agreement with the results of Guinee et al. (1998) and Rynne et al. (2004), who observed increased moisture and MNFS of Cheddar cheese with increasing levels of denatured whey protein.

Some studies also observed a decrease in the level of total calcium in cheese made from HHT milk (Guinee et al., 1998); however, such results were not observed in the current study. The mean levels of lactose and galactose were very low, below ~67 and ~36 mg/100 g of cheese, respectively, until 11 d of ripening, and lactose and galactose were not detected after warm-room ripening (Table 2). Low lactose and galactose contents within this cheese type were expected, as the lactose contents of cheese curd were reduced by curd washing.

Age-Related Changes in pH

The pH of all experimental cheeses increased ($P < 0.001$) over the 180 d of ripening (Figure 3A, Table 3) from a mean value of ~5.2 at 1 d to ~5.7 at 180 d. This trend is in agreement with that reported in a previous study of Gouda cheese by Lawrence et al. (1987), who also observed an increase in pH from ~5.15 at 1 d to ~5.5 to 5.9 at 150 d of ripening. The increase in the pH is attributed to several factors, including the proteolytic liberation of basic compounds, such as ammonia, free basic AA, and amines (Fenelon and Guinee, 2000; McSweeney, 2004). A reduction in lactate-to-protein ratio during maturation of cheese (Figure 3B) is known to increase the buffering capacity of cheese (Sheehan

et al., 2007a), which may contribute to some extent to the increase in cheese pH during ripening. The pH of the cheese curd was significantly ($P < 0.001$) negatively correlated (adjusted $R^2 = 0.53$) with lactate-to-protein ratio (Figure 3C).

In some brine-salted cheese types, such as Gouda, Edam, and Maasdam, the pH after brining is controlled by adjusting the residual curd lactose content by techniques such as curd washing or whey dilution (Lawrence et al., 1987). More recently, membrane separation techniques, such as ultrafiltration, have also been used for standardization of lactose content of cheese milk before cheesemaking (Moynihan et al., 2016). In the present study, ~34% (wt/wt) of whey was replaced with ~23% (wt/wt) warm reverse-osmosis water to control the postmanufacture reduction of pH. In contrast, O'Sullivan et al. (2016) observed a decrease in pH at the early stages of ripening in Swiss-type cheese (where whey was not replaced by warm water); however, the postbrining pH was higher (~5.6) than that of the cheese in the current study (~5.3). The decrease in the pH at the early stages of ripening has been attributed to continual metabolism of residual lactose and galactose to lactate by starter and non-starter lactic acid bacteria (**NSLAB**; O'Sullivan et al., 2016). Shakeel-Ur-Rehman et al. (2004) also observed a decrease in the pH during ripening when Cheddar cheese was made from milk supplemented with lactose. Regulation of pH is critical for proper eye development in some eye-forming cheese types, and a reduction in pH can reduce the levels of colloidal calcium, which are considered essential for elastic texture of cheese (Lucey and Fox, 1993), and inhibit the growth of PAB (Sheehan et al., 2008). Elastic texture is important in the case of eye-forming cheese types to accommodate gas produced during warm-room ripening for smooth eye formation (Daly et al., 2010). No significant effect of treatment on the mean cheese pH during maturation was observed (Table 3).

Levels of L-, D- and Total Lactate

The mean level of L-lactate of all experimental cheeses was ~1.5% (wt/wt) until 11 d of ripening, which is most likely due to fermentation of glucose, lactose, and galactose by starter lactic acid bacteria, including *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Lactobacillus helveticus*, during production of cheese (Beresford et al., 2001). However, the level of L-lactate decreased ($P < 0.001$) over ripening in all experimental cheeses, especially during warm room ripening from a mean of ~1.5% (wt/wt) at 11 d to ~0.4% (wt/wt) at 41 d (Figure 4A). This trend is in agreement with that reported in previous studies

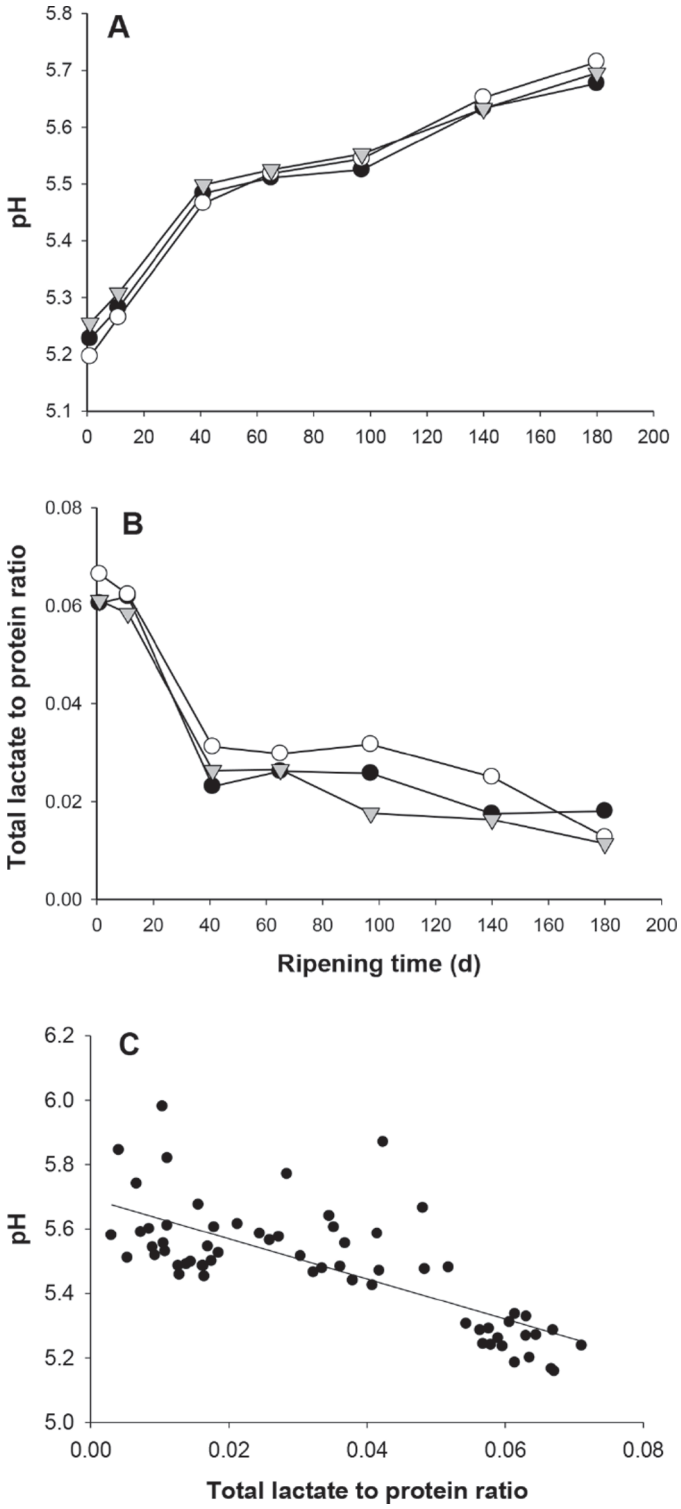


Figure 3. The effect of milk pretreatments on (A) pH and (B) lactate to protein ratio of Maasdam cheese during maturation. Milk pretreatments were control (●); centrifugation (▼); and centrifuged milk containing high-heat-treated centrifugate (○). Data presented are means of data from 3 replicate trials. (C) Relationship between pH and lactate-to-protein ratio; data were obtained from all experimental cheeses produced in 3 replicate trials and analyzed over a 180 d of ripening.

Table 3. Summary of the effects of treatment, time, and their interactions on properties of Maasdam cheeses[1]

Parameter[2]	Treatment	Time	Interactive effect (treatment × time)
pH	NS (0.81)	***	NS (1.00)
Total lactate-to-protein ratio	NS (0.33)	***	NS (0.99)
L-Lactate	NS (0.70)	***	NS (0.99)
D-Lactate	NS (0.32)	***	NS (0.99)
Total lactate	NS (0.53)	***	NS (0.99)
pH 4.6-SN (% TN)	NS (0.65)	***	NS (1.00)
Total FAA	NS (0.74)	***	NS (0.96)
Hardness	*	NS (0.29)	NS (0.97)
Springiness	NS (0.71)	*	NS (0.40)
Cohesiveness	NS (0.08)	***	NS (0.57)
Resilience	NS (1.00)	**	NS (0.96)

[1]Digits in parentheses after NS represent *P*-value.

[2]pH 4.6-SN (% TN) = soluble nitrogen at pH 4.6 as percentage of total nitrogen; FAA = free AA.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS = $P > 0.05$.

for different cheese varieties, such as Swiss-style cheese (Sheehan et al., 2008; O'Sullivan et al., 2016), Grevé (eye-forming, semihard cheese types; Rehn et al., 2011), and Cheddar (Rynne et al., 2007). This was expected, as L-lactate is metabolized by starter and nonstarter bacteria to different metabolites, such as propionate, acetate, butyrate, formate, succinate, DL-lactate, CO_2, H_2, and H_2O (McSweeney, 2004; Agarwal et al., 2006).

D-Lactate was virtually absent for all experimental cheeses at 1 and 11 d of ripening. However, unlike L-lactate, the level of D-lactate increased ($P < 0.001$) during warm room ripening, from a mean of ~0.01% (wt/wt) at 11 d to ~0.2 to 0.3% (wt/wt) at 41 d (Figure 4B). D-Lactate within cheese matrix typically arises either from fermentation of glucose, lactose, or galactose by microorganisms, including *Lactobacillus helveticus* and some *Leuconostoc* spp. (Beresford et al., 2001), or by racemization of L-lactate by NSLAB (Agarwal et al., 2006). As the levels of residual lactose and galactose were very low in all experimental cheeses before warm-room ripening (below 70 and 40 mg/100 g, respectively) due to curd washing, it may be assumed that the contribution of residual lactose and galactose for formation of D-lactate is minimal. The formation of D-lactate is most likely due to racemization of L-lactate to DL-lactate by NSLAB because the level of NSLAB reached ~10^8 cfu/g in all cheeses at 41 d of ripening (data not shown). Similar trends have previously been reported in different cheese varieties, such as half-fat Cheddar (Rynne et al., 2007) and Swiss-type cheese (O'Sullivan et al., 2016). Interestingly, the level of D-lactate decreased gradually after 65 d of ripening, probably due to its metabolism by microorganisms within the cheese matrix such as PAB (O'Sullivan et al., 2016). The degradation pathways of D-lactate in the cheese matrix are not yet fully understood. No significant effect of treatment was observed in the levels of L-lactate, D-lactate, and total lactate (Figure 4C) throughout ripening (Table 3).

Proteolysis

Nitrogen Soluble at pH 4.6. Primary proteolysis in cheeses was assessed by measuring the level of nitrogen soluble at pH 4.6, as a percentage of total nitrogen, which increased ($P < 0.001$) in all cheeses over the 180 d of ripening, especially during warm-room ripening, from ~5% of total nitrogen before warm-room ripening (11 d) to ~17% of total nitrogen at the end of warm-room ripening (41 d) in all experimental cheeses (Figure 5A). The levels in all cheeses reached ~22% at 180 d. The increase was of the same order of magnitude as that previously reported for semihard cheeses (Exterkate and Alting, 1995; Sheehan et al., 2007b; Huc et al., 2014). However, no significant effect of treatment on level of nitrogen soluble at pH 4.6, as a percentage of total nitrogen was observed (Table 3).

Total and Individual FAA. The mean levels of total FAA increased ($P < 0.001$) during ripening, especially when the cheeses entered the hot-room ripening phase, from ~3,000 mg/kg at 11 d to ~7,000 to 8,000 mg/kg at 41 d (Figure 5B). Enzymes from starter and nonstarter microorganisms and somatic cells, such as proteases and peptidases, contribute to primary and secondary proteolysis where present within the cheese matrix, and thereby to liberation of FAA during ripening (McSweeney, 2004; Kelly et al., 2006). No significant effect of treatment was observed (Table 3), although it was expected that the centrifugation process could alter secondary proteolysis, as the process can remove ~86 to 92% of total indigenous bacteria and ~95% of somatic cells from cheese milk (Te Giffel and Van Der

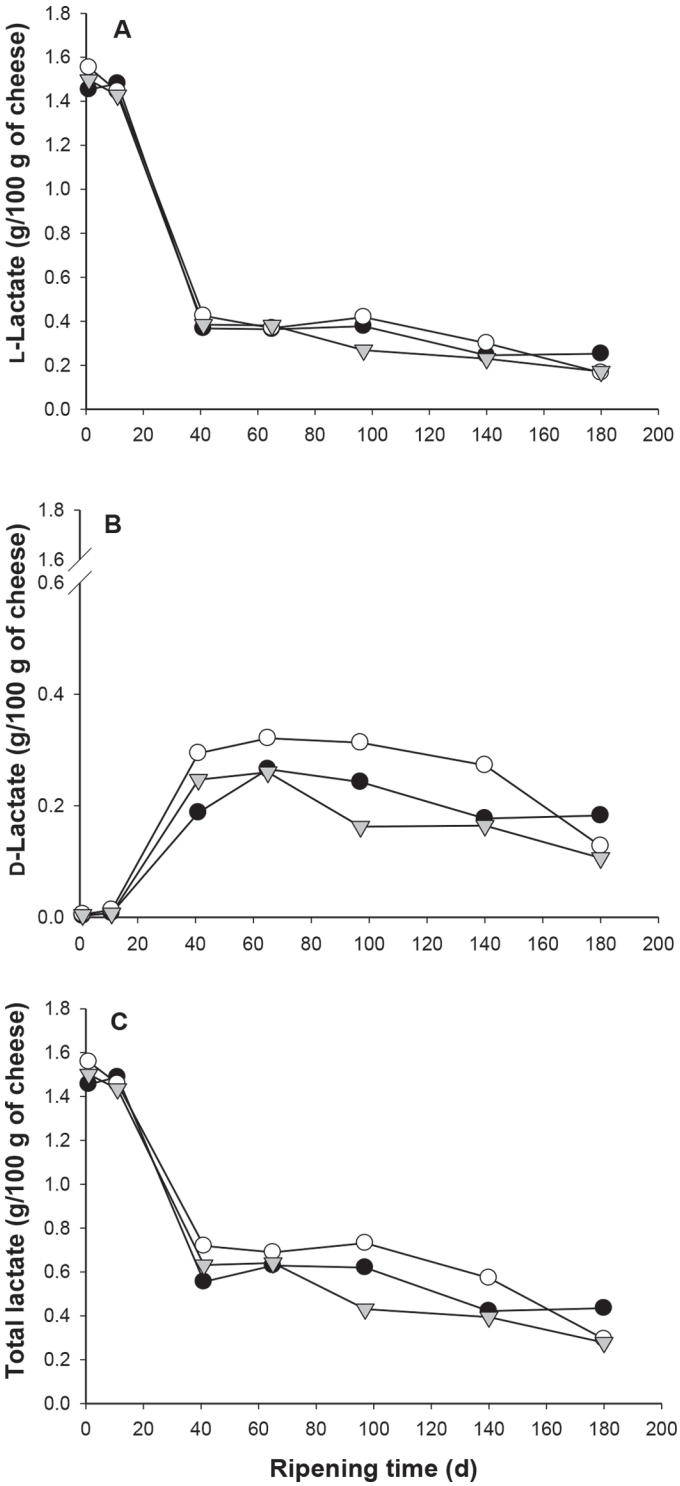


Figure 4. The effect of milk pretreatments on the mean level of (A) L-lactate, (B) D-lactate, and (C) total lactate of Maasdam cheeses during ripening. Milk pretreatments were control (●); centrifugation (▼); and centrifuged milk containing high-heat-treated centrifugate (○). Data presented are means of data from 3 replicate trials.

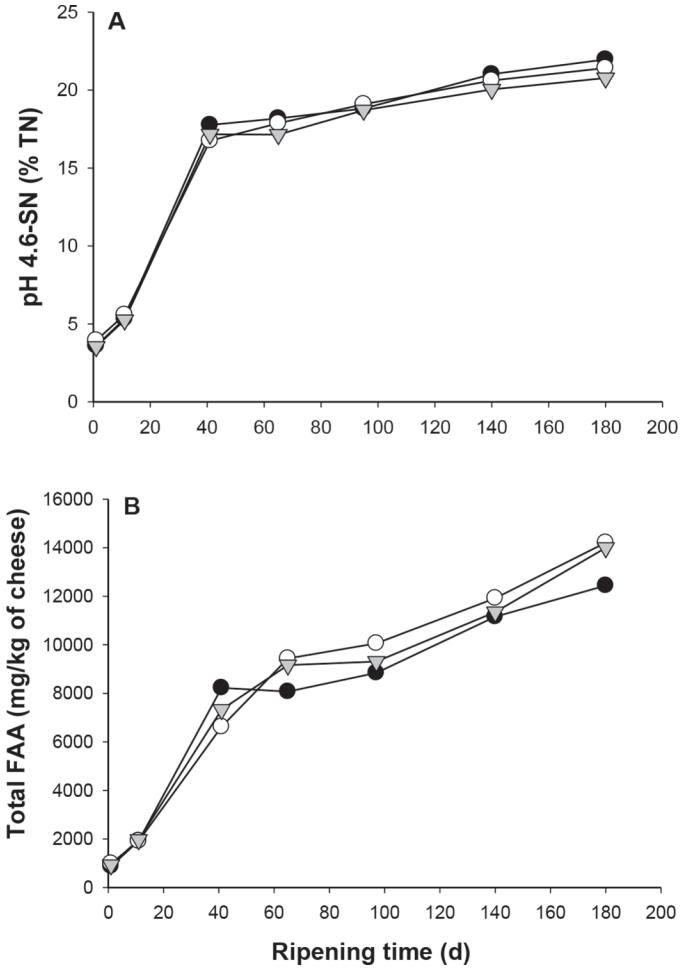


Figure 5. The effect of milk pretreatments on mean level of (A) pH 4.6-soluble nitrogen of percentage of total nitrogen (pH 4.6-SN, % TN), and (B) total free amino acids (FAA) of Maasdam cheeses during ripening. Milk pretreatments were control (●); centrifugation (▼); and centrifuged milk containing high-heat-treated centrifugate (○). Data presented are means of data from 3 replicate trials.

Horst, 2004; Wieking, 2004). In the current study, the centrifugation process also reduced the SCC by ~95%.

The concentrations of individual FAA (mg/kg) in cheeses at 140 d of ripening are shown in Figure 6. Leucine was the most abundant FAA found in all experimental cheeses, with ~2,300 mg/kg at 140 d, followed by Glu, Phe, Val, Lys, Pro, and Thr. Similar to this result, O'Sullivan et al. (2016) also observed a high level of Glu, Leu, Val, Lys, and Pro in Swiss-type cheese at 95 d of ripening. In contrast, the concentrations of Asp, Ser, Gly, Cys, Tyr, and Arg were among the lowest of the FAA. Free AA are important precursors for the formation of different classes of volatiles, such as amines, aldehydes, alcohols, acids, and sulfur compounds (Engels et al., 1997; Yvon and Rijnen, 2001).

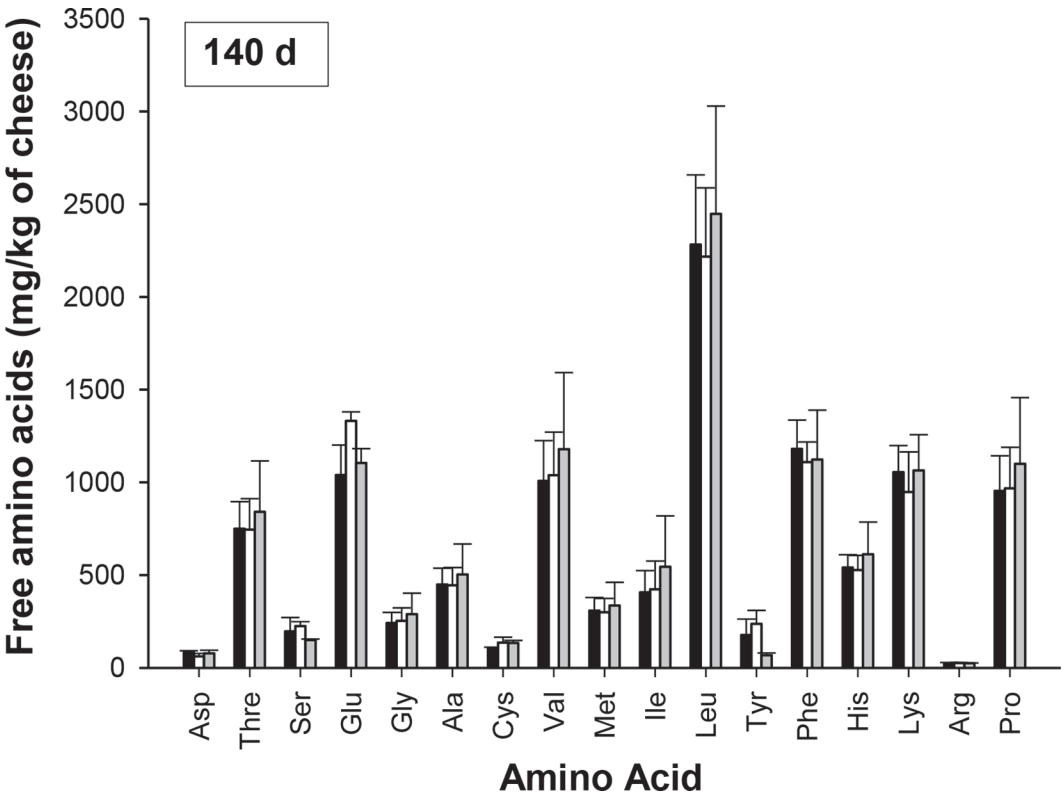


Figure 6. The effect of milk pretreatments on the mean levels of individual free AA in pH 4.6-soluble nitrogen extracts from Maasdam cheeses at 140 d of ripening. Milk pretreatments were control (black bars), centrifugation (white bars); and centrifuged milk containing high-heat-treated centrifugate (gray bars). Data presented are means of data from 3 replicate trials. Error bars show the SEM from 3 replicate trials.

No significant effect of treatment on the mean levels of individual FAA at 140 d of ripening was observed.

Texture Profile Analysis

The incorporation of HHT centrifugate into cheese milk decreased ($P < 0.05$) the mean level of instrumentally measured hardness of the resultant cheeses compared with CT and CF cheeses (Figure 7). This was attributed to significantly higher MNFS level in the CFHHT cheeses than CT and CF cheeses; MNFS is considered a good indicator of moisture associated with proteins (Lawrence et al., 1993). Moisture in the cheese matrix acts as a plasticizer between the protein strands, making cheese softer and more flexible. Moreover, during coagulation, the whey protein and whey protein-casein micelle aggregates may hinder the close approach of casein micelles during aggregation (fusion) of destabilized casein micelles; this may result in a weaker gel and curd texture (Waungana et al., 1996). From a materials science perspective, the strength of a material is known to be influenced by factors such as the extent of cross-linking and the orientation or the structural regularity of the constituents of the material (Pastorino et al., 2003; Lamichhane et al., 2018a). It may be assumed that denatured whey protein can alter the extent of cross-linking of casein micelles and the orientation or the structural regularity of casein networks within the cheese matrix.

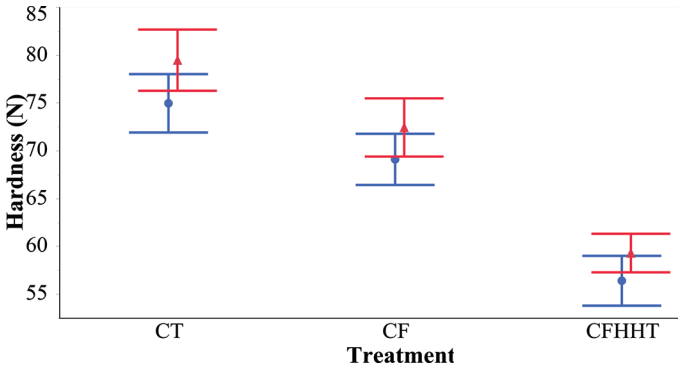


Figure 7. The effect of milk pretreatments on mean levels of hardness between 1 (●) and 11 d (▲) of ripening. Experimental cheese types were CT = control cheese, CF = cheese made from centrifuged milk, CFHHT = cheese made from centrifuged milk containing high-heat-treated centrifugate. Error bars show the SEM from 2 replicate trials. Color version available online.

No significant effects of treatment on mean levels of cohesiveness, resilience, and springiness in the cheeses were observed (Table 3); however, the values for these texture parameters decreased ($P < 0.05$) between 1 and 11 d of ripening (data not shown). Although the exact reasons for this are unknown, this may be attributed to solubilization of colloidal calcium during the early stages of ripening (O'Mahony et al., 2005). O'Mahony et al. (2005) also observed rapid decrease in the value for springiness and cohesiveness of Cheddar cheese between 1 and 21 d of ripening. Levels of insoluble calcium were not determined in cheeses in the present study, and we suggest that this should be a focus for future studies in Maasdam-type cheese. We found no significant difference in the mean level of hardness of cheese between 1 and 11 d of ripening, contrary to the results obtained by O'Mahony et al. (2005), who observed rapid decrease in the texture value within first 21 d of ripening of Cheddar cheese; this discrepancy may be attributed to different cheese types and different manufacturing steps. We were unable to analyze the texture profile of cheese after 11 d of ripening due to eye formation.

CONCLUSIONS

We demonstrated the effect of centrifugation and incorporation of HHT centrifugate on the composition, texture, and ripening characteristics of Maasdam cheese. Interestingly, centrifugation of cheese milk before cheesemaking appeared to have minimal effect on composition and age-related changes on texture, pH, proteolysis, and lactate levels of Maasdam cheese. However, incorporation of HHT centrifugate into cheese milk at levels of approximately 6 to 10% (wt/wt), depending on the protein content of centrifugate, into cheese milk significantly increased MNFS levels and also significantly decreased cheese hardness compared with control cheeses and cheeses made from centrifuged milk. Composition and strength of curd are considered important for eye-development characteristics of cheese without slits and cracks. In the current study, no clear trend for eye characteristics was observed between the treatments, and thus we were unable to draw a conclusion regarding the effect of the treatments applied on eye quality of cheese. We propose that this should be the focus of further research, possibly requiring analysis of a large number of commercial samples over the course of a manufacture season.

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Effect of milk centrifugation and incorporation of high heat-treated centrifugate on the microbial composition and levels of volatile organic compounds of Maasdam cheese

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ABSTRACT

Centrifugation is a common milk pretreatment method for removal of *Clostridium* spores which, on germination, can produce high levels of butyric acid and gas, resulting in rancid, gassy cheese. The aim of this study was to determine the effect of centrifugation of milk, as well as incorporation of high heat-treated centrifugate into cheese milk, on the microbial and volatile profile of Maasdam cheese. To facilitate this, 16S rRNA amplicon sequencing in combination with a selective media-based approach were used to study the microbial composition of cheese during maturation, and volatile organic compounds within the cheese matrix were analyzed by HPLC and solid-phase microextraction coupled with gas chromatography–mass spectrometry. Both culture-based and molecular approaches revealed major differences in microbial populations within the cheese matrix before and after warm room ripening. During warm room ripening, an increase in counts of propionic acid bacteria (by $\sim 10^{1.5}$ cfu) and nonstarter lactic acid bacteria (by $\sim 10^{8}$ cfu) and a decrease in the counts of *Lactobacillus helveticus* (by $\sim 10^{2.5}$ cfu) were observed. *Lactococcus* species dominated the curd population throughout ripening, followed by *Lactobacillus, Propionibacterium*, and *Leuconostoc*, and the relative abundance of these accounted for more than 99% of the total genera, as revealed by high-throughput sequencing. Among subdominant microflora, the overall relative abundance of *Clostridium sensu stricto* was lower in cheeses made from centrifuged milk than control cheeses, which coincided with lower levels of butyric acid. Centrifugation as well as incorporation of high heat-treated centrifugate into cheese milk seemed to have little effect on the volatile profile of Maasdam cheese, except for butyric acid levels. Overall, this study suggests that centrifugation of milk before cheesemaking is a suitable method for controlling undesirable butyric acid fermentation without significantly altering the levels of other volatile organic compounds of Maasdam cheese.

Key words: centrifugation, microbial composition, high-throughput sequencing, volatile profile, Maasdam cheese

INTRODUCTION

Centrifugation at $\sim$9,000 $\times$ *g* is a milk pretreatment method for removal of *Clostridium* spores. Some species of *Clostridium*, on germination, can produce gas and a high level of butyric acid via butyric acid fermentation, resulting in rancid, gassy cheeses (Su and Ingham, 2000; Le Bourhis et al., 2007). As well as removal of bacterial spores, centrifugation removes indigenous bacterial cells present in milk (Te Giffel and Van Der Horst, 2004). Some of these indigenous milk microorganisms can survive pasteurization and can grow during ripening of cheese (Grappin and Beuvier, 1997; Jordan and Cogan, 1999; Quigley et al., 2013; Sheehan, 2013). Therefore, it may be assumed that the reduction in microbial load in cheese milk by centrifugation may influence the microbial composition of cheese during maturation, as the environment would be less competitive, thus further favoring the growth of the most abundant bacteria. The microbial composition within the cheese matrix is known to play an important role in determining biochemical and ripening characteristics, including flavor development through production of enzymes and metabolites, of different varieties of cheese (Beuvier et al., 1997; Beresford et al., 2001; Montel et al., 2014; Guarrasi et al., 2017).

Although traditional culture-based approaches are effective for quantifying common starter or nonstarter

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bacteria, these approaches are not sensitive to those microorganisms that are difficult to culture, present as subdominant populations, or both (Quigley et al., 2013; O'Sullivan et al., 2015). Moreover, recent studies based on culture-independent approaches have suggested that some bacterial cells in a highly stressed condition are viable but not culturable (Quigley et al., 2013; Ruggirello et al., 2014; Hickey et al., 2018). Alternatively, molecular approaches, including high-throughput sequencing, can provide a detailed insight into the composition of both dominant and subdominant microflora. More recently, 16S rRNA amplicon sequencing has been increasingly used in the study of microbial composition within fermented food products, including cheese (Quigley et al., 2012; O'Sullivan et al., 2015; Alessandria et al., 2016). For the first time, we profiled the microbiota of cheese made from centrifuged milk, as well as cheese made from centrifuged milk containing high heat-treated (**HHT**) centrifugate compared with control cheeses, using high-throughput sequencing.

Maasdam is a washed-curd, brine-salted, large eye-forming, semihard cheese, which is developed by combining the cultures and technologies of Emmental and Gouda cheese. Apart from thermophilic lactobacilli, mesophilic mixed-strain cultures comprising *Lactococcus* and *Leuconostoc* are used as starters (as in Gouda cheese) and propionic acid bacteria (**PAB**) are used as secondary starters (as in Emmental cheese). To date, very little has been published regarding the microbial and volatile profile of Maasdam cheese, a better understanding of which will aid manufacturers to consistently achieve the desirable cheese aroma profile (Johnson and Lucey, 2006).

The objective of our study was to investigate the effect of (1) centrifugation and (2) the incorporation of the HHT centrifugate into cheese milk on microbial composition and levels of volatile organic compounds (**VOC**) of Maasdam cheese during maturation. In our study, centrifugation refers to the separation of bacteria and spores at a centrifugal force of ~9,000 × *g* (in some studies this is also referred as bactofugation; Te Giffel and Van Der Horst, 2004), whereas centrifugal separation refers to separation of milk into cream and skim milk. A parallel study was conducted investigating the effect of milk centrifugation and incorporation of HHT centrifugation on the composition, texture, and ripening characteristics of Maasdam cheese.

MATERIALS AND METHODS

Cheese Manufacture

Cheese milks were prepared as described by Lamichhane et al. (2018) and in Supplemental Figure S1 (https://doi.org/10.3168/jds.2017-14180). In summary, raw milk from a local dairy company (Dairygold Co Operative Society Limited, Cork, Ireland) was divided into 2 portions. One portion of the raw milk was separated into skim milk and cream using a cream separator. Control milk (**CT**) was prepared by adding a portion of cream and skim milk obtained from cream separator to achieve a protein to fat ratio of 1.13: 1. Another portion of the raw milk was centrifuged at 9,000 × *g* (at 50°C with flow rate of 1,000 L/h), resulting in centrifuged whole milk and centrifugate. Centrifuged whole milk was separated into cream and skim milk and high heat treatment (120°C for 26 s) was applied to centrifugate. A second cheese milk type (i.e., centrifuged milk; **CF**) was prepared by adding a portion of cream and skim milk obtained from separation of centrifuged whole milk, and a third cheese milk type was prepared by mixing a portion of cream and skim milk obtained from separation of centrifuged whole milk and HHT centrifugate (**CFHHT**; at a level of 6 to 10%, wt/wt, depending on the protein content of centrifugate). The protein-to-fat ratio of all cheese milks were standardized to 1.13: 1. All cheese milks were pasteurized before Maasdam cheese manufacture. Maasdam cheeses were manufactured as per Lamichhane et al. (2018). Three experimental Maasdam cheese types [i.e., cheese made from control milk (CT cheese), centrifuged milk (CF cheese) and centrifuged milk containing HHT centrifugate (CFHHT cheese)] were each manufactured on 3 different occasions in replicate cheesemaking trials over a 3-mo period as per Lamichhane et al. (2018). Starters and secondary starters (frozen direct vat inoculate, Chr. Hansen Ltd., Cork, Ireland) used for the manufacture of Maasdam cheese were (1) mesophilic mixed-strain (C950, 18 mg/kg of milk), consisting of *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Leuconostoc*; (2) *Lactobacillus helveticus* (LH-B01, 4.8 mg/kg of milk); and (3) PAB (PS-60, 7.0 mg/kg of milk).

Enumeration of Starter and Nonstarter Lactic Acid Bacteria and PAB

Samples were aseptically removed from cheese wheels using a cheese trier at 1, 11, 41, 65, 97, 140, and 180 d of ripening. The cheese samples (10 g) were placed in a sterile stomacher bag (Grade, Leicestershire, UK), diluted (10-fold) with 2% (wt/vol) trisodium citrate buffer (VWR, Dublin, Ireland), and stomached for 10 min using a stomacher (Iul Instruments, Barcelona, Spain). Serial dilutions of 10-fold diluted cheese samples were made using maximum recovery diluent, containing low levels of peptone (1 g/L) and sodium chloride (8.5 g/L). Total numbers of nonstarter lactic acid bacteria

(**NSLAB**) cells were enumerated on *Lactobacillus* selection agar (BD, Oxford, UK), with an overlay, after aerobic incubation for 5 d at 30°C. Viable cells of PAB were enumerated on sodium lactate agar, supplemented with kanamycin sulfate (Sigma-Aldrich, Arklow, Ireland) at a level of 4 mg/100 mL of sodium lactate agar, after anaerobic incubation for 7 d at 30°C, and only light brown colonies were counted as PAB (Rehn et al., 2011). *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa, and Sharpe agar (BD) at pH 5.4 after anaerobic incubation for 3 d at 42°C (Hickey et al., 2017). Anaerobic conditions were maintained through the use of anaerobic gas jars and AnaeroGen system (Oxoid, Basingstoke, UK).

Study of Microbial Composition Using High-Throughput Sequencing

Sampling and Nucleic Acid Extraction. Aseptic samples were removed using a cheese trier, at 1, 11, 41, 65, 97, and 180 d of ripening. Cheese samples (5 g) were homogenized in 45 mL of Ringer's solution (1/4 strength, Sigma-Aldrich) in a stomacher (BagMixer 400P, Interscience, Saint Nom, France). Enzymatic lysis of homogenized cheese samples was conducted before DNA extraction and included treatment with lysozyme (1 mg/mL, EC 3.2.1.17, Sigma-Aldrich) and proteinase K (5 mg/mL, EC 3.4.21.64, Sigma-Aldrich), followed by incubation at 37°C for 30 min and 55°C for 15 min, respectively. The DNA was extracted using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA).

PCR Amplification of the Microbial 16S rRNA Gene. Extracted DNA was amplified using primers targeting the V3 and V4 regions of the bacterial 16S rRNA gene. Illumina (San Diego, CA) adapter overhang nucleotide sequences were added to the primers. Therefore, the primer set used included the 16S amplicon PCR forward primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and the 16S amplicon PCR reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTAAGAGACAGGACTACHVGGGTATCTAATCC). Identification of individual sequences from the pooled samples was achieved by incorporating a dual indexing strategy, where 2 unique pairs of 8 base indices were attached to each sample. Prepared samples were purified by using AMPure XP purification system (Beckman Coulter, Takeley, UK) before sequencing.

Amplicon PCR reactions contained 25 μL of 2× KAPA HiFi HotStart ReadyMix (Roche Diagnostics, West Sussex, UK), 10 μL of each of the primers, and 5 μL of the DNA template. Therefore, the total volume of the reaction mix was 50 μL. The PCR amplification was carried out using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The amplification parameters were initial denaturation at 95°C for 3 min, followed by 30 cycles consisting of three 30-s steps including denaturation at 95°C, annealing at 55°C, and extension at 72°C. The process was completed by final elongation stage at 72°C for 2 min. Obtained amplicons were quantified by using Quan-It dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, CA). Additionally, samples were normalized by dilution to equimolar concentrations before library preparation and sequencing.

High-Throughput Sequencing. The 16S rRNA amplicons from the V3 and V4 regions were sequenced on a MiSeq (Illumina, San Diego, CA) platform in the Teagasc sequencing facility, in accordance with standard Illumina sequencing protocols (document no. 15044223; https://www.illumina.com/content/dam/illumina-marketing/documents/products/appnotes/16S-Metagenomic-Library-Prep-Guide.pdf). Paired-end reads were assembled using FLASH (fast length adjustment of short reads to improve genome assemblies). Denoising, chimera detection, and clustering into operational taxonomic units were performed using USEARCH (Version 7.0–64 bit; http://www.drive5.com/usearch/). Taxonomy was assigned using BLAST against the SILVA database release 123 (https://www.arb-silva.de/). Alpha diversity was calculated in QIIME (v1.9.0; http://qiime.org). Further data analysis was carried out using Phyloseq package in R (www.r-project.org).

Analysis of Acetic, Propionic, and Butyric Acid

Acetic, propionic, and butyric acids were recovered from cheese matrix by steam distillation and subsequently quantified by ligand exchange, ion-exclusion HPLC, as described by Kilcawley et al. (2001) with slight modification. Briefly, 5 g of grated cheese samples, 10 mL of 10% (wt/vol) H_2SO_4 (Sigma-Aldrich), 1 mL of valeric acid (Sigma-Aldrich) of concentration 1 mg/mL, 1 drop of silicon antifoaming agent (Sigma-Aldrich), and 10 mL of distilled water were added to a distillation tube before distillation (2100 Kjeltec Distillation unit, Foss Analytic, Hillerød, Denmark). The first 100 mL of distillate was collected into a flask, mixed gently, and representative distillate samples were filtered using 0.2-μm nylon syringe filters into HPLC vials (Agilent Technologies, Santa Clara, CA). Recovery of short-chain carboxylic acids from cheese matrix by steam distillation was checked based on recovery of valeric acid, which was added as an internal standard.

The filtered samples were then analyzed for acetic, propionic, and butyric acid content using HPLC (1260 Infinity, Agilient Technologies) equipped with Rezex

RHM-Monosaccharide H^+ (8%) column (Phenomenex, Cheshire, UK) under the following working conditions: sample injection volume, 40 μL; mobile phase, 0.01 *N* sulfuric acid (isocratic); flow rate, 0.7 mL/min; column temperature, 50°C; run time, 50 min; detection at 220 nm (UV detector). The quantification of analytes was based on the external standard method as described by Kilcawley et al. (2001). Results were expressed as milligrams per kilogram of cheese samples.

Analysis of Volatiles

Volatiles in cheese at 140 d of ripening were determined using GC-MS. Grated cheese samples (4 g) were placed in a 20-mL screw capped amber solid-phase microextraction vial (Apex Scientific, Maynooth, Ireland) and equilibrated to 40°C for 10 min with pulsed agitation of 5 s at 500 rpm. Sample introduction was accomplished using a Shimadzu AOC 5000 Autosampler (Shimadzu Corporation, Kyoto, Japan). A single 50/30 μm Carboxen/divinylbenzene/polydimethylsiloxane (Agilent Technologies) fiber was used. The solid-phase microextraction fiber was exposed to the headspace above the samples for 20 min at depth of 1 cm at 40°C. The fiber was retracted and injected into the GC inlet and desorbed for 2 min at 250°C. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60 m × 0.32 mm × 1.8 μm; Agilent Technologies, Cork, Ireland) column using a split/splitless injector with a 1/10 split. A Merlin microseal (Sigma-Aldrich) was used as the septum. The temperature of the column oven was set at 40°C, held for 5 min, increased at 5°C/min to 230°C, then increased at 15°C/min to 260°C, yielding total GC run time of 50 min. The carrier gas was helium held at a constant flow of 1.2 mL/min. The detector was a Shimadzu TQ8030 mass spectrometer detector (Mason Technology, Dublin, Ireland), run in single quadrupole. The ion source temperature was 220°C and the interface temperature was set at 260°C. The MS mode was electronic ionization (70 V) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library (https://www.nist.gov/srd/nist-standard-reference-database-1a-v14), a commercial flavor and fragrance library (FFNSC 2, Shimadzu Corporation), and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound. Linear retention indices were calculated as per Vandendool and Kratz (1963). Spectral deconvolution was also performed to confirm identification of compounds using an automated mass spectral deconvolution and identification system (AMDIS; http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis). Batch processing of samples was carried out using metaMS (Wehrens et al., 2014). An autotune of the GC-MS was carried out before the analysis to ensure optimal GC-MS performance. All analyses were performed in triplicate.

Statistical Analysis

Three experimental cheese types (CT, CF, and CF-HHT) were each manufactured on 3 different occasions in replicate cheese-making trials. An ANOVA, using IBM SPSS software version 24 (IBM Corp., 2016), was applied to determine the effect of treatment on formation of VOC. A split-plot design was used to determine the effect of treatment, ripening time, and their interactions on *Lactobacillus helveticus*, PAB, and NSLAB count and levels of short-chain carboxylic acids (acetate, propionate, and butyrate). Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple comparison test was used for paired comparison of treatment means at a 5% level of significance.

RESULTS AND DISCUSSION

Cheese Composition

The compositional parameters of cheeses were described in detail by Lamichhane et al. (2018) and in Supplemental Table S1 (https://doi.org/10.3168/jds.2017-14180). Briefly, except for levels of moisture in nonfat substances, all other compositional parameters of experimental cheeses were not statistically different. Cheeses made from cheese milk containing HHT centrifugate had higher ($P < 0.05$) levels of moisture in nonfat substances than cheeses made from centrifuged milk or control cheeses. This is attributed to the negative effect of HHT centrifugate (incorporated at levels of approximately 6 to 10% of the total cheese milk weight, depending on the protein content of centrifugate) on syneresis of rennet-induced milk gels. The mean moisture content of CFHHT cheese was ~3% higher than that of CT and CF cheeses; however, the data were not differ statistically ($P = 0.057$).

Growth and Viability of Lactobacillus helveticus, PAB, and NSLAB

Mean viable counts of *Lactobacillus helveticus* in all experimental cheeses decreased ($P < 0.001$) from ~10^7 cfu/g at 1 and 11 d to ~$10^{4.5}$ cfu/g at 41 d (Figure 1A), indicating lysis during warm room ripening. Similar trends have previously been reported in Swiss-

type cheese (White et al., 2003; Sheehan et al., 2008; O'Sullivan et al., 2016). As the increased number of NSLAB would influence the accuracy of *Lactobacillus helveticus* counts (O'Sullivan et al., 2016), we did not enumerate *Lactobacillus helveticus* beyond 41 d. As expected, the mean viable count of PAB increased ($P < 0.001$) in all experimental cheeses during the warm room ripening, from $\sim 10^{6.5}$ cfu/g at 11 d to $\sim 10^{8}$ cfu/g at 41 d (Figure 1B). The increase in PAB count during the warm room ripening stage is consistent with previous results for Grevé (Rehn et al., 2011) and Swiss-type cheeses (O'Sullivan et al., 2016). The count decreased slightly thereafter to $\sim 10^{7}$ cfu/g during cold storage (4°C).

Although counts of NSLAB were very low at 1 and 11 d of ripening, their levels in all cheeses increased to $\sim 10^{8}$ cfu/g at 41 d of ripening and leveled off during further storage (Figure 1C); Sheehan et al. (2008) also observed a similar trend in Swiss-style cheeses. The NSLAB in cheese can originate from milk, processing equipment, or the processing environment, and counts are reported to be less than 10^{2} cfu/g in young cheese made under good sanitary conditions with high-quality milk (Steele et al., 2006). Pasteurization of milk drastically reduces the number of indigenous milk flora; however, some of these indigenous milk microorganisms can survive pasteurization and can grow during ripening of cheese (Grappin and Beuvier, 1997; Jordan and Cogan, 1999; Johnson, 2001; Quigley et al., 2013). Contrary to our results, O'Sullivan et al. (2016) observed high levels of NSLAB ($\sim 10^{6}$ cfu/g) at 1 d of ripening in Swiss-type cheese, and those authors speculated that they might have originated from the processing environment during cheese manufacture. The rapid increase in numbers of NSLAB during warm room ripening is attributed to elevated temperature (23°C), which accelerates the metabolic activities of microorganisms (Beresford et al., 2001; De Filippis et al., 2016), and availability of substrates, such as sugars, nucleic acids and lactate, from metabolism of starters and their cell lysate (Steele et al., 2013; Ortakci et al., 2015). The NSLAB contribute to cheese maturation through production of enzymes and metabolites (Settanni and Moschetti, 2010). No significant effect of treatment was observed for mean counts of NSLAB, *L. helveticus*, and PAB during ripening (Table 1).

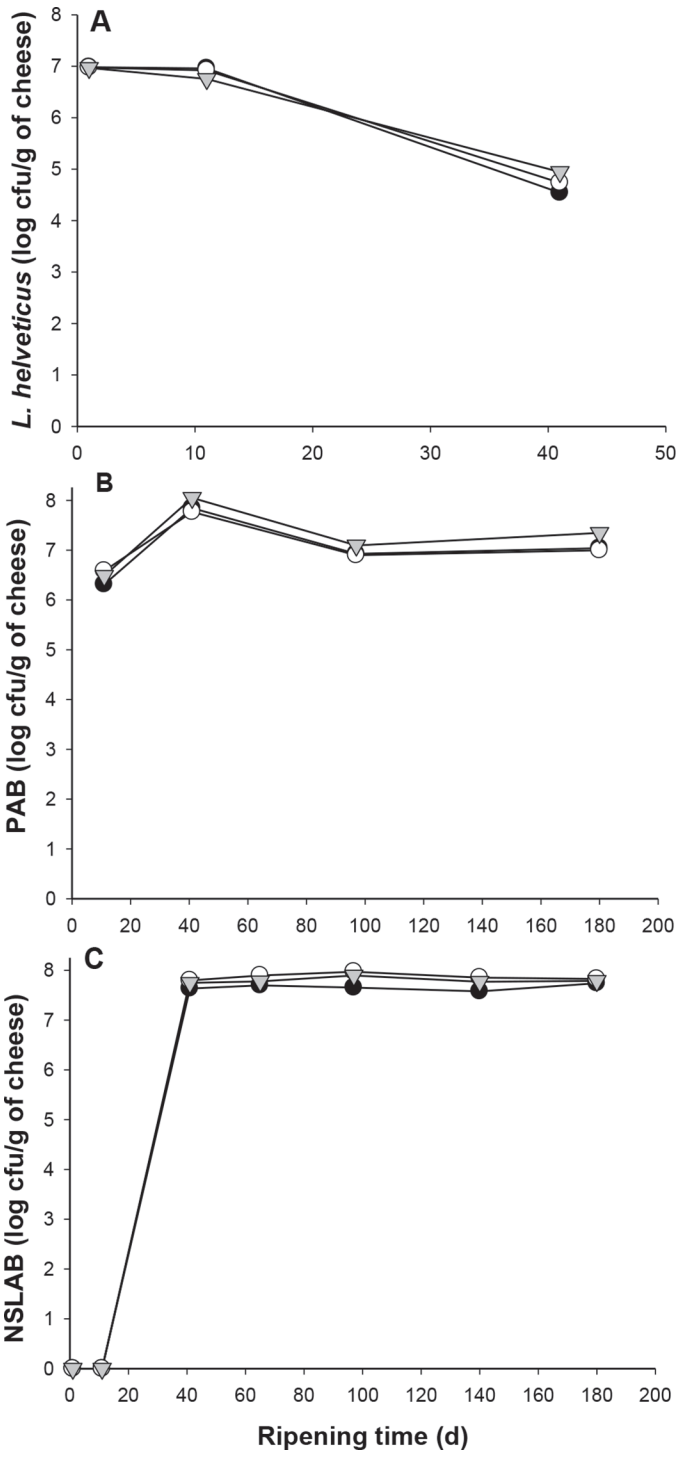


Figure 1. The effect of milk pretreatments on average count of (A) *Lactobacillus helveticus*, (B) propionic acid bacteria (PAB), and (C) nonstarter lactic acid bacteria (NSLAB) of Maasdam cheeses during ripening. Milk pretreatments were control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data are means of data from 3 replicate trials.

Microbial Composition of Maasdam Cheese

After DNA extraction, amplicons of the bacterial 16S rRNA gene were generated by PCR. These amplicons were then subjected to next-generation sequencing, generating an average of 253,870 good-quality reads per sample. Alpha diversity was calculated for each

Table 1. Summary of the effects of treatment, ripening time, and their interactions on microbiology and short-chain carboxylic acids profile of Maasdam cheeses[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
Lactobacillus helveticus	NS (0.71)	***	NS (0.37)
PAB	NS (0.52)	***	NS (0.99)
NSLAB	NS (0.37)	***	NS (0.99)
Acetic acid	NS (0.49)	***	NS (0.99)
Propionic acid	NS (0.55)	***	NS (1.00)
Butyric acid	***	***	NS (0.28)

[1]Digits in parentheses after NS represent *P*-values; abbreviations: PAB = propionic acid bacteria; NSLAB = nonstarter lactic acid bacteria.
***$P < 0.001$, NS = $P > 0.05$.

sample to analyze species richness and diversity within each sample. Chao 1 values, which represented species richness, ranged from 16 to 65, whereas the Shannon index ranged from 0.20 to 2.66. Analysis of these data revealed that the bacterial diversity fluctuated throughout the ripening process; however, an overall increase in diversity was observed, in contrast to a similar study conducted by O'Sullivan et al. (2015) in Swiss-type cheese with thermophilic starters *Streptococcus thermophilus* and *Lactobacillus helveticus*.

Differences in microbial taxa and shifts in relative abundance of the population were revealed between CT, CF, and CFHHT cheeses. Phylogenetic assignment of the sequences revealed presence of bacteria belonging to 7 phyla: *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, and *Saccharibacteria*. As expected, *Firmicutes* dominated across all samples, with relative abundance ranging between 78.72 and 99.96% in the CT cheeses, 67.83 to 99.86% in the CF cheeses, and 76.63 to 99.89% in the CFHHT cheeses. The second most abundant phylum was *Actinobacteria*, followed by *Proteobacteria*. In addition, a rapid increase in abundance of the bacteria belonging to *Actinobacteria* phylum can be observed after 41 d of ripening (after warm room stages) in all experimental cheese types.

Lactococcus, *Lactobacillus*, *Propionibacterium*, and *Leuconostoc* were the dominant genera of Maasdam cheese throughout ripening, accounting for more than 99% relative abundance altogether (Figure 2). Before warm room ripening (i.e., until 11 d postproduction), the relative abundance of major microorganisms (at genus level) were similar between treatments; *Lactococcus* spp. (ranging from 86.8 to 94.5%) dominated the curd population followed by *Lactobacillus* (4.8–12.4%). *Leuconostoc* (0.15–0.4%) and *Propionibacterium* (0.14–0.35%) were detected in very small proportions during this period. These results were expected, as all these genera were added as starters or secondary starters at a similar proportion for all experimental cheeses.

In agreement with the results from the culture-based approach, the molecular approach also revealed major differences in microbial populations within the cheese matrix before and after warm room ripening. As expected, the relative abundance of *Propionibacterium* was higher after warm room ripening than before warm room ripening in all experimental cheeses. An increase in the level of PAB by $\sim 10^{1.5}$ cfu/g during warm room ripening was also observed in the culture-based method. Although the overall relative abundance of *Lactococcus* decreased after warm room ripening, it was still the most dominant microflora in all experimental cheeses; *Lactobacillus* and *Propionibacterium* were the second most abundant genera, followed by *Leuconostoc*. During maturation, in general, some lactic acid bacteria (**LAB**) within the starter cultures die off and their metabolites (e.g., lactate) and carbon sources from cell lysate (e.g., ribose) favor the growth of secondary starter, such as PAB and NSLAB (Ortakci et al., 2015). Moreover, the elevated temperature (23°C) during warm room ripening accelerates the metabolic activity of microorganisms (Beresford et al., 2001; De Filippis et al., 2016).

Subtle differences were observed in the composition of dominant microflora (i.e., *Lactococcus*, *Lactobacillus*, *Propionibacterium*, and *Leuconostoc*) between treatments; however, the differences were not consistent throughout ripening. This suggests that milk centrifugation as well as incorporation of HHT centrifugate into cheese milk had minimal effect on the composition of major genera of Maasdam cheese, which is consistent with the results from selective media-based approach.

Apart from major microflora, many (~40) other genera were also detected; however, their relative abundances were very low, ranging from 0.04 to 0.95%. Among these subdominant genera, *Enterococcus*, *Stenotrophomonas*, *Paenibacillus*, *Pseudomonas*, and *Acinetobacter* were detected in relatively higher abundance. The presence of *Enterococcus* was detected at all time points in CT and CF cheeses, and its population increased rapidly from 41 d of ripening. This genus was not detected

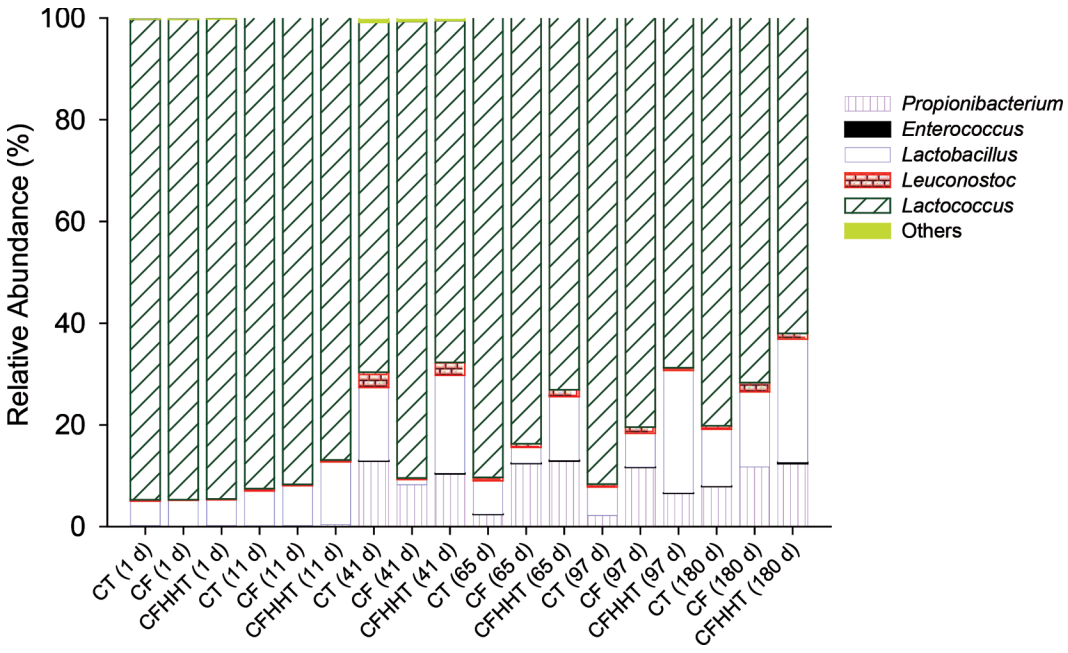


Figure 2. Relative abundance of bacteria at genus level within the 3 experimental cheese types during maturation: CT = control cheese, CF = cheese made from centrifuged milk, CFHHT = cheese made from centrifuged milk containing high heat-treated centrifugate. Data are means of data from 3 replicate trials. Color version available online.

in CFHHT cheeses at 1 d of ripening; however, it was detected thereafter. Enterococci have been previously isolated from traditional cheeses produced with raw or pasteurized milk and are considered to originate from bulk tank, milking machine, and processing equipment or the processing environment (Gelsomino et al., 2002; Nieto-Arribas et al., 2011). Although enterococci have shown a potential role in ripening and flavor development in some artisanal cheeses and cheeses made from raw milk (Beuvier et al., 1997; Beresford et al., 2001; Nieto-Arribas et al., 2011), the significance of the presence of this genus within Maasdam cheese matrix is not yet fully understood and requires further investigation.

Pseudomonas, *Stenotrophomonas*, and *Acinetobacter* were present in all experimental cheese types but in different proportions. The highest abundance of *Pseudomonas* was observed in CT cheeses, particularly at 1, 11, and 97 d of ripening. *Pseudomonas* (psychrotrophic bacteria) have previously been isolated from cheese matrix (O'Sullivan et al., 2015), which can cause flavor and texture defects in cheese if they are present in high numbers (Champagne et al., 1994). *Stenotrophomonas* was present uniformly across the sample groups but not across time points. Although *Acinetobacter* has frequently been detected in several types of cheese, such as Camembert (Addis et al., 2001) and Swiss-style (O'Sullivan et al., 2015), its role on ripening of cheese is not fully understood.

Clostridium sensu stricto is a subset of the species of *Clostridium* that form a distinct cluster in the 16S rRNA tree (cluster I; Gupta and Gao, 2009). Nearly all species within this genus produce butyric acid as a major fermentation product (Wiegel, 2009). *Clostridium* spp. associated with late blowing defect (**LBD**) of cheese, including *Clostridium tyrobutyricum* and *Clostridium butyricum*, also fall within this genus (Collins et al., 1994; Brändle et al., 2016). Although the overall percentage relative abundance of *Clostridium sensu stricto* was very low (below 0.05%), the overall percentage relative abundance in CT cheeses (ranging between 0.00 and 0.02% throughout ripening) was relatively higher than those in CF (0.00 to 0.002%) and CFHHT (0.00 to 0.003%) cheeses; this may be explained by the removal of *Clostridium* spores from milk by centrifugation. It is well known that the centrifugation can remove more than 97% of *Clostridium* spores from milk (Su and Ingham, 2000; Te Giffel and Van Der Horst, 2004).

Levels of Acetic, Propionic, and Butyric Acids

Short-chain carboxylic acids contribute to the aroma profile of most cheese varieties (Kilcawley et al., 2001). Starter, secondary starter, and nonstarter bacteria present in the cheese matrix can produce short-chain volatile carboxylic acids, including propionic, acetic, and butyric acids. Propionic acid is one of the major

products of lactate metabolism by PAB; hence, it was detected in all experimental cheeses at high levels (Figure 3A), particularly during warm room ripening, with a mean level of ~50 mg/kg of cheese at 11 d (start of warm ripening) and ~4,000 mg/kg of cheese at 41 d (end of warm room ripening). Similar trends have been reported in other studies (Huc et al., 2014; O'Sullivan et al., 2016). No significant effect of treatment was observed (Table 1). In Swiss-type cheese, propionic acid contributes to sweet or nutty notes characteristic of these varieties (Kilcawley et al., 2001).

The production of acetic acid followed a similar trend to that of propionic acid during ripening of cheese. Acetic acid in cheese can be formed from several pathways, including propionic acid fermentation by PAB, and metabolism of lactate and citrate by LAB (Sheehan et al., 2008; Huc et al., 2014). The mean level of acetic acid at 1 and 11 d of ripening was only ~200 mg/kg of cheese; however, the level increased rapidly during warm room ripening to ~2,200 mg/kg of cheese at 41 d (Figure 3B). The production of acetate in all experimental cheeses during warm room ripening is most likely due to activity of starter LAB, NSLAB, and PAB. No significant effect of treatment was observed (Table 1).

Significant effects of treatment and time were observed for mean levels of butyric acid during maturation (Table 1). The mean levels of butyric acid in CT cheeses were higher ($P < 0.05$) than in CF and CFHHT cheeses (Figure 3C), which coincided with the higher relative abundance of *Clostridium sensu stricto* in CT cheeses than CF and CFHHT cheeses, as revealed by high-throughput sequencing. Higher levels of butyric acid in the CT cheeses are most probably due to butyric acid fermentation by *Clostridium*, which may be removed during centrifugation in CF and CFHHT milks.

A high level of butyric acid can influence the flavor profile of cheese and may result in down-graded cheese. Some species of *Clostridium* produces carbon dioxide and hydrogen via butyric acid fermentation, which can impair the quality of eyes and also increase the risk of slits and crack formation in cheese (Sheehan, 2011; Gómez-Torres et al., 2015). The level of butyric acid in late-blown cheeses varies among studies; in semihard (Bogovič Matijašić et al., 2007) and Gouda cheese (Klijn et al., 1995), butyric acid contents higher than 200 mg/kg have been found to be associated with the LBD, and the severity of this defect was greater when the level of butyric acid was higher. However, it should be noted that butyric acid within the cheese matrix can also originate from lipolysis during ripening (Le Bourhis et al., 2007; Garde et al., 2012). Although the level of butyric acid was significantly higher in CT cheeses than CF and CFHHT cheeses, we did not observe LBD in the CT cheeses. In the current study, the level of butyric

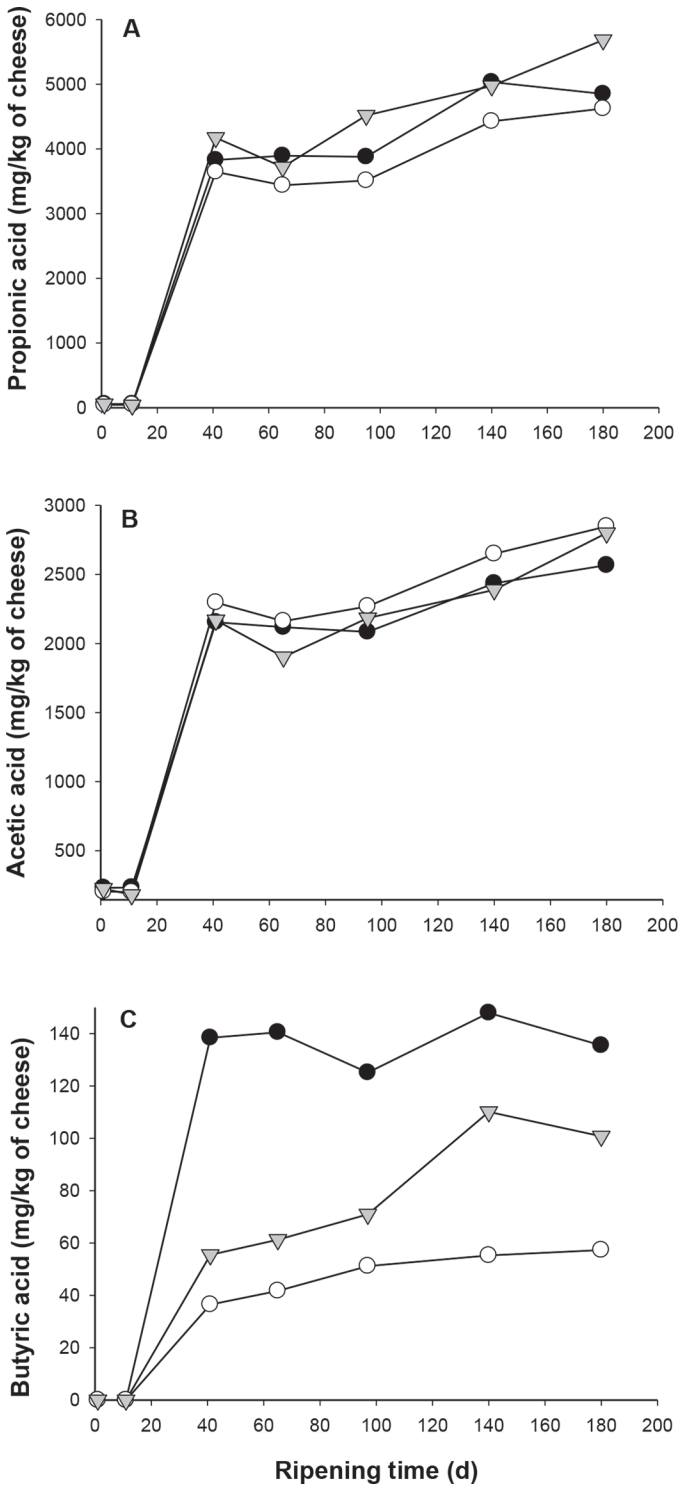


Figure 3. The effect of milk pretreatments on the mean level of (A) propionic acid, (B) acetic acid, and (C) butyric acid during ripening of Maasdam cheeses. Milk pretreatments were control (●); centrifugation (▼); and centrifuged milk containing high heat-treated centrifugate (○). Data are means of data from 3 replicate trials.

acid was below 200 mg/kg in all experimental cheeses over 6 mo of ripening. These results are in agreement with the studies of Le Bourhis et al. (2007) and Beuvier et al. (1997), who also observed butyric acid contents of less than 200 mg/kg in normal Swiss-type cheeses. The comparatively low level of butyric acid in the CT cheeses is most probably due to low levels of *Clostridium* spores in the cheese milk obtained from a local dairy company during spring-summer (May–July); during this period, contamination of milk with *Clostridium* is less likely, as the milk supply was from spring-calving herds fed on pasture grass rather than silage (the main source of clostridial spore contamination in milk; Sheehan, 2011). The levels of butyric acid between CF and CFHHT cheeses were not statistically different.

Volatile Profile of Maasdam Cheese

In total, 28 major volatile compounds were identified at 140 d of ripening, consisting of 8 ketones, 7 acids, 4 alcohols, 4 esters, 2 aldehydes, 2 sulfur compounds, and a hydrocarbon (Table 2). The volatile flavor compounds in cheese are the result of complex biochemical reaction during maturation, such as proteolysis, lipolysis, and glycolysis (McSweeney, 2004). The correct balance and concentration of a wide range of flavor compounds gives the characteristic flavor of different cheese varieties. Centrifugation of cheese milk and incorporation of HHT centrifugate into centrifuged milk had virtually no effect on the formation of volatile compounds at 140 d of ripening of Maasdam cheese. However, as expected, the mean relative abundance of butanoic acid (butyric acid) was lower ($P < 0.05$) in CF and CFHHT cheeses than in CT cheeses (Table 2), attributed to the removal of butyrate-fermenting *Clostridium* spores from cheese milk by the centrifugation process. Although butanoic acid at low levels contributes positively to the aroma of the cheese, it gives an undesirable rancid note at high concentration (Curioni and Bosset, 2002). Propionic and acetic acid were also detected in higher abundance in all experimental cheeses, as expected. The presence of hexanoic and octanoic acids within the experimental cheeses is attributed to lipolytic activity of enzymes (Delgado et al., 2010).

Only 2 aldehydes (i.e., benzaldehyde and 2-methylbutanal) were detected in all experimental cheeses, and these are derived from Phe and Ile, respectively (Yvon and Rijnen, 2001), via α-keto acids by the transaminase pathway (Smit et al., 2005). Branched-chain aldehydes, including 2-methylbutanal, are generally detected in high levels in cheese containing PAB (Thierry et al., 2005) and are responsible for dark chocolate/malty aroma notes (Singh et al., 2003; Bertuzzi et al., 2017). Aldehydes are relatively unstable compounds and can further catabolize to other groups of volatile compounds, such as alcohols or carboxylic acids. It has been reported that 3-methylbutanal and 2-methylbutanal can oxidize to 3-methylbutanoic acid and 2-methylbutanoic acid, respectively, and these acids contribute to cheesy, sweaty, or rancid notes in cheese (Yvon and Rijnen, 2001). Alcohol dehydrogenase from lactic acid bacteria can convert 2-methylbutanal to 2-methyl-1-butanol, which has fruity, waxy, or sweaty-fatty acid aroma notes (Singh et al., 2003).

Ketone flavor compounds, such as 2,3-butanedione (responsible for creamy/buttery aroma note), acetoin, and 2-butanone, are considered important volatile compounds in Maasdam cheese and are likely generated from metabolism of citrate by *Lactococcus lactis* and *Leuconostoc* spp. (Engels et al., 1997; Le Bars and Yvon, 2008). Detection of these ketone flavor compounds in all experimental cheeses at higher abundance is not surprising because *Lactococcus* and *Leuconostoc* spp. were added as starter culture, and these genera were also detected within the cheese matrix throughout ripening, as revealed by high-throughput sequencing. Interestingly, 2,3-pentanedione was only detected in CF and CFHHT cheeses, whereas 2-hexanone was only detected in CT cheeses; however, these compounds were detected in only 1 out of 3 trials. Pentane-2,3-dione has been suggested to be produced from intermediate of Ile metabolism (Imhof et al., 1995). Heptan-2-one, one of the important methyl ketones in Parmigiano-Reggiano cheese types (Qian and Reineccius, 2002), derived from the oxidation of octanoic acid, was also present in all experimental cheeses and likely contributes to cheesy or fruity aroma notes.

Ethanol and 2-butanol were the 2 most abundant alcohols detected in all experimental cheeses. Ethanol may be produced by the heterofermentative LAB present within the cheese matrix (Thierry et al., 2006). The abundance of ethanol and short-chain acids, such as propionic, butyric, and hexanoic acids, inevitably results in ethyl esters via esterification or alcoholysis from microbial activity (Hong et al., 2018). Ethanol is considered as the limiting factor for ethyl ester formation in different cheese types, including Swiss cheese (Thierry et al., 2006). Therefore, modulation of ethanol level can potentially alter the fruity flavor of Swiss (Thierry et al., 2006) and Camembert (Hong et al., 2017) cheeses.

Esters, especially ethyl esters, are responsible for fruity aroma notes in some cheese varieties, such as Parmesan and Swiss-type cheeses (Engels et al., 1997; Thierry et al., 2006). Ethyl propanoate was one of the most abundant esters in all experimental cheeses,

in agreement with the results previously reported by other authors for cheeses containing PAB (Thierry et al., 2005; Thierry et al., 2006). Ethyl butanoate, propyl propanoate, and ethyl hexanoate were also detected.

Volatile sulfur compounds are considered to be an important contributor to the flavor of different cheese types, and these compounds are reported to have very low-odor threshold values (Martínez-Cuesta et al., 2013). Dimethyl sulfide (responsible for rotten cabbage/cheese/vegetative/sulfur aroma notes; Smit et al., 2005) and carbon disulfide were the 2 sulfur compounds present in all experimental cheeses at 140 d of ripening, which are considered important flavor compounds of Swiss cheese released from metabolic activity of PAB (Adda et al., 1982). These sulfur-containing compounds are derived from the catabolism of sulfur AA (Met and Cys) by microorganisms during ripening (Smit et al., 2005; Liu et al., 2012).

Toluene was detected in all experimental cheeses and may originate from the degradation of β-carotene (Verzera et al., 2010; O'Callaghan et al., 2017). Some studies observed high levels of toluene in milk of cows fed on pasture grass (Villeneuve et al., 2013) and also in cheese made from milk of cows fed on pasture grass (O'Callaghan et al., 2017).

It is well known that heat treatment changes the flavor profile of milk through production of volatile compounds from proteins (e.g., sulfur compounds from heat

Table 2. Mean volatile compound peak areas from Maasdam cheese samples at 140 d of ripening[1]

			Experimental cheese groups[3]				
Volatile compound	LRI	Ref. LRI[2]	CT	CF	CFHHT	SEM	*P*-value
Acid							
Acetic acid	690	720	937,050[a]	892,939[a]	863,495[a]	42,910	NS
Propionic acid	784	813	1,780,403[a]	1,796,587[a]	1,574,464[a]	138,686	NS
Butanoic acid	864	883	507,815[a]	200,039[b]	187,989[b]	63,928	*
3-Methylbutanoic acid	917	924	17,441[a]	19,946[a]	15,435[a]	2,355	NS
2-Methylbutanoic acid	924	945	34,971[a]	38,769[a]	27,595[a]	5,155	NS
Hexanoic acid	1,052	1,074	109,023[a]	102,471[a]	93,513[a]	10,345	NS
Octanoic acid	1,244	1,264	9,755[a]	10,307[a]	9,382[a]	811	NS
Alcohol							
Ethanol	506	—	328,148[a]	277,956[a]	295,035[a]	30,099	NS
1-Propanol	612	611	11,263[a]	11,044[a]	25,842[a]	3,461	NS
2-Butanol	648	624	229,554[a]	69,075[a]	614,349[a]	179,338	NS
2-Methyl-1-butanol	789	794	22,266[a]	32,545[a]	25,350[a]	2,937	NS
Aldehyde							
2-Methylbutanal	700	700	8,163[a]	12,268[a]	10,572[a]	1,239	NS
Benzaldehyde	1,032	1,016	151,680[a]	101,440[a]	90,813[a]	32,465	NS
Ester							
Ethyl propanoate	737	744	44,917[a]	38,659[a]	42,970[a]	4,493	NS
Ethyl butanoate	826	830	24,229[a]	18,161[a]	17,146[a]	2,645	NS
Propyl propanoate	835	—	10,310[a]	6,582[a]	8,226[a]	1,274	NS
Ethyl hexanoate	1,024	1,028	6,041[a]	5,502[a]	4,455[a]	615	NS
Ketone							
Acetoin	778	782	2,423,512[a]	2,345,157[a]	1,829,257[a]	370,152	NS
Acetone	533	529	20,569[a]	12,568[a]	7,144[a]	3,736	NS
2,3-Butanedione	631	632	339,191[a]	308,814[a]	246,050[a]	44,948	NS
2-Butanone	639	630	1,705,995[a]	1,780,078[a]	4,570,336[a]	857,979	NS
2-Pentanone	730	733	18,254[a]	22,489[a]	20,644[a]	1,033	NS
2,3-Pentanedione	736	740	0.00[a]	5,465[a]	17,682[a]	5,943	NS
2-Hexanone	834	834	10,146[a]	0.00[a]	0.00[a]	3,378	NS
2-Heptanone	936	933	58,171[a]	38,408[a]	37,236[a]	6,968	NS
Sulfur compound							
Dimethyl sulfide	538	532	593[a]	1,099[a]	814[a]	195	NS
Carbon disulfide	546	538	10,454[a]	10,360[a]	6,091[a]	1,212	NS
Hydrocarbon							
Toluene	794	788	42,593[a]	48,265[a]	41,576[a]	4,741	NS

[a,b]Values within a row not sharing common superscripts differ significantly ($P < 0.05$); data presented are the means of data from 3 replicate trials.

[1]LRI = linear retention index; Ref. LRI = reference linear retention index.

[2]Reference LRI for ethanol or propyl propanoate were not found; however, they have been identified correctly.

[3]CT = control cheese; CF = cheese made from centrifuged milk; CFHHT = cheese made from centrifuged milk containing high heat-treated centrifugate.

*$P < 0.05$, NS = $P > 0.05$.

denaturation of whey protein), carbohydrates (via the nonenzymatic browning reactions), and lipids (e.g., formation of methyl ketones, lactones, and aldehydes from degradation of milk fat; Calvo and de la Hoz, 1992). However, the volatile profile of cheese made from CF and CFHHT was not statistically different, suggesting that the VOC solely generated by the heat-treatment given to the centrifugate have minimal effects on the volatile profile of final cheese.

Overall, the treatments applied to the milk had minimal effect on the volatile profile of Maasdam cheese, except for butyric acid levels, suggesting that centrifugation is a suitable method for controlling undesirable butyric acid fermentation without significantly altering the other VOC in Maasdam cheese. The level of butyric acid in the current study was not high (below 200 mg/kg of cheese) in all experimental cheeses, suggesting that the milk was contaminated by low levels of *Clostridium* spores. Although levels of *Clostridium* spores may be higher in milk supplies other than those studied, this would not influence the findings of the current study, as it was focused on the influence of the milk pretreatments (i.e., centrifugation and high heat treatment of centrifugate) on the microbial and volatile profile of the cheese and not on the influence of clostridia per se.

Based on the relative abundance of volatiles present in the current study and the previous study of Engels et al. (1997), acetoin, 2-butanone, propionic acid, acetic acid, 2,3-butanedione, and butyric acid (at low levels) can be considered as key aroma compounds of Maasdam cheese. Sensory analysis of the cheeses would complement the volatile results, and this could be the focus for future studies.

CONCLUSIONS

High-throughput sequencing in combination with a selective media-based approach revealed distinct differences in the composition of microbiota before and after warm room ripening. High-throughput sequencing facilitated a more detailed insight into the complexity of microbes within the Maasdam cheese matrix and revealed subtle changes in both dominant and subdominant microbiota between treatments. Interestingly, except for butyric acid, treatments applied had minimal effect on other VOC in the fully ripened Maasdam cheeses. Overall, high-throughput sequencing proved to be a useful method to profile the complex microbial population structure of Maasdam cheese during maturation; moreover, the centrifugation of milk before cheesemaking can potentially control the level of butyric acid in Maasdam cheese without significantly altering the levels of other VOC.

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Microstructure and fracture properties of semi-hard cheese: Differentiating the effects of primary proteolysis and calcium solubilization

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ABSTRACT

The individual roles of hydrolysis of α_{S1}- and β-caseins, and calcium solubilization on the fracture properties of semi-hard cheeses, such as Maasdam and other eye-type cheeses, remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low temperature (8 °C) decreased the hydrolysis of α_{S1}-casein by ~95%, ~45%, or ~30%, respectively, after 90 d of ripening, whereas ~35% of β-casein was hydrolysed in that time for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of insoluble calcium as a percentage of total calcium decreased significantly from ~75% to ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to the expectation that shortness of cheese texture is associated with α_{S1}-casein hydrolysis, fracture strain was significantly positively correlated with the level of intact β-casein and insoluble calcium content, indicating that the cheeses with low levels of intact β-casein or insoluble calcium content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this study suggests that the fracture properties of cheese can be modified by selective hydrolysis of caseins, altering the level of insoluble calcium or both. Such approaches could be applied to design cheese with specific properties.

1. Introduction

Knowledge of fracture properties of cheese is important for understanding breakdown properties of cheese during mastication, in designing cheese texture suitable for size reduction operations (e.g., slicing, dicing or grating), and in understanding the reasons for formation of undesirable texture defects within the cheese matrix, such as slits and cracks (Luyten, 1988).

Development of undesirable slits and cracks within the cheese matrix is an international problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to downgrading of the product, resulting in lost revenue to manufacturers (Grappin, Lefier, Dasen, & Pochet, 1993; Guggisberg et al., 2015; White, Broadbent, Oberg, & McMahon, 2003). To date, the exact reasons for development of such defects are not known. However, excessive production of gas, an unsuitable cheese texture or both have been considered as root causes for occurrence of this defect (Daly, McSweeney, & Sheehan, 2010; Rehn et al., 2011). If the cheese texture is short or brittle (i.e., fracturing of cheese matrix at a relatively small deformation), the cheese matrix is no longer able to withstand increased gas pressure during eye-formation or storage, leading to formation of cracks and splits. Although the exact reasons for a cheese to become short or brittle during ripening are not yet fully understood, proteolysis, partial solubilization of colloidal calcium phosphate associated with *para*-casein matrix of the curd during ripening, or both, have been considered as possible reasons (Daly et al., 2010; Lucey, Johnson, & Horne, 2003). However, the role of primary proteolysis and level of insoluble calcium on fracture behaviour of brine-salted semi-hard cheese has not yet been fully elucidated.

From a structural perspective, α_{S1}-casein and β-casein are the two important caseins within the cheese matrix, and these undergo varying degree of hydrolysis during ripening in different cheese varieties

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through the action of residual coagulant and plasmin, respectively (Kelly, O'Flaherty, & Fox, 2006; Lamichhane, Kelly, & Sheehan, 2018b; Sheehan, O'Sullivan, & Guinee, 2004). Studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas β-casein has a hydrophilic and a hydrophobic region at its N- and C-terminal, respectively (Lucey et al., 2003). Thus, these caseins are held together by various molecular forces within the cheese matrix. Moreover, calcium associated with casein enhances the cross-linking of casein within the cheese matrix. Therefore, it is reasonable to assume that both hydrolysis patterns of casein and solubilization of colloidal calcium during ripening alter casein-casein interactions, which may in turn influence the textural, rheological and fracture behaviour of cheese. A better understanding of the individual contribution of such factors may allow the development of strategies to design cheese with specific properties.

Unlike high maximum scald temperatures (~55 °C) in Emmental cheese manufacture, cheese curds are cooked only to ~40 °C during manufacture of most semi-hard cheeses, such as Maasdam and Jarlsberg (Fröhlich-Wyder et al., 2017), which is not sufficient to inactivate or reduce the residual chymosin activity, resulting in extensive breakdown of α_{S1}-casein during ripening (McGoldrick & Fox, 1999). The role of chymosin-mediated proteolysis on texture properties of Cheddar cheese has previously been studied by inhibition of the residual chymosin by the addition of a chymosin inhibitor to the curd-whey mixture (O'Mahony, Lucey, & McSweeney, 2005). However, little is known about the role of chymosin-mediated proteolysis on the fracture behavior of semi-hard Swiss, Dutch and related eye-type cheeses. Some semi-hard eye-type cheeses are ripened in a warm room (~23 °C) for 4–6 weeks for the development of eyes. However, the effect of such elevated ripening temperature on solubilization of calcium and hydrolysis of casein is also not fully understood.

The aim of this study was to decouple and explore the individual role of primary proteolysis (both of α_{S1}- and β-casein) and insoluble calcium on the fracture properties of washed-curd brine-salted semi-hard cheese.

2. Materials and methods

2.1. Milk supply and cheese manufacture

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using a bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52% (*w*/w) and 3.21% (w/w), respectively. The standardized cheese-milks were then pasteurized at 72 °C for 15 s (MicroThermics, USA) and stored at 4 °C overnight prior to cheese manufacture.

Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk, for each replication. Each vat contained automated variable speed cutting and stirring equipment. All cheese milks were inoculated at 32 °C with frozen direct vat inoculation cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*. Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-formation during ripening of cheese which would not permit measurement of texture parameters.

All cheese milks were pre-acidified to 6.55 using 4% (*w*/*v*) lactic acid (Sigma-Aldrich) prior to rennet addition. After 40 min of pre-ripening, the coagulant, fermentation-produced bovine chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 2 mL/11 kg cheese milk in 3 out of 4 vats, whereas fermentation-produced camel chymosin (FPCC; CHY-MAX M, ~200 IMCU/mL; Chr. Hansen Ltd., Cork, Ireland), was added at a level of 1.5 mL/11 kg cheese milk in the fourth vat. Coagulants were diluted ~1:10 with deionized water prior addition. The addition rates of both FPBC and FPCC to milk were predetermined through a series of rheological experiments where the levels of the coagulants were adjusted to achieve coagula of similar gel strength (35 Pa) after a set period of ~45 min.

All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments) and the resultant curd/whey mixture was allowed to heal for 5 min before being stirred continuously for another 10 min. Stirring was then stopped and a portion of whey (0.35 kg/kg cheese milk) was removed. Just after whey removal, in one vat out of four vats, Pepstatin A (synthetic; Enzo life science, Exeter, UK) was added to the curd/whey mixture at a rate of 10.0 μmol/kg cheese milk and evenly distributed by continuous stirring during cooking. Pepstatin A is an inhibitor of aspartic proteases, including chymosin, pepsin, cathepsin D, and renin (Marciniszyn, Hartsuck, & Tang, 1976). After whey removal, reverse osmosis water at ~50 °C (0.25 kg/kg cheese milk) was added to each cheese vat to cook the curd to 37 °C at a rate of 0.2 °C/min with continuous stirring.

Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 h. When the pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a saturated brine solution (23%, *w*/w, NaCl, 0.56%, w/w, $CaCl_2$, and pH 5.2) for 7.5 h at 8 °C. After brining, cheese wheels were vacuum-packed (Falcon 52, Original Henkelman vacuum system, the Netherlands), and transferred to the ripening room. Cheese wheels were ripened at 8 °C for 20 d (pre-ripening), and then at 23 °C for 28 d (warm-room ripening) or 8 °C for 28 d (without warm room ripening), and finally stored at 4 °C for 42 d. A summary of the experimental plan is shown in Table 1.

2.2. Milk and cheese composition

The composition of raw and pasteurized (72 °C for 15 s) cheese milks were analyzed as described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed in duplicate at 20 d of ripening for moisture, fat, protein and salt as described by Hickey et al. (2018). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon, Wilkinson, and McSweeney (2007).

Table 1
General overview of the treatments and ripening regimens used in the study[a].

Treatment	Cheese type[b]			
	Control	NoWR	CC	PepA
Rennet type	FPBC	FPBC	FPCC	FPBC
Chymosin inhibitor	Not added	Not added	Not added	Added
Ripening regimen	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 8 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d	8 °C for 20 d 23 °C for 28 d 4 °C for 39 d

[a] FPBC, fermentation-produced bovine chymosin; FPCC, fermentation-produced camel chymosin.

[b] NoWR, cheese without warm room ripening; CC, cheese made using fermentation- produced camel chymosin as a coagulant; PepA, cheese containing chymosin inhibitor, i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

2.3. Enumeration of starter and nonstarter lactic acid bacteria

Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of ripening. Cheese samples were prepared as described by Lamichhane et al. (2018). Viable *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium, supplemented with 0.5% (*w/v*) lactose, after aerobic incubation at 25 °C for 3 d (Ruggirello et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42 °C (Lamichhane, Pietrzyk, et al., 2018). Nonstarter lactic acid bacteria (NSLAB) cells were enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation for 5 d at 30 °C (Lamichhane, Pietrzyk, et al., 2018).

2.4. Proteolysis

2.4.1. pH 4.6-soluble nitrogen (% of total nitrogen)

The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 were measured after 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

2.4.2. Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd., Watford, Herts, UK), as described by Sheehan and Guinee (2004). Briefly, grated cheese samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55 °C for 10 min and each sample was loaded at a level of 12 μL per well. Sodium caseinate powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples ran initially through the stacking gel at 280 V and then through the separating gel at 300 V. The resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee (2017). Densitometry analysis was performed on the scanned images using image analysis software, i.e., ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Eight major bands corresponding to caseins or its breakdown products were used for calculation: 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). The area of each protein band was expressed as a percentage of total band area of these eight major bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage of their level at 1 d.

2.5. Determination of total and insoluble calcium content

The total calcium content of milk and cheese samples (after 20 d) was determined using atomic absorption spectroscopy (IDF, 2007). The cheese insoluble calcium contents, expressed as percentage of total calcium, were determined after 1, 20, 48, and 90 d of ripening using an acid-base titration method as described by Hassan et al. (2004).

2.6. Fracture properties

Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples were wrapped in tin foil; half of the cylindrical cheese samples were stored at 4 °C and the remainder was stored at 23 °C for at least 4 h. Cheese samples (at 4 °C or 23 °C) were compressed at a rate of 60 mm/min until fracture. True stress (σ; Eq. 1) and Hencky strain (ε_H; Eq. 2) were calculated, assuming a constant volume deformation (Rehn et al., 2011):

$$\sigma = \frac{FH_t}{A_0 H_0} \quad (1)$$

$$\varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \quad (2)$$

where F is a load applied, H_t is the sample height at time t, and A_0 and H_0 are the initial cross-sectional area and height of sample, respectively. Fracture stress (σ_f) and fracture strain (ε_f) values of cheese samples were determined from the inflection point of the stress-strain curve (Rehn et al., 2011).

2.7. Visualization of cheese microstructure

Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission, Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto 2500, Gatan UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (−200 °C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high vacuum cryo-preparation chamber at −185 °C, etched at −95 °C over a period of 15 min, sputter-coated at −125 °C and finally transferred onto the SEM cold stage at −125 °C. Cryo-SEM images were acquired at −125 °C.

The microstructure of cheese samples was also visualised using confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany). Rectangular cheese samples (5 mm × 5 mm × 2 mm) were removed from cheeses using a sharp scalpel. Solutions of the fat specific dye Nile Red (Sigma Aldrich) and protein specific dye Fast Green (Sigma Aldrich) were prepared at a concentration of 0.01% (*w/v*) in 1,2-propanediol (Sigma Aldrich) and deionized water respectively, which were then mixed at a ratio of 3:1. The prepared dye mixture (40 μL) was applied to the surface of cheese samples; a cover slip was gently placed on top and the sample was held at 4 °C for 10 min prior to imaging. The protein and fat phases of the cheese samples were visualised by exciting the Fast Green dye (using a He—Ne laser; excitation wavelength of 633 nm and emission wavelength range of 650–700 nm) and Nile Red dye (using an Argon laser; excitation wavelength of 488 nm and emission wavelength range of 500–580 nm) respectively as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica Microsystems, Baden-Württemberg, Germany).

2.8. Statistical analysis

One way ANOVA, using SPSS software version 24 (IBM Corp., Armonk, NY), was performed to determine the effect of treatment on cheese composition. A split-plot design was used to determine the effect of treatment, ripening time, and their interactions on pH, counts of *Lactococcus lactis* and *Lactobacillus helveticus*, levels of pH 4.6-SN (% TN), insoluble calcium (% of total calcium) and fracture properties (stress and strain at fracture) of cheese. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc, 2011). Tukey's multiple comparison tests was used for paired comparison of treatment means at a 5% level of significance. Pearson correlation analysis was performed between fracture parameters, pH 4.6-SN (% TN), insoluble calcium (% of total calcium), intact β-casein level and intact α_{S1}-casein level using SPSS software version 24 (IBM Corp., Armonk, NY).

3. Results and discussion

3.1. Milk and cheese composition

The average fat, protein, and lactose contents of the standardized and pasteurized cheese-milk used for the 3 replicate cheese-making

Table 2
Compositional parameters at 20 d and pH at 1 d of ripening in semi-hard cheeses[1].

Compositional factors	Cheese types[2]				P-value
	Control	noWR	CC	PepA	
Moisture (%, w/w)	40.83 ± 1.61[a]	40.89 ± 2.47[a]	40.95 ± 3.36[a]	41.84 ± 2.51[a]	0.96
MNFS (%, *w*/w)	56.24 ± 1.54[a]	56.11 ± 2.20[a]	56.26 ± 3.11[a]	57.05 ± 2.28[a]	0.96
Protein (%, w/w)	25.32 ± 0.90[a]	25.57 ± 1.47[a]	25.44 ± 1.56[a]	25.39 ± 1.48[a]	0.98
Fat (%, w/w)	27.42 ± 1.06[a]	27.16 ± 1.77[a]	27.29 ± 1.98[a]	26.70 ± 1.65[a]	0.95
FDM (%, w/w)	46.33 ± 0.91[a]	45.92 ± 1.46[a]	46.18 ± 0.85[a]	45.88 ± 1.28[a]	0.96
Salt (%, w/w)	1.34 ± 0.12[a]	1.38 ± 0.14[a]	1.38 ± 0.09[a]	1.36 ± 0.08[a]	0.96
S/M (%, w/w)	3.28 ± 0.31[a]	3.39 ± 0.51[a]	3.38 ± 0.41[a]	3.25 ± 0.08[a]	0.95
Total calcium (mg/100 g cheese)	867 ± 30[a]	861 ± 30[a]	837 ± 34[a]	842 ± 27[a]	0.60
pH (1 d)	5.18 ± 0.03[a]	5.21 ± 0.03[a]	5.19 ± 0.03[a]	5.23 ± 0.03[a]	0.28

[1] MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio; Control, control cheeses; noWR, cheeses without warm room ripening; CC, cheeses made using fermentation-produced camel chymosin; PepA, cheeses containing chymosin inhibitor i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

[2] Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean ± standard deviation of data from three replicate trials.

trials were 3.21, 3.52, and 4.87% (*w*/w), respectively. The composition of the experimental cheeses at 20 d of ripening is shown in Table 2. The cheeses had a composition similar to those of Maasdam-type cheese reported by Lamichhane, Kelly, and Sheehan (2018a). The treatments applied had no significant effect on the mean levels of moisture, moisture in non-fat substance, protein, fat, fat-in-dry matter, salt, salt-in-moisture and pH (at 1 d of ripening) of the experimental cheeses.

3.2. pH

The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 3) during ripening from 5.18–5.23 at 1 d to 5.35–5.40 at 90 d (Fig. 1a). The pH trend during ripening is consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane, Kelly, & Sheehan, 2018a). No significant effect of treatment was observed for the mean value of pH during ripening.

3.3. Growth and viability of *Lactococcus lactis*, *Lactobacillus helveticus* and NSLAB

A significant effect of ripening time and treatment was observed for the counts of *Lactococcus lactis* (Table 3). The counts of *Lactococcus lactis* decreased in all cheeses during ripening from $10^{9.4}$–$10^{9.7}$ cfu/g at 1 d to $10^{7.4}$–10^{9} cfu/g at 90 d, indicating cell death and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses, suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the warm room ripening.

No significant effect of treatment and ripening time was observed

Table 3
Summary of the effects of treatment, time and their interactions on properties of semi-hard cheeses[a].

Parameter	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
Lactococcus lactis count	**	***	NS
Lactobacillus helveticus count	NS	NS	NS
pH 4.6-SN (% TN)	***	***	***
Insoluble Ca (% of total Ca)	NS	***	NS
Fracture stress (kPa, measured at 4 °C)	***	**	NS
Fracture stress (kPa, measured at 23 °C)	***	***	NS
Fracture strain (measured at 4 °C)	**	***	NS
Fracture strain (measured at 23 °C)	**	**	NS

NS, $P > 0.05$.
** $P < 0.01$
*** $P < 0.001$
[a] pH 4.6-SN (% TN), soluble nitrogen at pH 4.6 as percentage of total nitrogen.

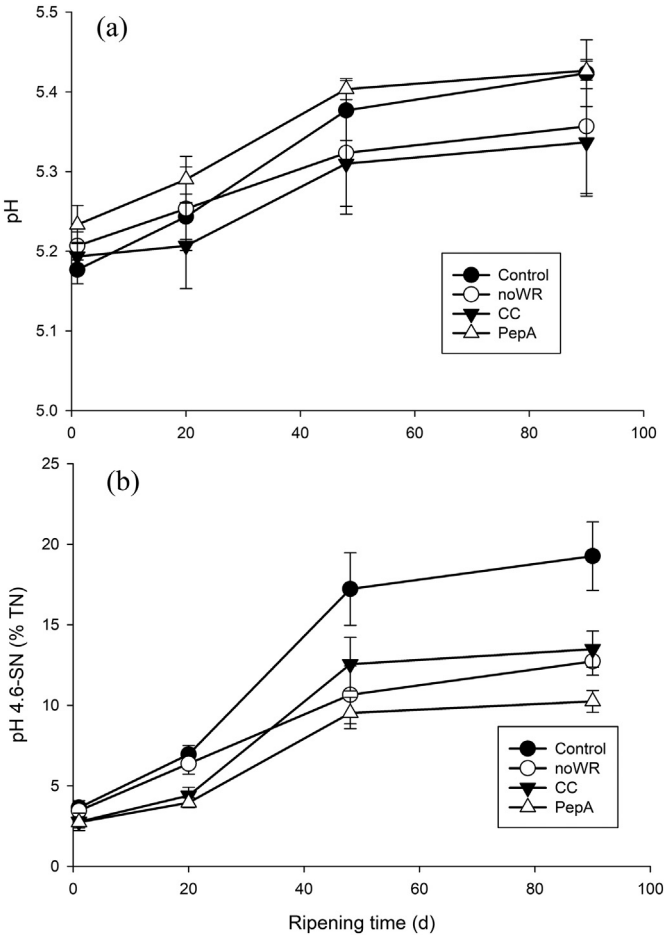


Fig. 1. Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6 in experimental cheeses, expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from three replicate trials; Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

for counts of *Lactobacillus helveticus* until 20 d of ripening, at which time the average count was 10^5–$10^{6.5}$ cfu/g. After warm-room ripening (48 d), the typical colonies of *Lactobacillus helveticus* were not observed, suggesting that either the cells were in a stressed condition which may be viable but not culturable, or may have lysed due to changes in the cheese-ripening environment, such as microbial composition, depletion of energy sources (e.g., low residual lactose), production of metabolites (Steele, Broadbent, & Kok, 2013) or inward diffusion of salt (Hickey, Fallico, Wilkinson, & Sheehan, 2018).

NSLAB counts were variable between trials, although one trial did show that the average counts of NSLAB increased during ripening from $10^{4.3}$–10^5 cfu/g at 20 d (before warm room ripening) to $10^{6.7}$–$10^{7.7}$ cfu/g at 48 d (after warm-room ripening). Moreover, the average count of NSLAB was ~1 log lower in noWR cheeses than for the other cheeses at 48 d of ripening.

3.4. Proteolysis

3.4.1. Nitrogen soluble at pH 4.6 (% of total nitrogen)

A significant ($P < 0.001$, Table 3) interaction was observed between the effect of treatment and ripening time for levels of nitrogen soluble at pH 4.6 [% of total nitrogen; pH 4.6-SN (% TN)] in all experimental cheeses. The mean levels of pH 4.6-SN (% TN) increased with increasing ripening time in all experimental cheeses (Fig. 1b). However, the extent of the increase in pH 4.6-SN (% TN) level during ripening was higher in control cheeses than for other experimental cheese variants, which increased from 6.95 at 20 d to 19.27 at 90 d. The level of pH 4.6-SN (% TN) in control cheeses is in close agreement with that previously reported for semi-hard (Huc, Challois, Monziols, Michon, & Mariette, 2014) and Maasdam (Lamichhane, Kelly, & Sheehan, 2018a) cheeses.

Although propionic acid bacteria were not inoculated into the cheese milks of the current study, the levels and trend of pH 4.6-SN (% TN) during ripening of cheeses were found to be similar to semi-hard cheeses with propionic acid bacteria, suggesting that propionic acid bacteria have a minor role in the proteolysis of washed-curd brine-salted semi-hard cheese (Gagnaire, Thierry, & Léonil, 2001). Moreover, the autolysis of propionic acid bacteria and the release of proteases from their cell have been shown to be limited in cheese (Valence, Richoux, Thierry, Palva, & Lortal, 1998).

As expected, the mean level of pH 4.6-SN (% TN) in PepA cheeses was approximately two-fold lower that of control cheeses at 90 d; O'Mahony et al. (2005) has previously reported a similar trend for Cheddar cheese. The low level of proteolysis in the PepA cheeses is due to inhibition of residual chymosin by pepstatin A (which was added to the curd-whey mixture at a level of 10 μmol/L). The level of pH 4.6-SN (% TN) in PepA cheese was found similar to that reported for Emmental cheese at 90 d of ripening (O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016); in Emmental, residual coagulant is largely or wholly inactivated by use of a high cook temperature during cheese manufacture.

The mean levels of pH 4.6-SN (% TN) in noWR and CC cheeses were 12.73 and 13.49, respectively, after 90 d of ripening, which were significantly lower than in the control cheeses. A higher average level of proteolysis in control cheeses compared to the noWR cheeses is attributed to an increase in the rate of proteolysis due to elevated ripening temperature (Sheehan et al., 2004; Soodam, Ong, Powell, Kentish, & Gras, 2017). The lower levels of pH 4.6-SN (% TN) in CC cheese compared to control cheeses is attributed to the lower general proteolytic activity of FPCC compared to FPBC (Bansal et al., 2009; Kappeler et al., 2006).

3.4.2. Urea-polyacrylamide gel electrophoresis

During ripening, α_{S1}- and β-caseins were hydrolyzed progressively to an extent dependent on the treatment applied and ripening temperature, while breakdown products accumulated simultaneously (Fig. 2 and Supplementary Fig. 1). Extensive hydrolysis of α_{S1}-casein was observed for control cheeses during ripening (i.e., > 90% of levels at 1 d), with the rate of hydrolysis being most rapid during the warm room ripening stages, whereas the hydrolysis of α_{S1}-casein was ~30% and ~45% less in noWR and CC cheeses at 90 d, respectively, compared to control cheeses (Fig. 2b).

Less hydrolysis of α_{S1}-casein in noWR cheeses compared to control cheeses was attributed to the influence of temperature on the residual coagulant activity (Sheehan et al., 2004). Less extensive breakdown of α_{S1}-casein in CC cheeses compared to control cheese is attributed to the lower proteolytic activity of FPCC compared to FPBC (Bansal et al., 2009; McCarthy et al., 2017).

Limited breakdown of α_{S1}-casein, i.e., ~5%, was observed in PepA cheeses in agreement with the previous studies (O'Mahony et al., 2005; Shakeel-Ur-Rehman, Feeney, McSweeney, & Fox, 1998), suggesting that the addition of chymosin inhibitor, i.e., pepstatin A, to the curd/whey mixture during cheese manufacture was an effective means for greatly reducing the chymosin-mediated hydrolysis of α_{S1}-casein within the semi-hard cheese during ripening.

Hydrolysis of β-casein was observed in all cheeses during ripening (Fig. 2c), most likely due to plasmin activity (Kelly et al., 2006). The extent of hydrolysis of β-casein was similar for control, CC and pepA cheeses (i.e., ~35% of levels at 1 d), suggesting that neither the substitution of FPBC with FPCC nor addition of chymosin inhibitor to the curd/whey mixture influenced the hydrolysis of β-casein in agreement with the previous studies (Bansal et al., 2009; O'Mahony et al., 2005). However, the extent of breakdown was relatively lower in noWR cheeses (i.e., < 20% of levels at 1 d) than other cheeses, suggesting that warm room ripening accelerates the degradation of β-casein. Overall, these results suggest that the various hydrolysis patterns of casein can be achieved by using different coagulant types, modulating ripening temperature or inhibiting residual chymosin activity, although inhibition of the latter using pepstatin A is obviously not commercially viable.

3.5. Insoluble calcium contents of cheeses

The mean level of insoluble calcium (percentage of total calcium) decreased significantly ($P < 0.001$, Table 3) during ripening (Fig. 3), especially at the early stage of ripening, from ~75% at 1 d to ~66% at 20 d. After 20 d of ripening, the rate of decrease in the level of insoluble calcium was slower than at the early stages of ripening, which is in agreement with the previous studies in different cheese types (Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010; O'Mahony et al., 2005).

The effect of warm-room ripening on solubilization of colloidal calcium in brine-salted cheese varieties has not previously been studied. Therefore, the rate of calcium solubilization was compared between cheeses subjected to warm room ripening (control cheeses) and without warm room ripening (noWR cheeses). Interestingly, the mean insoluble calcium content of noWR cheeses was ~3% higher than that of the control cheese after 48 d of (after warm room ripening); however, the difference observed was not statistically significant, suggesting that, at best, the warm room ripening had only a minor effect on the solubilization of calcium. Hydrolysis of β-casein is known to release phosphopeptides (Gagnaire, Mollé, Herrouin, & Léonil, 2001), which could contribute to decreases in the level of casein-bound calcium. As expected, substitution of FPBC with FPCC as a coagulant or addition of pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on insoluble calcium content.

3.6. Fracture properties

The fracture properties of experimental cheeses were studied at two different temperatures, i.e., 4 °C or 23 °C (Fig. 4). The stress at fracture (σ_f) and strain at fracture (ε_f) were significantly influenced by treatment and ripening time (Table 3).

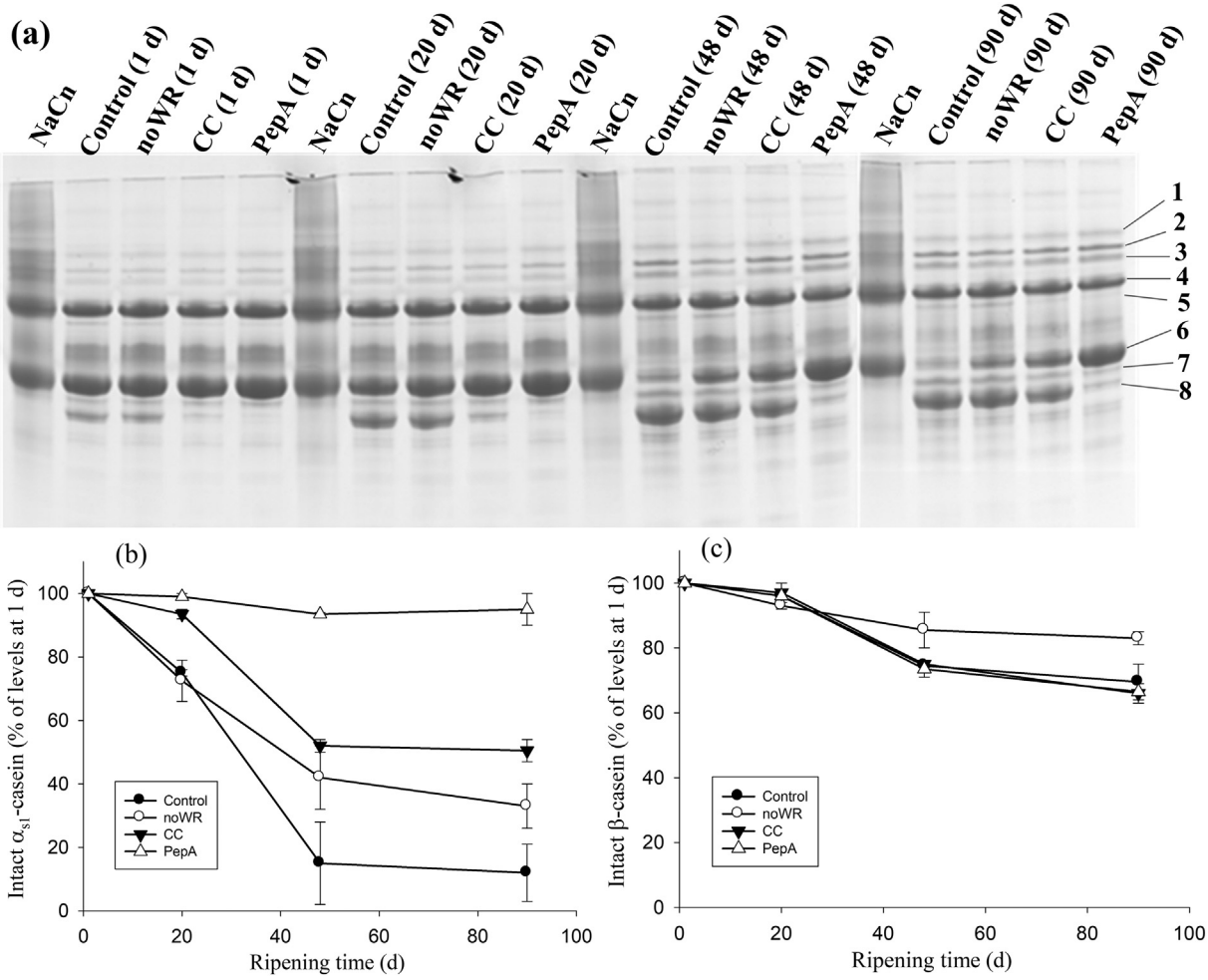


Fig. 2. (a) Urea-polyacrylamide gel electrophoretograms of semi-hard cheeses after 1, 20, 48 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands were identified according to McCarthy et al. (2017): 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). Level of (b) intact α_{S1}-casein and (c) intact β-casein as a percentage of the level at 1 d. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength or rigidity of the cheese matrix. The σ_f measured at 4 °C or 23 °C decreased significantly (Fig. 4a–b; Table 3) in all cheeses over maturation. However, the σ_f was significantly higher ($P < 0.05$) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control cheeses compared to other experimental cheese types was attributed to higher levels of protein breakdown in the control compared to PepA, noWR and CC cheeses (Fig. 1b). A significant negative correlation (Table 4) between pH 4.6-SN (% TN) and σ_f was observed for the experimental cheeses, which is in agreement with previous studies on Cheddar cheese (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly positively (Table 4) correlated with level of intact α_{S1}-casein. Intact β-casein level was also significantly positively correlated with the value of σ_f; however, the correlation coefficient (r) value was lower for intact β-casein (Table 4) as compared to intact α_{S1}-casein. This suggests that intact α_{S1}-casein is the principle load-bearing protein within the semi-hard cheese matrix. No significant correlation was found between the σ_f and insoluble calcium content (Table 4), indicating that the extent of solubilization of calcium after 20 d of ripening had no pronounced influence on the strength of the cheese matrix.

Fracture strain (ε_f) represents the shortness or brittleness of cheese texture; cheeses with a lower fracture strain value are susceptible to fracture at small deformation (Grappin et al., 1993; Sharma, Munro, Dessev, Wiles, & Foegeding, 2018). The ε_f measured at 4 °C or 23 °C decreased significantly for control, CC and PepA cheeses, especially during warm room ripening, from 1.0–1.2 at 20 d to 0.75–0.8 at 48 d (Fig. 4c-d).

Although α_{S1}-casein was hydrolyzed to varying degrees among the control, CC and PepA cheeses after 48 d of ripening (ranging from ~5% in PepA to ~90% in control cheeses; Fig. 2), no significant difference in ε_f was observed among these cheeses. In the current study, hydrolysis of α_{S1}-casein mainly occurred at Phe_{23}-Phe_{24} during ripening, yielding peptides α_{S1}-casein (f1–23) and α_{S1}-casein (f24–199). The former peptide may be hydrolyzed rapidly by proteinases of the starter microorganisms (Shakeel-Ur-Rehman et al., 1998), whereas the latter peptide accumulated during ripening (Fig. 2a). Therefore, the results of this study suggest that the primary breakdown of α_{S1}-casein into the large peptide fragment, i.e., α_{S1}-casein (f24–199) had no pronounced effect on the ε_f in semi-hard cheese during ripening. Since the peptide fraction α_{S1}-casein (f24–199) is so large, it is likely that this fraction may remain attached to the protein network rather than becoming part of the serum phase (Lucey et al., 2003; Luyten, 1988). Further breakdown of α_{S1}-casein (f24–199) (secondary breakdown) into small peptides may decrease the ε_f of cheese (Luyten, 1988). In the current study, no noticeable breakdown of α_{S1}-casein (f24–199) was observed during 90 d of ripening (Fig. 2a); therefore, the role of secondary breakdown of α_{S1}-casein (f24–199) on shortness of cheese could not be elucidated. Similar

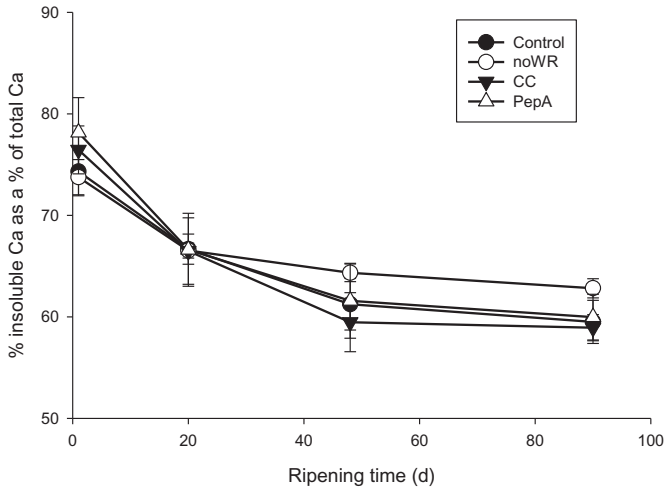


Fig. 3. Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca) as a function of ripening time in semi-hard cheeses. Data are the mean of data from three replicate trials and error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

to the current study, Luyten (1988) also didn't observe a clear link between the primary breakdown of α_{S1}-casein and ε_f in Gouda cheese. A significant decrease in ε_f in control, CC and PepA cheeses during warm-room ripening may be due to other age-related changes within the cheese matrix rather than primary breakdown of α_{S1}-casein.

Interestingly, the ε_f for the noWR cheeses remained almost the same or decreased slightly over the ripening period (Fig. 4c–d). Moreover, the ε_f for noWR cheeses was significantly higher ($P < 0.05$) at 48 and 90 d as compared to control, PepA and CC cheeses (which were subjected to warm room ripening stage). Similarly, Luyten (1988) also observed considerably lower ε_f in Gouda cheeses ripened at higher temperature (i.e., 18 °C) than ripened at lower temperature (i.e., 8 °C) during ripening. Furthermore, similar to the current study, ε_f of the Gouda cheeses ripened at 8 °C decreased slightly from 1.3 at 14 d to 1.2 at 42 d of ripening, whereas ε_f of the Gouda cheese ripened at 18 °C decreased considerably from 1.3 to 0.8 over the same ripening period. Although the exact reasons for such an influence of ripening temperature on fracture behaviour of cheese are unknown, it may be assumed that temperature-induced changes within the cheese matrix, such as rate of solubilization of colloidal calcium, specific hydrolysis patterns of casein and the resultant peptide profiles, could be possible reasons.

In the current study, insoluble calcium (expressed as a percentage of total calcium) and intact β-casein were significantly positively correlated with ε_f (Table 4). Furthermore, levels of intact β-casein (Fig. 2c) and insoluble calcium (Fig. 3) were on average ~15% and ~3% higher, respectively, in noWR cheeses than in the other cheeses after 48 d of

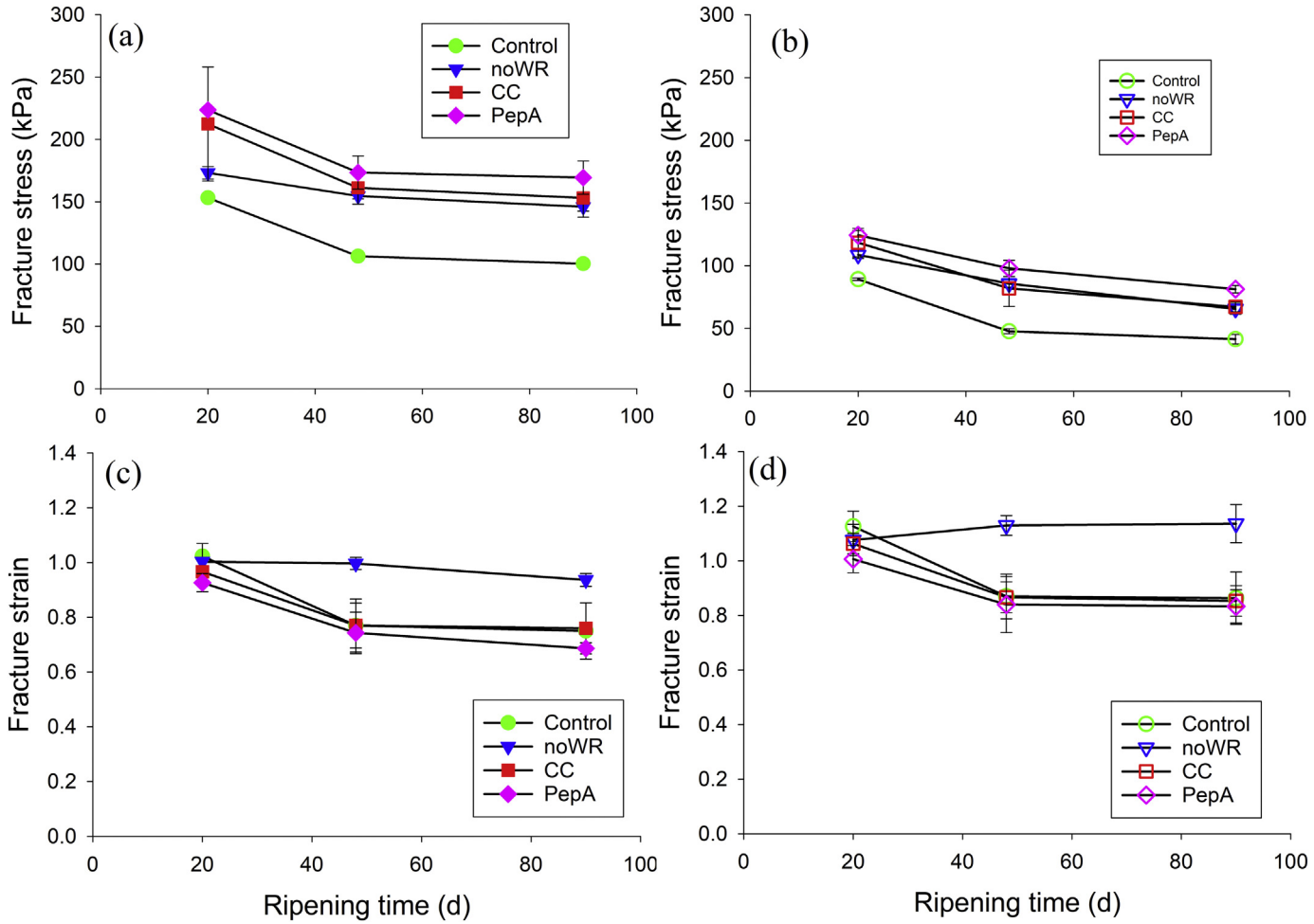


Fig. 4. Changes in (a-b) fracture stress (σ_f, n = 2) and (c-d) fracture strain (ε_f, n = 3), measured at 4 °C (closed symbols) and 23 °C (open symbols), in semi-hard cheese during ripening. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Table 4
Pearson correlation coefficients between fracture parameters, primary proteolysis, insoluble calcium, and intact β-casein and α_{S1}-casein[a].

	σ_f (measured at 4 °C)	ε_f (measured at 4 °C)	σ_f (measured at 23 °C)	ε_f (measured at 23 °C)
pH 4.6-SN (% TN)	−0.77**	−0.62**	−0.88**	−0.54**
Insoluble Ca (% of total Ca)	0.23^{NS}	0.66**	0.33^{NS}	0.53**
Intact β-casein	0.50*	0.75**	0.68**	0.60**
Intact α_{S1}-casein	0.79**	0.25^{NS}	0.83**	0.19^{NS}

[a] σf, stress at fracture; ε_f, strain at fracture; data were obtained from all experimental cheeses over a 90 d of ripening.
** $P < 0.01$
* $P < 0.05$
^{NS} $P > 0.05$.

ripening. This suggests that the breakdown of intact β-casein, solubilization of colloidal calcium during ripening, or both may contribute to a shorter texture (i.e., lower ε_f) observed in control, CC and PepA than noWR cheeses. Therefore, the results from this study suggest that the influence of varying degrees of hydrolysis of β-casein or level of colloidal calcium on shortness of cheese texture merits further research.

It is now well established that the calcium associated with casein is an important structural component, which enhances the cross-linking of caseins within the cheese matrix (Lamichhane, Kelly, & Sheehan, 2018b; Lucey et al., 2003; O'Mahony et al., 2005). Thus, it is reasonable to assume that the solubilization of colloidal calcium during ripening within the cheese matrix is one of the possible reasons for shorter texture of cheese. Moreover, studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N and C termini, respectively (Lucey et al., 2003). Therefore, it is likely that the specific hydrolysis of caseins during ripening may alter their molecular interactions within cheese matrix which in turn may influence the texture, rheological and fracture behaviour of cheese. For example, Bogenrief and Olson (1995) observed a degree of melt of Cheddar cheese which was more closely related to the extent of β-CN hydrolysis than the hydrolysis of α_{S1}-CN.

Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein, modulation of colloidal calcium associated with casein, or both. Such knowledge is particularly important for designing cheese with desired texture profiles or for designing cheese texture suitable for withstanding increased gas pressures during ripening in some eye-type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is higher for cheeses with lower ε_f (short or brittle texture) (Grappin et al., 1993; Rehn et al., 2011). However, it should be noted that unsuitable cheese texture is one possible contributing factor among other factors for the development of undesirable splits or cracks, such as; rate and extent of gas production and its behavior (e.g., solubility and diffusivity) within the cheese matrix; late gas production; and the presence of micro-defects within the cheese matrix (Daly et al., 2010).

The σ_f of cheeses measured at 4 °C (Fig. 4a) was considerably higher as compared to same cheeses measured at 23 °C (Fig. 4b) at all stages of ripening, which is attributed to the temperature-induced changes on the components of cheese and their interactions (Lamichhane, Kelly, & Sheehan, 2018b). At low temperature (~4 °C), more than half of the milk fat present within the cheese matrix is in a crystallized form, and acts as a reinforcing filler, contributing to the elastic texture of cheese (Lamichhane, Kelly, & Sheehan, 2018b; Lopez, Briard-Bion, Camier, & Gassi, 2006). However, the test temperature (4 °C or 23 °C) had no pronounced effect on the ε_f of cheeses at all stages of ripening.

3.7. Microstructure

The microstructure of cheese (at 90 d of ripening) observed by cryo-SEM is shown in Fig. 5. The microstructure of the control cheese is clearly different from that of the other experimental cheese types; the microstructure observed for the control cheese was more open than that of the other experimental cheeses. The open structure may be attributed to significantly higher levels of proteolysis in the control cheeses compared to the other cheese types. For other experimental cheeses, the microstructure looks visually similar. During proteolysis, the intact caseins, which are responsible for network formation, breakdown into small and medium size peptides and free amino acids and these peptides and amino acids are released into the serum fraction of the cheese (Sousa, Ardö, & McSweeney, 2001). Soodam, Ong, Powell, Kentish, and Gras (2015) also observed a less open structure of cheese with low levels of primary proteolysis than in cheeses with high levels.

The microstructure of the cheeses (at 90 d ripening) was also visualised using CLSM (Supplementary Fig. 2). In agreement with the previous studies (Lopez, Camier, & Gassi, 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese matrix, which is attributed to the aggregation, coalescence, and disruption of the fat globules due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al., 2007). The microstructures of all experimental cheeses were visually similar.

4. Conclusions

The roles of primary proteolysis and calcium solubilization on the fracture properties of washed-curd brine-salted semi-hard cheese were investigated. Addition of a chymosin inhibitor i.e., pepstatin A, to the curd/whey mixture during cheese manufacture, substitution of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in all cheeses during ripening.

The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to expectation, shortness or brittleness (as indicated by lower strain at fracture) of cheese texture was negatively associated particularly with the level of intact β-casein and also with insoluble calcium content.

The results from this study suggest that modulation of hydrolysis of α_{S1}-casein is an effective means for maintaining the strength of the cheese matrix during ripening. This could be achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or modulating ripening temperature. However, shortness or brittleness of cheese texture could potentially be altered by maintaining higher levels of intact β-casein or insoluble calcium content or both within the cheese matrix. Shortness or brittleness of cheese has previously been associated with undesirable slits or cracks. Therefore, the role of intact β-casein or insoluble calcium content on fracture behaviour, especially fracture strain, merits further research.

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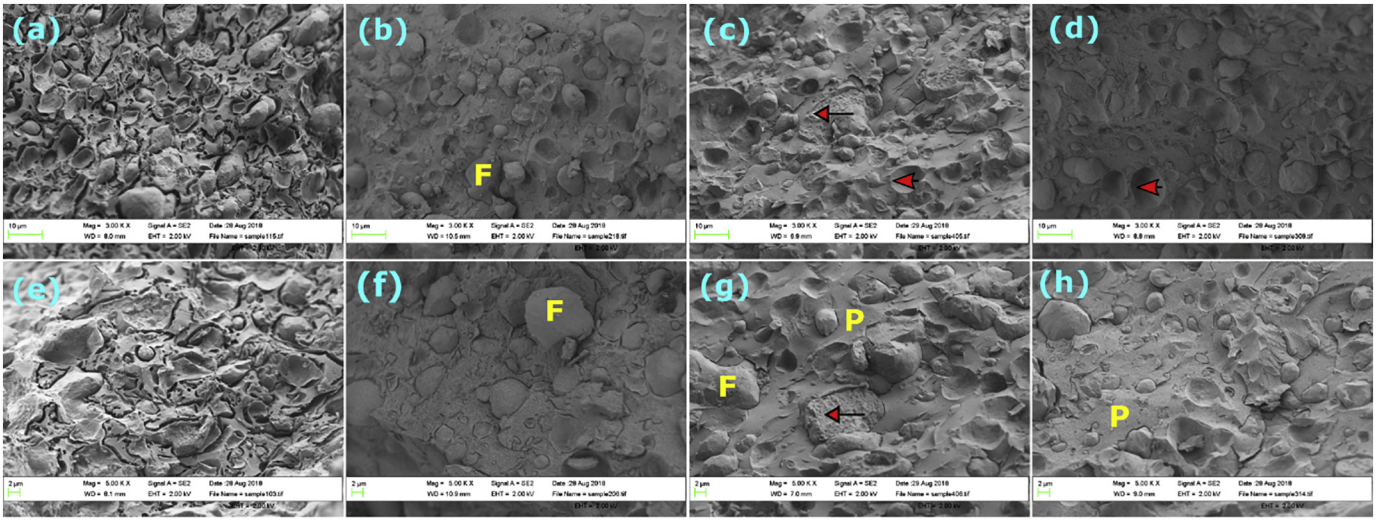


Fig. 5. Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h) PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows = spherical imprints in the protein matrix left by fat globules that were removed during sample preparation, and long arrows = remnant fat from globules partially removed during sample preparation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108525.

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