Effects of raw milk quality on UHT milk instability

Enzymatic factors associated with destabilization of UHT milk

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Chapter 1

General introduction

The demand for ultra-high-temperature (UHT) processed milk is steadily increasing worldwide. This demand is driven by many factors, such as continued urbanization, increased desire for dairy-based products in some countries, and the economic and environmental unviability of refrigerated storage (Chavan, Chavan, Khedkar, & Jana, 2011; Gaur, Schalk, & Anema, 2018). UHT treatment has little effect on the nutritional value of milk, but can render milk free from pathogens, spoilage microorganisms and most of the thermoresistant spores and enzymes, thus resulting in a long shelf life of the UHT milk at ambient storage conditions. The absence of necessity of refrigeration significantly reduces the cost for storage and transportation.

Considering the increasing demand for UHT milk in the international dairy market, destabilization during transportation and storage is becoming an increasingly important issue. This destabilisation can lead to several undesirable changes, such as age gelation, fat separation and bitterness, which may bring about consumer's complaints, product recalls, significant financial loss and a negative brand image to the dairy companies. Destabilization of UHT milk is considered to be promoted by residual proteolytic activity, which is mainly attributed to native milk proteases and psychrotrophic bacterial proteases (Chavan et al., 2011; Datta & Deeth, 2001). There is, however, no yet a practical procedure for determining which type of protease is responsible for proteolysis in UHT milk, mainly because the scientific understanding of the different proteolytic degradation pathways is incomplete. Consequently, control strategies in the dairy production chain for the improvement in the quality and shelf life of UHT milk have not yet been established.

The work described in this thesis focuses on exploring and comparing the effects of bacterial and native proteases on the protein and fat stability of skim and full-fat UHT milk. This will provide insights in the mechanisms of various types of enzyme-induced destabilization in UHT milk. Moreover, this may lead to a better shelf life of UHT milk, which may for instance be achieved by setting specific raw milk quality criteria for UHT processing.

1. UHT milk

To provide a safe and shelf-stable milk, the most commonly applied technique is heat treatment. Ultra-high-temperature (UHT) is a process involving heating of raw milk at a high temperature (typically 135-150 ˚C) for a short holding period (typically 2-10 s), followed by aseptic filling into sterile containers (Datta & Deeth, 2001). The aim of UHT processing is to achieve commercial sterility, which means the product is free of microorganisms that can grow under non-refrigerated storage conditions (Deeth & Lewis, 2016).

1.1   Development history and market status of UHT milk

UHT processing is a well-established and successful technology to prolong the shelf life of milk. The forerunner of continuous flow sterilisers had been constructed and patented before the end of the 19th century (Chavan et al., 2011). The development history of UHT process, along with the aseptic packaging is shown **Fig. 1**.
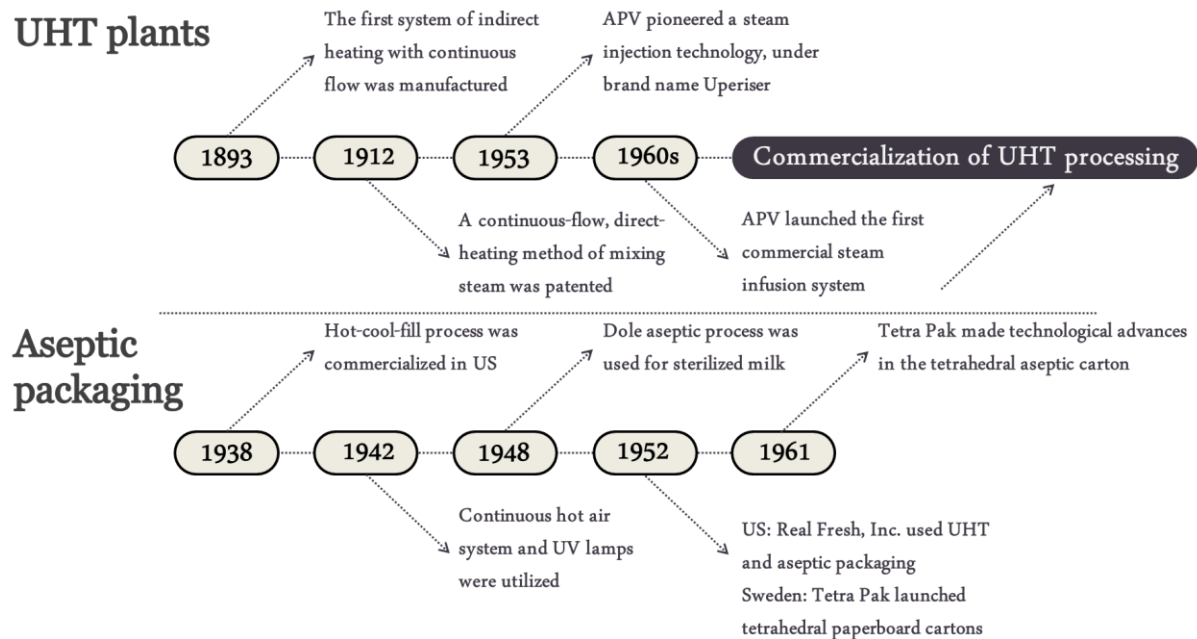
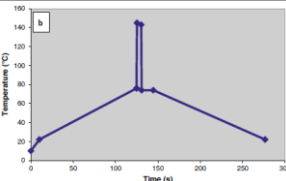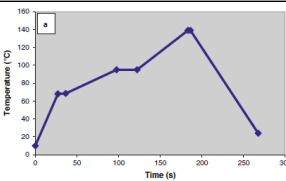**Fig. 1**. Development history of UHT plants and aseptic packaging. Data are from Chavan et al. (2011); David, Graves, and Szemplenski (2012); Zadow (1998).

From this figure, we can deduce the two main challenges considerably impacted the development of UHT milk. The first challenge was producing a sterile product with acceptable flavour and organoleptic quality, which necessitated a heat treatment at ultra-high temperature. This was achieved by the design and manufacture of indirect and direct heating systems for UHT treatment. The development of the UHT process was then hindered due to possible contamination without commercial aseptic systems, which was the second main challenge. This was first solved by filling the sterilised milk in cans under superheated steam. But to decrease costs this was later changed to systems where milk and package were separately sterilised, allowing more economical packaging materials. Therefore, the laminated paperboard based aseptic packing system by Tetrapak emerged in response, and marked the real turning point in the commercialisation of UHT processed dairy products. Since then, the challenge for UHT milk turned from the production process itself, to increased stability of UHT products during shelf life, which is the topic of this research.

1.2   Processing systems

Table 1. Comparison of processing and product quality characteristics of direct and indirect heating systems used for UHT milk production.

	Parameter	Direct heating	Indirect heating
PROCESSING CHARACTERISTICS	Principle	The treated product comes in direct contact with the heating medium	Heat is transferred from the heating media to the product through a partition
	Systems	Steam injection nozzle; Steam infusion vessel	Plate heat exchanger; tubular heat exchanger; scraped surface heat exchanger
	Temperature–time profiles (Deeth and Lewis, 2016)		
	Heat load (with equivalent bactericidal effectiveness)	Lower	Higher
	Preheat hold (at ~90℃)	Uncommon	Common
	Heating rate from preheating to high heat	Fast (<0.5s)	Slow (~30-120s)
	Flouling/ burn-on	Usually minimal	A major problem
	Steam quality requirement	Very high (due to contamination risks)	No specific requirement
	Heat regeneration	~50%	≥90%
	Energy and water requirement	Higher	Lower
	Ability to reach very high temperature (i.e.>145℃)	Capable	Limited
PRODUCT QUALITY	Flavour	Mild cooked flavour	Strong cooked flavour
	Oxygen level	Lower	Higher
	Heat indices (eg. maltitol, furosine)	Lower	Higher
	Undenatured β-lactoglobulin	> 700 mg/L	< 200 mg/L
	Total soluble proteins	Higher	Lower
	Plasmin level	Not completely inactivated	Plasmin generally inactivated

Adapted from Lewis and Deeth (2009).

As shown in the development history of UHT plants (**Fig. 1**), UHT heating can be either "indirect" or "direct", and the stability of UHT milk products during storage can be influenced by the heating method. The differences between these two methods are summarized in **Table 1**, their different heating principles, and the resulting temperature-time profiles, determine that a higher heat load is applied in indirect heating systems. On the one hand, due to the higher heat load, more thermostable proteases are inactivated in indirectly heated UHT milk (Deeth & Lewis, 2016; Thu Tran, Saveyn, Hoang Dinh, & Meeren, 2008). In addition, the extent of whey

protein denaturation is greater for indirect UHT, and the formed denatured whey proteins can inhibit plasmin activity. Due to both these reasons, as reported by Nieuwenhuijse and van Boekel (2003) and Malmgren (2007), proteolysis by plasmin should not be a problem for indirectly heated UHT milk. Nevertheless, indirect heating of milk causes more pronounced cooked flavour arising from sulfhydryl groups exposed upon protein denaturation (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015), and more stale or oxidized flavours due to the higher oxygen level (Lewis & Deeth, 2009). Therefore, in spite of the fact that directly heated UHT milk is more susceptible to enzyme-induced destabilization during shelf life, it is also widely adopted for the better flavour.

1.3   Structural changes of casein micelles and fat globules in UHT milk

The destabilization of UHT milk is closely related to the changes in the casein micelles and fat globules during storage. Casein micelles are highly hydrated colloidal structures which are mainly composed of α_{s1}-, α_{s2}-, β-, and κ- (molar ratio: 4:1:4:1.6), colloidal calcium phosphate and water (Jeurnink & Dekruif, 1993). The exact structure of casein micelles has been extensively debated (Douglas G Dalgleish, 2011; Holt, De Kruif, Tuinier, & Timmins, 2003; Horne, 2006; Huppertz et al., 2017; Pieter Walstra, 1990). A representation of the casein micelle based on the model proposed by C. G. De Kruif, Huppertz, Urban, and Petukhov (2012) is shown in **Fig. 2**. The internal structure of the casein micelle consists of matrixes of α_{s1}-, α_{s2}-, β-caseins, which are linked together by calcium phosphate nanoclusters. The external region is characterized by the presence of κ-caseins as a "hairy layer", which protrude from the micellar surface and provide the steric & electric stabilization of the casein micelles (C. De Kruif, 1999).

Even though casein micelles are highly heat-stable and the micellar conformation is preserved even during severe heat treatments (Pieter Walstra, Walstra, Wouters, & Geurts, 2005), the casein micelles in UHT milk are, from the beginning of storage, different from those in raw milk (Nieuwenhuijse & van Boekel, 2003). Because during UHT heating, β-lactoglobulin irreversibly unfolds at temperatures above about 70 ˚C, and α-lactalbumin above about 65 ˚C (Boye & Alli, 2000). Upon unfolding of whey proteins, the free cysteine residues and hydrophobic residues become exposed and are able to react via thiol-thiol and hydrophobic interactions with other proteins. As a result, whey proteins denature and form aggregates, besides, whey proteins can react with κ-casein on the surface of the casein micelle via thiol-disulphide interchange reactions (Anema, 2008), as ***bolded*** in **Fig. 2**. The aggregation of β-lactoglobulin-κ-casein complexes ($\beta\kappa$-complexes) forms during heating and subsequently dissociates from the casein micelle during storage. A longer heating time or higher heating

temperature also causes more dissociation of βκ-complexes from casein micelles (Metwalli &
Van Boekel, 1996). The βκ-complexes have been considered to be key elements for one of the
pathways of age gelation in UHT milk (McMahon, 1996).
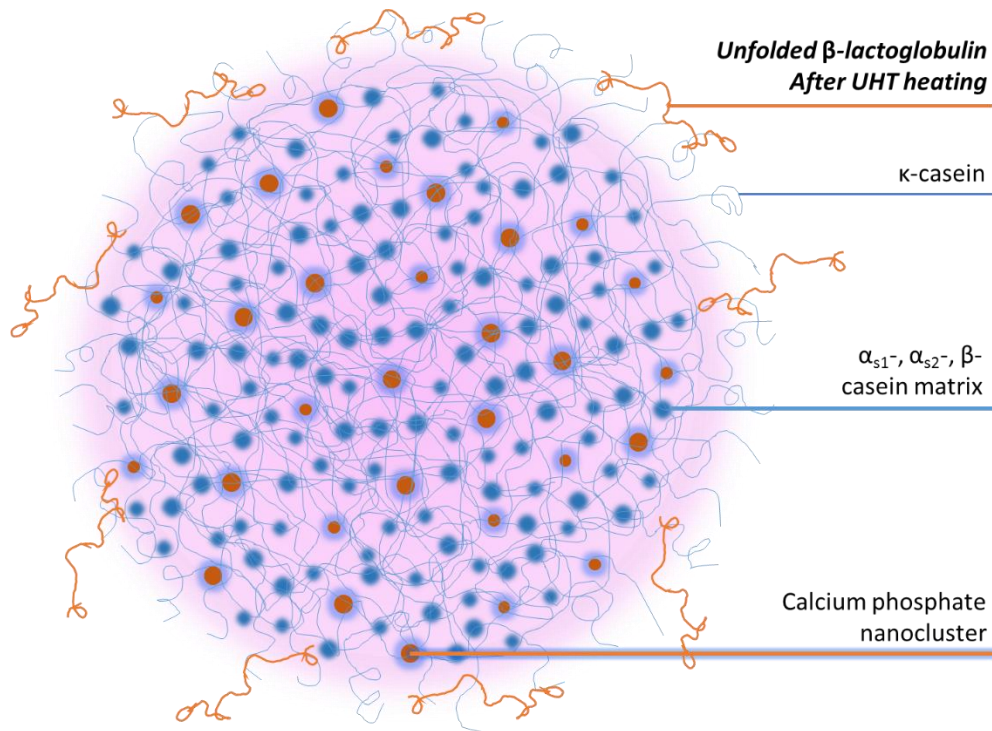
Fig. 2. Schematic representation of a casein micelle (not to scale) after UHT heattreat, adapted
from De Kruif et al. (2012).

The other important milk component, fat, is present in globules, which in UHT milk are
homogenized and heated. Hence, the structure and composition of fat globules are greatly
altered compared to raw milk (Cano-Ruiz & Richter, 1997). Natural milk fat globules are
entirely covered by a multi-layered membrane, known as the milk fat globule membrane
(MFGM) (McPherson, Dash, & Kitchen, 1984). MFGM acts as a natural emulsifier that
prevents flocculation and coalescence of milk fat globules in raw milk (Lee & Sherbon, 2002).
In UHT milk, due to high-pressure homogenization, the average milk fat globule diameter is
reduced to less than 1 μm, which causes the surface area to increase. The native MFGM is not
present in sufficient quantity to cover the newly-formed membrane after homogenisation.
Consequently, surface active milk proteins, both whey proteins and caseins either as semi-intact
micelles or as micellar fragments, are adsorbed on the newly-formed surface (**Fig. 3**) (Lopez,
2005). Subsequent UHT heating promotes the adsorption of whey proteins directly on the
surface of fat globules (Sharma & Dalgleish, 1993). This layer of absorbed milk proteins are
prone to enzymatic hydrolysis during the storage of UHT milk.
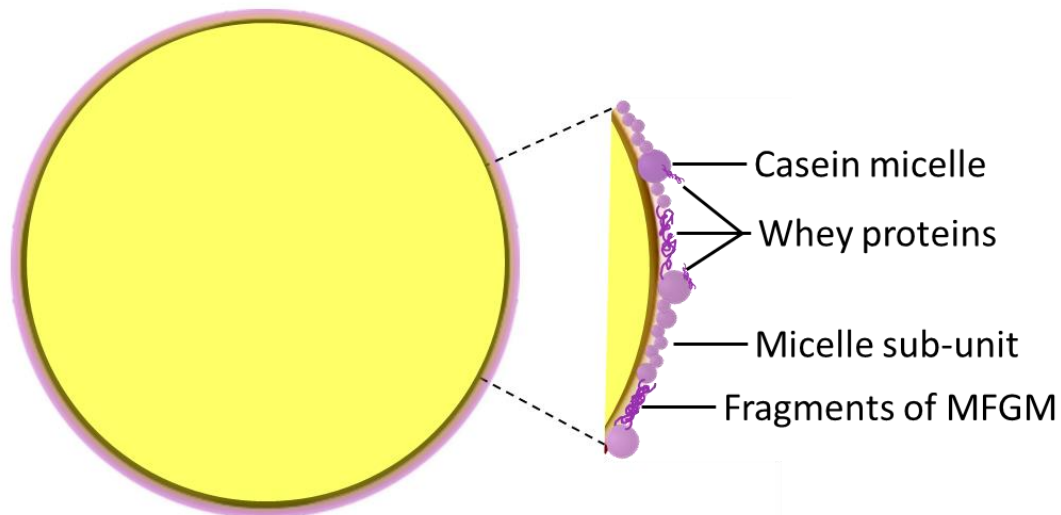
Fig. 3. Schematic representation of the supramolecular structure of milk fat in dairy products (not to scale), adapted from Lopez (2005).

2. Instability of UHT milk during storage

2.1 Raw milk quality associated with UHT milk products

The quality of incoming raw milk has a great influence on the quality and stability of UHT milk products. In this study, we mainly focused on two aspects of raw milk quality, which were the microbiological quality and the somatic cell count (SCC), because they are associated with the proteases produced by psychrotrophic bacterial contaminants and native proteases, respectively.

Raw milk with a high microbiological quality standard can only be maintained based on good hygiene and cooling operations at each step of the dairy production chain. Low microbiological quality will seriously increase the risk of UHT milk instability during shelf life caused by bacterial proteases, of which AprX produced by *Pseudomonas* species is the most well-known example. Detailed information about AprX will be provided in **Chapter 2**.

Besides microbiological quality, SCC is also used as an indicator of raw milk quality, reflecting the udder health status of the cows. Increased milk SCC is correlated with increased indigenous enzyme activity (Somers, O'Brien, Meaney, & Kelly, 2003). Milk contains two main indigenous protease systems, plasmin and cathepsin, of which plasmin has been extensively studied due to its high heat stability and impact on dairy products (A. Kelly & Fox, 2006). Plasmin (EC 3.4.21.7) is a serine protease with a pH optimum of 7.5 at 37 ˚C (Grufferty & Fox, 1988). As shown in **Fig. 4**, plasmin is part of a complex system consisting of plasminogen, plasminogen activators (PAs), PA inhibitors (PAIs), and plasmin inhibitors (PIs) (Bastian & Brown, 1996).

In fresh milk, plasmin is mainly present in its inactive form, plasminogen, which can be activated by tissue-type PAs (tPAs) associated with caseins or urokinase type PAs (uPAs) associated with the somatic cells. Therefore, high SCC is a sign of greater plasmin activity. The system is also mediated by the presence of inhibitors for plasmin and plasminogen activators, i.e. PIs and PAIs (Nielsen, 2002). Plasmin, plasminogen and the activators are all known to be heat stable, whereas the inhibitors are heat labile (Chavan et al., 2011). Consequently, heat treatment strikes the natural balance between the activators and inhibitors in favour of the activators, which thus can lead to enhanced plasmin-induced proteolysis in UHT milk.
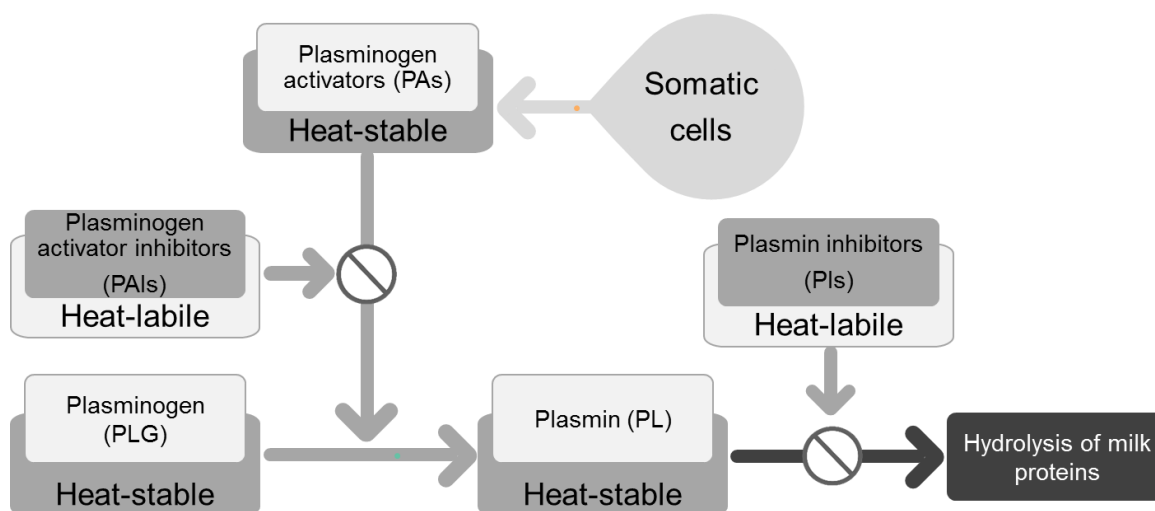
Fig. 4. The plasmin system in milk, adapted from Ismail and Nielsen (2010).

2.2   Effects of raw milk quality on UHT milk instability

The shelf life of UHT milk can be restricted by a suite of negative sensory attributes such as sedimentation, gelation, fat separation, and bitterness. The most significant factor that results in these unacceptable changes is the raw milk quality, which is primarily related to the enzymatic proteolysis by psychrotrophic bacterial proteases and native proteases, as mentioned above. Below, the current theories of the effects of these two categories of proteases on the protein and fat stability of UHT milk will be introduced.

2.2.1   Protein destabilization

Irreversible gelation of UHT milk (age gelation) is a major factor that limits its shelf life and commercial exploitation. There have been many attempts at elucidating the mechanisms of age gelation in sterilised milk, for which the theories have been summarised by Nieuwenhuijse and van Boekel (2003). They identified four types of gel that can be formed through different

pathways, as shown in **Fig. 5**. Pathways A and B, which are closely related to raw milk quality, will be introduced in this chapter, while the influence of storage conditions (pathways C and D) will be discussed in **Chapter 6**.
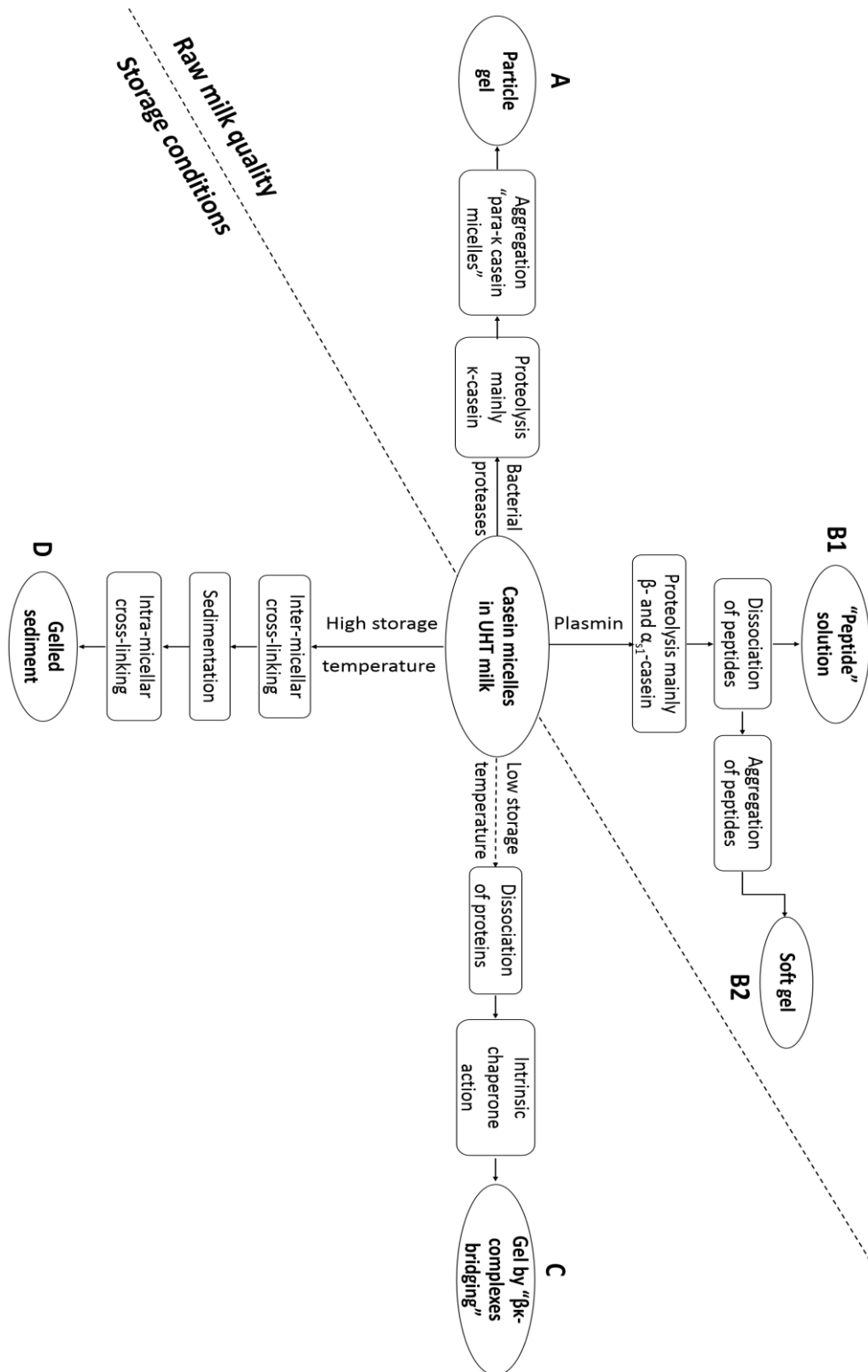
Fig. 5. Simplified scheme of various pathways for destabilization of proteins in UHT milk, adapted from Nieuwenhuijse and van Boekel (2003).

Various forms of gel were reported in UHT milk, ranging from soft to strong. The rennet curd-like "particle gel" has been found to be associated with bacterial proteases (pathway A). There is ample evidence showing that either growth of proteolytic bacteria in milk before sterilisation, or aseptic addition of bacterial proteases to milk after sterilisation can result in such gelation during storage (Keogh & Pettingill, 1984; Law, Andrews, & Sharpe, 1977; Matéos et al., 2015; Recio, López-Fandiño, Olano, Olieman, & Ramos, 1996; Stuknytė et al., 2016). It is considered that bacterial proteases can not only induce age gelation by hydrolysing κ-casein in a similar fashion as chymosin (Nieuwenhuijse & van Boekel, 2003), but they are also able to destabilize casein micelles by proteolysis of caseins in general (Gaucher et al., 2009; Machado, 2015; Marchand, Duquenne, Heyndrickx, Coudijzer, & De Block, 2017).

In contrast to bacterial proteases, plasmin can hardly hydrolyse κ-casein, but it can destabilize casein micelles by breaking down α- and β-caseins (**Fig. 5**, pathway B) (Grufferty & Fox, 1988). Although intensive research has shown that plasmin is involved in age gelation, the conditions under which gelation occurred varied considerably (Enright, Bland, Needs, & Kelly, 1999; A. L. Kelly & Foley, 1997; Manji & Kakuda, 1988; Manji, Kakuda, & Arnott, 1986; V. M. Rauh, Sundgren, et al., 2014a, b). A high rate of proteolysis, either by the addition of plasmin or somatic cell extracts, or by high residual plasmin activity, results in a "peptide" solution, but not in gelation (**Fig. 5**, pathway B1) (Kohlmann, Nielsen, & Ladisch, 1987, 1991). Addition of low concentration of plasmin or somatic cell extracts, however, resulted in gel formation (A. L. Kelly & Foley, 1997; Valentin Maximilian Rauh, 2014b). The plasmin-induced gel has been proposed to be formed by the aggregation of a large number of amphiphilic and charged polypeptides which are produced during the proteolysis by plasmin (**Fig. 5**, pathway B2) (V. M. Rauh, Johansen, et al., 2014a).

Although age gelation has been extensively studied, and different factors which promote gelation have been identified and proposed (Chavan et al., 2011; Datta & Deeth, 2001; Nieuwenhuijse & van Boekel, 2003), the precise biochemical and physical changes which can be correlated with the gelation have not been elucidated. Besides, for the enzyme-induced instability, feasible and effective diagnostic approaches are needed to identify which enzyme is responsible for the gelation.

2.2.2   Fat destabilization

Besides gelation, creaming in UHT milk also receives frequent customer complaints, however, less research has focussed on creaming compared to gelation. If homogenisation during

processing results in sufficiently small fat globules, then enzymatic hydrolysis of the proteins absorbed on fat globule surface could lead to fat instability during storage. Based on the limited reports, creaming is usually accompanied by sedimentation in heat-treated homogenised milk, resulting in a cream layer and/or a sediment layer that contains both protein combined with a relatively high concentration of fat (Nieuwenhuijse & van Boekel, 2003). The rate of creaming and sedimentation depends on the difference in mass density and on the size of the particles (D. G. Dalgleish, 1992; P Walstra & Oortwijn, 1975). It has been reported that the fat separation in full-fat UHT milk differed in appearance when occurring as a result of proteolysis by bacterial proteases or plasmin (Deeth & Lewis, 2016; Visser, 1981). The presence of bacterial proteases in whole milk led to the formation of a custard-like gel (Harwalkar, 1992), whereas plasmin proteolysis causes a creamy surface layer with a more or less clear serum layer (Hardham, 1998; Kohlmann et al., 1991). However, in-depth exploration on the mechanisms of how the proteases destabilize fat globules in UHT milk has not been carried out. Due to limitations in current understanding, studies in this thesis were carried out to attain further knowledge on the protease-induced fat destabilization in full fat UHT milk.

3. Aim and outline of this thesis

This thesis aims at increasing the understanding of the role of raw milk quality, especially the thermostable enzymes originating from raw milk, in UHT milk destabilization during its shelf life. This knowledge can then be used to improve the stability of UHT milk by a targeted control of the enzymatic factors in raw milk.
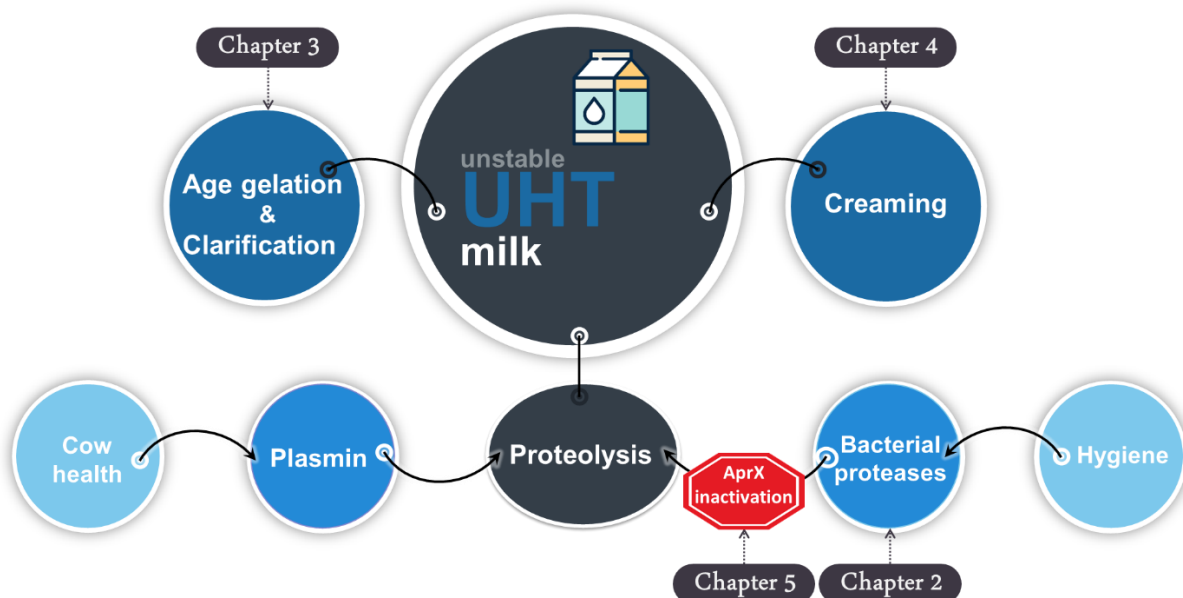
Fig. 6. Schematic overview of the outline of the thesis.

The structure of this thesis is shown in **Fig. 6**. **Chapter 2** contains comprehensive background information on the major bacterial protease AprX, including its biological properties, regulation, proteolytic activity, spoilage potential in UHT milk, detection as well as its inactivation.

To fully understand the mechanism of enzyme-induced destabilization of UHT milk, the effects of different proteases should be separated. Furthermore, the effects of proteolysis and the effects of storage temperature and time should be separated. To achieve this, we built skim and full-fat UHT milk-based model systems to which either AprX or plasmin was added, in order to cause fast destabilization of either protein and/or fat without the interference from non-enzymatic physicochemical changes during storage. Destabilization of protein and fat in UHT milk by AprX and plasmin is compared in **Chapter 3** and **Chapter 4**, respectively.

As a potential strategy to reduce the AprX-induced destabilization of UHT milk, AprX inactivation using a specific low temperature inactivation (LTI) was evaluated in **Chapter 5**. In the end, the general discussion (**Chapter 6**) summarises how the results obtained in this thesis contribute to the understanding of UHT milk instability by proteases. It is also discussed how the results can be applied in practice and used for diagnosing the cause of UHT milk instability.

Chapter 2

The extracellular protease AprX from *Pseudomonas* and its spoilage potential for UHT milk: a review

Abstract

The negative effects of proteases produced by psychrotrophic bacteria on dairy products, especially UHT milk, are drawing increasing attention worldwide. These proteases are especially problematic, because it is difficult to control psychrotrophic bacteria during cold storage and to inactivate their heat-resistant proteases during dairy processing. The predominant psychrotrophic species with spoilage potential in raw milk, *Pseudomonas,* can produce a thermostable extracellular protease, AprX. A comprehensive understanding of AprX on the aspects of its biological properties, regulation, proteolytic potential, and its impact on UHT milk can contribute to finding effective approaches to minimize, detect, and inactivate AprX. AprX also deserves attention as a representative of all extracellular metalloproteases produced by psychrotrophic bacteria in milk. The progress of current research on AprX is summarized in this review, including a view on the gap in current understanding of this enzyme. Reducing the production and activity of AprX has considerable potential for alleviating the problems that arise from the instability of UHT milk during shelf life.

Introduction

Refrigerated storage of raw milk selects for psychrotrophic bacterial genera, predominantly *Pseudomonas* (Stoeckel et al., 2016a). Some *Pseudomonas* strains can produce a specific caseinolytic extracellular protease, AprX, that is resistant to the heating conditions used in ultra-high-temperature (UHT) processing (Dufour et al., 2008; Marchand et al., 2009a). The residual AprX activity can lead to the development of quality defects in UHT milk, including increased viscosity, sedimentation, age gelation, fat separation, and bitterness, all of which cause shelf life reduction during ambient storage and transportation of UHT milk (Baglinière et al., 2013; Matéos et al., 2015; Vithanage, 2017).

After the commercial availability of UHT milk in the 1960s, the observance of age gelation followed, after which the study on proteolytic activity in UHT milk started. Age gelation was first ascribed to the proteases produced by psychrotrophic microorganisms during refrigerated storage of milk, after which the basic biochemical properties, the impact on UHT milk, and the inactivation of bacterial proteases were explored (Adams, Barach & Speck, 1976; Law, Andrews & Sharpe, 1977; Möller, Andrews & Cheeseman, 1977; Keogh & Pettingill, 1984). Later on, some work suggested that proteolytic action of the indigenous milk protease plasmin could also be a causative factor for age gelation (Dekoning, Kaper, Rollema & Driessen, 1985; Kohlmann, Nielsen & Ladisch, 1988), which was then intensively studied in the 1980s-1990s, as reviewed by Bastian and Brown (1996); Datta and Deeth (2001); Fox and Kelly (2006); Ismail and Nielsen (2010). Moreover, a preheating step for plasmin inactivation at around 85-95 ℃ for around 3 minutes has been developed for UHT milk production (Newstead, Paterson, Anema, Coker & Wewala, 2006; Van Asselt, Sweere, Rollema & De Jong, 2008; Rauh et al., 2014b). By contrast, the studies on the effects of bacterial proteolytic enzymes on UHT milk have not been followed up as vigorously as those on plasmin. Before the 21st century, even though there were many studies on UHT milk gelation caused by bacterial proteases, as reviewed by Law (1979), Cousin (1982), and Sørhaug & Stepaniak (1997), a clear recognition of specific bacterial proteases was not reached based on the observational results.

Recently, UHT milk stability during transport regained considerable interest from dairy manufacturers and researchers, because the UHT milk market is blossoming in countries like China, where long-distance transportation is needed, combined with a general growth in the international demand for exporting UHT milk (Chavan, Chavan, Khedkar & Jana, 2011). In order to address the problem of proteolysis in UHT milk, the study of various aspects of the responsible bacterial proteases is becoming an active area of research. Proteases produced by

Pseudomonas species were brought into focus because they are the most important psychrotrophs that dominate the microflora in raw milk, and are known to produce many extracellular enzymes.

Recent advances in molecular biology have allowed the investigation of genetic and regulatory mechanisms underlying AprX production. The first biochemical and genetic characterization of an extracellular protease from *Pseudomonas* was reported by Liao and McCallus (1998). They designated this enzyme as AprX, named after the conserved *aprX* gene cluster encoding an extracellular caseinolytic metalloprotease in *Pseudomonas fluorescens*. Woods, Burger, Beven and Beacham (2001) presented a phenotypic and molecular study of the *aprX–lipA* operon of *Pseudomonas fluorescens* and an analysis of its regulation by temperature. The regulation of the same operon was further studied by McCarthy, Woods and Beacham (2004). Dufour et al. (2008) isolated and characterized three strains of *Pseudomonas* species from a dairy plant, and they found that their different extracellular caseinolytic potentials may result from different levels of AprX expression. However, a large heterogeneity was also revealed on the DNA and the amino acid level when Marchand et al. (2009a) developed an AprX-screening PCR test. Simultaneously, AprX from several *Pseudomonas* strains started to be identified, purified, and characterized (Mu, Du & Bai, 2009; Zhang, Hu, Wang & Sun, 2009; Jankiewicz, Szawłowska & Sobańska, 2010). The spoilage potential of AprX in UHT milk was studied by Matéos et al. (2015); Stuknytė et al. (2016); Machado et al. (2017); and Marchand, Duquenne, Heyndrickx, Coudijzer and De Block (2017).

Studying AprX is also of wider significance in understanding bacterial extracellular metalloproteases of dairy origin. The microbiota in raw milk mainly consists of Gram-negative psychrotrophs (i.e., *Pseudomonas, Serratia, Aeromonas,* and *Enterobacter*), and Gram-positive spore-formers (i.e., *Bacillus, Aneurinibacillus, Brevibacillus,* and *Geobacillus*) (Machado et al., 2015; Vithanage, 2017). These two types of bacteria produce bacterial zinc-metalloprotease of the serralysin subfamily (EC 3.4.24.40, particularly AprX) and the serine subfamily (EC 3.4.21, particularly substilisin and thermolysin), respectively. These two subfamilies of zinc-metalloprotease exhibit significant amino acid homology in their primary structure, regardless of their sources, due to their necessity for zinc for catalytic functions, leading to similar spoilage mechanisms in UHT milk (Vithanage, 2017).

Because AprX is an excellent representative of bacterial extracellular metalloproteases, existing studies were reviewed and summarized. These studies give an overview of the current state of

knowledge of the biological properties, regulation, proteolytic activity, spoilage potential of UHT milk, and detection and inactivation of AprX. These insights can be used as tools to improve UHT milk quality.

1. Biological properties of protease AprX

The biochemical properties of the protease AprX are determined by the expression and regulation of relevant genes, as well as its protein structure. Understanding the biochemical properties under different environmental conditions would enable alterations in certain storage conditions and processing criteria of raw milk, to ultimately improve the quality of UHT milk.

1.1   *aprX* gene, amino acid sequence, and protein structure

The *aprX* gene, which encodes the AprX protein, is located at the beginning of a polycistronic operon in the gene cluster *apr*, found in pseudomonads. This operon, (*aprX- inh- aprDEF- prtAB- lipA,* see **Fig.1**) contains an *inh* gene, coding for a protease inhibitor, type I secretion system genes (*aprDEF*), two autosecreted serine-protease homologues (*prtAB*) and a lipase gene (*lipA*) (Duong et al., 2001; Woods et al., 2001). In previous studies, it is found that part of the genes in this operon are not included in some strains strains (Ahn, Pan, & Rhee, 1999; Johnson, Beacham, MacRae, & Free, 1992), the absence of any of these genes in bacteria will result in loss or relatively low proteolytic or lipolytic activity (Liao & McCallus, 1998; et al., 2001).

5' —[aprX]—[inh]—[aprD]—[aprE]—[aprF]—[prtA]—[prtB]—[lipA]— 3'
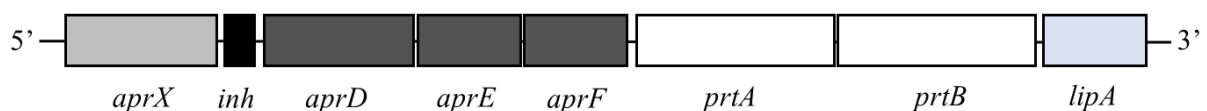
Fig. 1. Structure of the *aprX–lipA* operon.

Genes *aprDEF* encoded the secretion apparatus including a so-called ABC (ATP binding cassette) protein (AprD), a membrane fusion protein (AprE) and an outer membrane protein (AprF). These proteins were believed to be involved in organizing the secretion of AprX, exclusively by the type I secretion (also called the ABC transporter) process (Duong et al., 2001).

Due to the operon structure, the simultaneous occurrence of lipolytic and proteolytic activities is likely (Vithanage, 2017). While, unlike AprX, whose optimal production temperature is slightly above the optimum for bacterial growth, LipA is optimally produced well below the optimal growth temperature (Merieau, Gugi, Guespin-Michel, & Orange, 1993). The

differences in the regulation of lipase and protease may be related to the location of *aprX* and *lipA* at opposite ends of the operon (McCarthy et al., 2004; Woods et al., 2001).

Due to variations in the exact genes present, intra- and interspecies variation in protease and lipase activity is possible, which may be related to genetic and/or expression variability (von Neubeck et al., 2015). The genetic information can be used for the development of molecular-based assays for rapid and reliable detection in dairy industry, which will be discussed in **Section 4.1.1**.

The nucleotides in the *aprX* gene were found to be highly conserved within *Pseudomonas* species that were isolated from raw milk (Martins, de Araújo, Mantovani, Moraes & Vanetti, 2005; Marchand et al., 2009a; Matéos et al., 2015). The conserved domains in the amino acid sequence, which belong to zinc metalloproteases of the metzincin superfamily, determine these features of the primary structure of the AprX protein:

1) A catalytic domain (Zn^{2+}-binding domain) in the N-terminal: a Zn^{2+}-binding motif (xxx**QTLTHEIGH**xxGLxxGLx**HP**x, where x stands for an arbitrary amino acid) is essential in the catalytic domain. Zn^{2+} ions (yellow in **Fig. 2**) bind to histidine residues (blue in **Fig. 2**), and glutamic acid (red in **Fig. 2**) is presumed to be the catalytic residue. The methionine residue (magenta in **Fig. 2**) close to the Zn^{2+}-binding domain is also important for the structure of the active site (Hege & Baumann 2001; Matéos et al., 2015). The influence of Zn^{2+} on AprX activity is further discussed in **Section 1.3.1**;

2) A Ca^{2+}-binding domain in the C-terminal: this domain contains an extended parallel β-roll structure, in which successive strands are twisted in a right-handed spiral (Baumann, Wu, Flaherty & McKay, 1993). The turns between strands are composed of a repeated GGxGxD motif. In these repeats, Ca^{2+} is coordinated to the carboxylate groups of the aspartic acid side chains and the carbonyl groups of the glycine backbones, thereby being stabilized by these six-residues-long repeats (Ertan et al., 2015). This motif is characteristic of proteins secreted via the type I secretion process (Chabeaud et al., 2001; Matéos et al., 2015);

3) A high content of hydrophobic amino acids;

4) No cysteine residues and a high content of glycine residues (Matéos et al., 2015; Marchand et al., 2017).

Fig. 2. X-ray structure of extracellular alkaline metalloprotease from *Pseudomonas aeruginosa* (1KAP). Zn^{2+} ions in the N-terminal catalytic domain are shown in yellow; Zn^{2+} binding histidine residues are in blue; catalytic glutamic acid residue is in red; putative methionine residues in the Met-turn characteristic of metzincins are in magenta, with Ca^{2+} ions in the C-terminal domain shown in green, reproduced with permission from Ertan et al. (2015).

These features in the amino acid sequence create the special protein structure of AprX, which is known to be stabilized by the involvement of Ca^{2+} ions (Liao & McCallus 1998; Ahn et al., 1999). In addition, the lack of cysteine residues and the high content of glycine residues (~15%) in its sequence provide the conformational flexibility of AprX, because the lack of cysteine residues avoids the steric constraints of disulfide bonds. On top of that, the high content of glycine close to the Ca^{2+} binding site would lead to a weak coordination of Ca^{2+} and thus increase local flexibility (Davail, Feller, Narinx & Gerday, 1994). The flexible structure enables reversible unfolding during heat treatments at higher temperatures, such as UHT processing, thus probably causing its thermostability (Adams, Barach & Speck, 1976; Glück et al., 2016), as further discussed in **Section 1.3.2**. The flexible structure also allows AprX to pass easily through the cell wall during its secretion (Pollock & Richmond, 1962).

Comparing different enzymes from the bacterial zinc-metalloprotease family, Vithanage (2017) found all studied proteases contain 1) α/β fold domains with one catalytic Zn^{2+} coordinated in the active site; 2) several Ca^{2+} ions for structural stabilization; 3) C-terminal extension composed of β-sheets. These analogous protein structures cause similar biochemical properties of these proteases.

1.2   Regulation of AprX production

Being a free-living organism, pseudomonads produce various extracellular proteases and lipases that are presumably required for utilizing the available macromolecular nutrients. Of all proteases produced, AprX is an important protease in psychrotroph-contaminated milk that may lead to spoilage. Understanding the regulation of AprX by environmental and nutritional factors is thus of significance.

1.2.1   Environmental factors

Overall, neutral pH is optimal for AprX synthesis (Malik, Prasad & Mathur, 1985; Fairbairn & Law, 1987). The influence of two other main environmental factors, temperature and oxygen, is discussed in this section.

1.2.1.1   Temperature
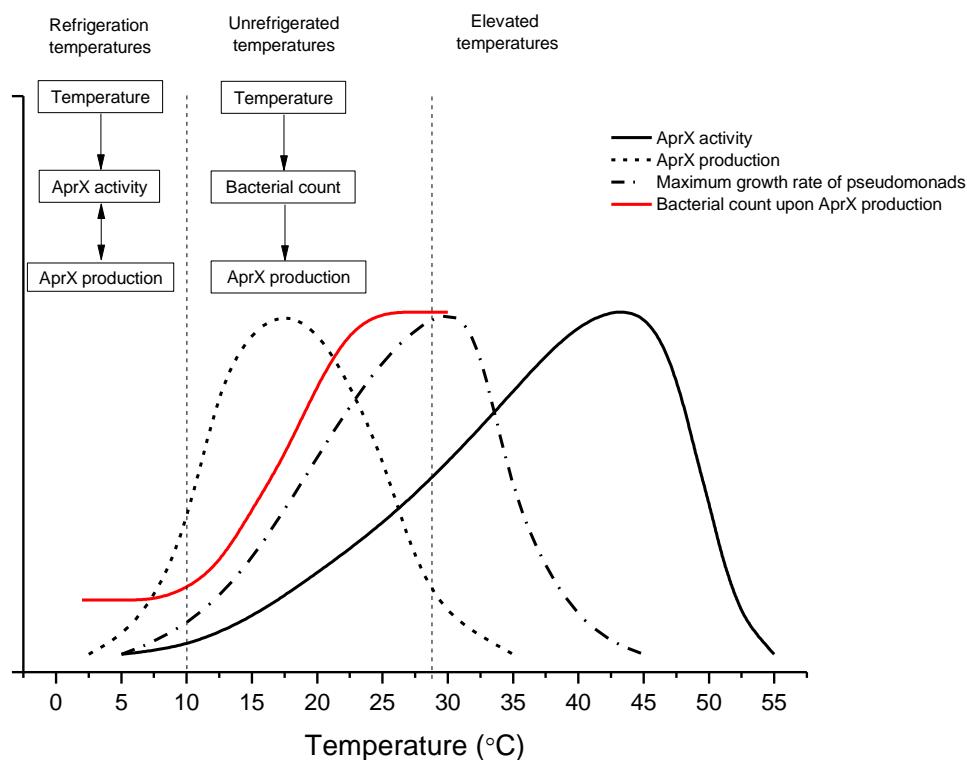
Fig. 3. Schematic illustration (dimensions not to scale) of the maximum growth rate of *Pseudomonas* (dash dot line), bacterial count upon AprX production (red solid line), AprX production (dash line), and AprX activity (solid line) as a function of temperature in milk, drawn based on the literature data reviewed. The temperature range is divided into three parts. I: refrigeration temperatures (4-10 ˚C), II: unrefrigerated temperatures (10-30 ˚C), III: elevated temperatures (higher than 30 ˚C).

The production and activity of AprX, as well as the growth rate of pseudomonads, are strongly temperature-dependent (Buchon, Laurent, Gounot & Guespin-Michel, 2000; Alves et al., 2018). The production of protease at different temperatures neither strictly depends on the bacterial counts, nor the genus of the strains (Gügi et al., 1991; Buchon et al., 2000; Nicodème, Grill, Humbert & Gaillard, 2005). The regulation of AprX production by temperature may happen at the level of post-transcription or post-translation (Woods et al., 2001), and may be related to a specific mechanism of temperature regulation which might be specific to the psychrotrophy (Gügi et al., 1991; Buchon et al., 2000; Nicodème et al., 2005). As sketched in **Fig. 3**, the optimal temperatures for growth of pseudomonads, AprX production and AprX activity are different.

At refrigeration temperatures, both the growth rate of pseudomonads and the AprX activity are low. The AprX production, despite being low in total, has been found to proceed earlier and from a lower bacterial count at refrigeration temperatures than unrefrigerated temperatures (**Fig. 3**) (Haryani, Datta, Elliott & Deeth, 2003; Zhang et al., 2015). Alves et al. (2018) observed greater expression of AprX produced by *Pseudomonas fluorescens* 07A in 10% (w/v) sterile reconstituted skim milk at 12 h when the strain was in the lag phase at 4 ℃ and 10 ℃, than when the logarithmic and stationary phases were reached. The early production of AprX may be closely associated with the low enzymatic activity at low temperatures (Dufour et al., 2008; Baur et al., 2015; Zhang et al., 2015). To effectively utilize the nutrients in the medium and realize proliferation, pseudomonads probably compensate for the decreased activity with greater AprX production. As the temperature increases, there is no need for extensive AprX production, because the enzymatic activity is high enough (Alves et al., 2018).

At unrefrigerated temperatures, both AprX activity and growth rate of pseudomonads increase with increasing temperature. AprX production reaches its maximum at 17.5-20 ℃, which is lower than the optimal growth temperature of pseudomonads at around 30 ℃ (**Fig. 3**) (Gügi et al., 1991; Buchon et al., 2000). At the same time, a dramatic increase in AprX production or total enzymatic activity, due to booming AprX production, was observed in the late exponential or the early stationary phase, only if the cell density reached 10^7–10^8 cfu/mL in milk (Guinot-Thomas, Al Ammoury, Le Roux & Laurent, 1995; Matselis & Roussis, 1998; Stevenson, Rowe, Wisdom & Kilpatrick, 2003). The growth phase-dependent regulation of AprX is the result of a dramatically increased activity of the AprX promoter in the late exponential phase of growth (Liu, Wang & Griffiths, 2007). The close relation with high bacterial count indicates that induction of these enzymes may be a candidate for quorum sensing, a phenomenon by which

bacteria can sense and respond to cell population size by means of chemical signals based on the homoserine lactone molecule (Fuqua, Winans & Greenberg, 1994).

At elevated temperatures higher than 30 ℃, as shown in **Fig. 3**, the growth rate of pseudomonads decreases with increasing temperature, whereas the AprX activity increases until reaching its maximum at around 45 ℃ after which it decreases rapidly until reaching its autolysis temperature at around 55 ℃ (Dufour et al., 2008; Baur et al., 2015).

1.2.1.2   Oxygen

Some controversy exists regarding the role of oxygen in AprX production. On the one hand, *Pseudomonas* are obligate aerobes, which means they require oxygen for growth and extracellular enzyme synthesis (Malik et al., 1985). In the study by Matselis and Roussis (1998), culture agitation was found to increase the ability of *Pseudomonas fluorescens* MR1 to produce protease at 5 ℃ in skim milk. Protease production started earlier in agitated culture than in static culture, and the maximum protease production was higher in the agitated than static cultures.

On the other hand, a rapid drop in dissolved oxygen tension has been observed prior to the onset of AprX production (Griffiths & Phillips, 1984; Rowe & Gilmour, 1986). This large decrease in oxygen tension resulted from the increased metabolic demands for cell proliferation during logarithmic growth, and enzyme synthesis is likely to be initiated by this decrease in oxygen availability (McKellar, 1989), which may also underlie the aforementioned relation between bacterial cell count and AprX production.

1.2.2   Nutritional factors

The enzyme production can be influenced by many nutritional factors, for instance, fermentable carbohydrates, amino acids, and minerals. Because of variations in these nutrients in different milk samples, it is relevant to consider their effects on the induction and/or repression of extracellular bacterial production of proteases.

1.2.2.1   Minerals

Two minerals have been found to be relevant for AprX production, calcium and iron. Calcium/ Ca^{2+} has been implicated as the most important inorganic compound in modulating the protease production from pseudomonads, because Ca^{2+} is necessary for the protein structure of AprX (Ertan et al., 2015). Calcium-stimulated protease production has also been reported for many *Pseudomonas* strains (Nicodème et al., 2005; Ertan et al., 2015). Ca^{2+} supplementation at a

concentration of 1 mmol/L has been shown to drastically promote the extracellular protease production compared with absence of Ca^{2+} in a minimal salt medium (Liao & McCallus, 1998) and LB medium (Liao & McCallus, 1998; Zhang et al., 2009; Ertan et al., 2015). In milk, the calcium concentration is as high as 26-32 mmol/L (Gaucheron, 2005). Even though only a small fraction of calcium is present as free ions, the concentration of free calcium can still reach 2 mmol/L (Lucey & Horne, 2009), which means that milk contains sufficient free Ca^{2+} to stimulate protease production.

Next to calcium, also iron has been found to significantly influence AprX production (McKellar, 1989; Woods et al., 2001). The regulation of AprX synthesis by iron differs with the iron concentration, i.e., stimulation at low concentrations but repression at high concentrations (Maunsell, Adams & O'Gara, 2006). As an important element of many enzymes related to metabolism and respiration, iron is required by pseudomonads for growth and development (Brown & Luke, 2010). To utilize the iron in the environment, ferric iron will be scavenged from the environment by the low-molecular-weight iron chelators called siderophores (Brown &Luke, 2010). The siderophore and AprX were found to be jointly produced under low environmental iron conditions (Maunsell et al., 2006). During the transcription of *aprX*, PbrA, an iron starvation extracytoplasmic function alternative sigma factor, is required (Sexton, Gill, Dowling & O'Gara, 1996; Woods et al., 2001). On the other hand, the transcription of the promotor *pbrA* was found to be repressed by the ferric uptake regulator at high concentrations of free ferric iron (100 µmmol/L), which may account for the strong repression of *aprX* transcription under high iron conditions (Maunsell et al., 2006). Bovine milk is a poor source of iron, containing only 3.6-12.5 µmol/L, with most iron present as bound iron (Hunt & Nielsen, 2009). Therefore, the iron concentration in milk will stimulate, rather than repress, AprX production.

1.2.2.2   Carbon and nitrogen sources

The intermediates of the citric-acid cycle are important carbon sources for the biochemical reactions of aerobic microorganisms (McKellar, 1989). But, except for succinate and pyruvate, the use of citric acid cycle intermediates mostly results in repression of AprX production (Jaspe, Palacios, Matias, Fernandez & Sanjose, 1994). Of all the intermediates, citric acid was found to be the strongest repressor of AprX production (McKellar, 1989). The inhibitory action of citrate is not due to its chelating property, because the repression of citrate on the activity of pre-formed AprX was ruled out (Fairbairn & Law, 1987). Therefore, the inhibitory action of

citrate is more related to its effects on the metabolism of the organism. The repression is of particular significance in relation to pseudomonads growing in milk if *Pseudomonas fluorescens* is present as the typical strain. Because lactose is not utilized by most pseudomonads (Jaspe et al., 1994), citrate is the major carbon source for this organism. The concentration range of citrate in milk is 7-11 mmol/L, and was found to be related to the season (Gaucheron, 2005). Therefore, the variations in citrate concentration in milk may partially explain the wide variations in AprX levels associated with similar numbers of pseudomonads.

Because the onset of protease production could depend on a low energy status in pseudomonads cells, as a result of the exhaustion of the more easily used substrates (Jaspe et al., 1994), addition of more easily metabolizable carbon sources, for instance citrate, will repress the protease production (McKellar, 1989). However, glucose cannot act as an easily metabolizable carbon source, because phosphofructokinase, a key enzyme of the glycolytic pathway, is lacking in most *Pseudomonas* strains, glucose is therefore a poor repressor of AprX production (Fairbairn & Law, 1987; Rojo, 2010).

Even though some inorganic nitrogen compounds, such as ammonium, nitrate, and urea, have been found to be good nitrogen sources for pseudomonads (Daniels et al., 2010), their regulatory effect on the production of AprX has been rarely studied. Based on the existing research, conflicting results have been obtained, especially for ammonium ions. Both stimulation and repression of AprX production by ammonium ions have been reported (Whooley, O'Callaghan & McLoughlin, 1983; Fairbairn & Law, 1987). Urea was also shown to be an effective nitrogen supply for the production of AprX (McKellar, 1989). Overall, organic nitrogen is preferred over inorganic nitrogen for the production of AprX.

The contribution of amino acids in modulating AprX production depends on the use of amino acids as sole or alternative sources of carbon, nitrogen, or both (Jaspe et al., 1994). All the amino acids which can be utilized as a sole source of carbon by pseudomonads (Pro, Ala, Glu, Gln, His, Arg, Asp and Asn) could induce AprX production to various degrees (Fairbairn & Law, 1987; Rojo, 2010), while the others do not enhance AprX production.

Besides the inorganic nitrogen compounds and amino acids, some proteins and their small-molecular-weight hydrolysis products can also be used as inducers and nitrogen sources (Fairbairn & Law, 1987). McKellar (1989) found that it was the low-molecular-weight (<5,000 Da) compounds from skim milk that were responsible for inducing AprX synthesis in *Pseudomonas fluorescens*.

Overall, even though there are many free amino acids and small endogenous peptides existing in bovine milk as inducers and as a nitrogen source, AprX is not produced when there are adequate carbon sources, Instead, AprX is produced by pseudomonads only when a rapid drop in oxygen tension, iron starvation, and exhaustion of easily metabolizable carbon sources happens. All these changes will result in a low metabolic energy state, i.e., catabolite repression. Therefore, AprX is synthesized by pseudomonads to ensure a supply of carbon rather than protein synthesis.

1.3   Biochemical properties of AprX

Pseudomonas is a very large and heterogeneous genus of gram-negative bacteria. The majority of *Pseudomonas* species produce only one protease, AprX. However, different biochemical properties of AprX from different strains of *Pseudomonas* have been reported. There are some biochemical variations among AprX, although general trends are also apparent. Some of the biochemical properties have been reviewed by Fairbairn and Law (1986), and some results from reports after that year are summarized in **Table 1**. Two main biochemical properties, factors affecting AprX activity and its thermal stability, are discussed in this section.

1.3.1   Factors affecting AprX activity

1.3.1.1   Inhibitors and stimulators

Three types of agents have been evaluated on their inhibitory or stimulatory effects on AprX activity:

1) Chelators: EDTA (a chelator of divalent metals), 1,10-phenanthroline (a chelator with a high affinity for Zn^{2+}) and EGTA (a Ca^{2+} chelator) have been frequently tested for their inhibitory effects on AprX activity. EGTA inhibited activity to a lesser degree than EDTA and 1,10-phenanthroline (Jankiewicz et al., 2010; Baglinière et al., 2013). These results indicate that the divalent cations Zn^{2+} and Ca^{2+} are both required for the activity of AprX (Liao & McCallus, 1998), but the stronger inhibition caused by 1,10-phenanthroline, compared to EGTA, suggests that especially the zinc ions are required (Jankiewicz et al., 2010). DTT has also been reported to inhibit AprX activity (Yang, Wang & Zhou, 1996; Paoletti, Ascher & Neyton, 1997), maybe through acting as a metal ion chelator other than a disulfide bonds reducing agent, considering the absence of cysteine residues in the protease sequence, as discussed in **Section 1.1**;

2) Inhibitors for amino-acid-specific proteases: as a metalloprotease, the activity of AprX is only negligibly affected by the inhibitors of aspartyl protease (pepstatin A), serine

protease (PMSF and benzamidine), and cysteine peptidases (E-64) (Baur et al., 2015; Alves, Salgado, Eller, Vidigal & de Carvalho, 2016);

3) Ions: the findings about effects of free ions on AprX activity are contradictory, except for Zn^{2+}, Ca^{2+}, and Mn^{2+}. Zn^{2+} is able to both activate and inhibit AprX activity, depending on the enzyme/substrate ratio. As a zinc-dependent metalloprotease, some Zn^{2+} is needed for the enzyme activity without doubt. Zn^{2+} could also efficiently restore the activity of the apoenzyme, which is an inactive form of enzyme lacking the association of coenzyme and/or cofactors, to 80% of the original level (Karadzic, Masui & Fujiwara, 2004). However, Alves et al. (2016) found that Zn^{2+} inhibited the protease activity proportionally with its concentration. The inhibitory effect of Zn^{2+} may be due to the formation of a zinc mono-hydroxide bridge when there is an excess of Zn^{2+} (Larsen & Auld, 1989).

Besides Zn^{2+}, Ca^{2+} and Mn^{2+} have also been reported to stimulate AprX activity in some cases (Mu et al., 2009; Jankiewicz et al., 2010; Alves et al., 2016). Ca^{2+} ions were discovered to stimulate AprX activity by regulating the calcium-binding domain (Miyajima et al., 1998; Zhang et al., 2009; Alves et al., 2016), while Mn^{2+} may reinforce AprX activity by playing a role in maintaining its active conformation (Kuddus & Ramteke, 2008), possibly by replacing the role of Zn^{2+}, although Mn^{2+} can probably not fully replace Zn^{2+}, as it was not able to reactivate the apoenzyme (Karadzic et al., 2004).

1.3.1.2   pH

AprX activity is not sensitive to pH. The isoelectric point for AprX has been reported to be between 3.95 and 4.5 (Dufour et al., 2008; Matéos et al., 2015). At a pH higher than the isoelectric point, the proteolytic activity of AprX remains high across the pH range of 5-10 (**Table 1**), because the ionization and deprotonation generated by increasing the pH do not disrupt the flexible enzyme structure of AprX (Alves et al., 2016).

1.3.2   Thermal stability of AprX in milk

Pseudomonas proteases are well known for their very high heat stability, for example being able to survive at UHT temperatures. First-order inactivation was found in milk or whey in the UHT region (heating at 135-150 ℃ for 1-10 s) using proteases isolated from *Pseudomonas* species (Kroll & Klostermeyer, 1984; Vercet, Lopez & Burgos, 1997; Stoeckel et al., 2016a). The most probable mechanism of thermal inactivation of AprX was suggested to be the deamidation of asparagine or glutamine residues (Schokker & van Boekel, 1999a). Succinimide

formation from aspartate residues, oxidation of amino acid side chains, and aggregation of proteins may also lead to the thermal inactivation (Daniel, Dines & Petach, 1996).

Many studies report remaining protease activity after arbitrary temperature-time combinations, which makes comparisons between studies difficult. But kinetic parameters, for example activation energy (E_a) and D-value, can be used to compare the heat resistance of enzymes. The E_a here means the minimum energy required to start inactivation of the enzyme; and the D-value is the time required to reduce the enzyme activity to 10% of its original value at a certain temperature. Kinetic parameters for the thermal inactivation of AprX have been calculated in the review by Stoeckel et al. (2016b). The average reported E_a is 96.1±9.3 kJ/mol, which is much higher than the 36.3±12.2 kJ/mol reported for plasmin in milk (>90 ℃), indicating a higher energy barrier to inactivate AprX than plasmin. The D-value for the inactivation of AprX and plasmin in milk was calculated to be 124 s and 13 s at 140 ℃, respectively (Kroll & Klostermeyer, 1984; Saint Denis, Humbert & Gaillard, 2001), suggesting the time required for inactivating both AprX and plasmin are several times longer than regular UHT treating time (a few seconds), especially for AprX. The high D-value renders the inactivation of the enzyme using current UHT regimes difficult, without causing detrimental effects of increased heating to the milk protein and its sensory properties.

As mentioned in **Section 1.1** on protein structure, the thermal stability of AprX is mainly attributed to its flexible tertiary structure, which can refold quickly and accurately with the formation of calcium salt bridges when the temperature is lowered again (Barach, Adams & Speck, 1978; Ertan et al., 2015). This property gives rise to the enhanced heat resistance in the presence of calcium, although this unfortunately makes AprX more difficult to be thermally inactivated in milk.

**Table 1**. Properties of AprX from *Pseudomonas* species

Organism	Optimal pH	Medium	Optimal temperature (℃)	Molecular weight (kDa)	Inhibitors of protease activity	Residual activity after heat treatment	References
Pseudomonas fluorescens 07A	7.5	LB broth	37	49.5	EDTA, E_a , Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Mg^{2+}	40% (100 ℃, 5 min)	Alves et al. 2016
Pseudomonas fluorescens BJ-10	NS	nutrient broth	NS	NS	NS	39.36% (130 ℃, 3 min)	Zhang et al. 2015
Pseudomonas LBSA1	5~10	synthetic medium	40	49	EDTA, EGTA, 1,10-phenanthroline, DTT	40% (90 ℃, 30 min)	Matéos et al. 2015; Nicodème et al. 2005
Pseudomonas fluorescens 041	6~6.5	TYEP broth	37	49	EDTA, SDS, Urea, DTT, β-mercaptoethanol, Co^{2+}	70% (75 ℃, 20 s)	Martins, Pinto, Riedel & Vanetti, 2015
Pseudomonas panacis	8.1	mineral salt medium	40	49.4	1,10-Phenanthroline, EDTA	88.0 ± 7.7% (138 ℃, 18 s)	Baur et al. 2015
Pseudomonas fluorescens BJ-10	7	nutrient broth	30	47	$Cu^{2+,}$ DTT	94.8±0.88% (100 ℃, 2 min)	Zhang & Lv, 2014
Pseudomonas fluorescens	9	mineral salt medium	42	50	EDTA, EGTA and 1,10 phenanthroline, Zn^{2+}, Co^{2+}, Cd^{2+}	NS	Jankiewicz et al. 2010
Pseudomonas fluorescens TSS	8	LB broth	50	47	Co^{2+}	NS	Zhang et al. 2009
Pseudomonas Xuorescens Rm12	7.5	nutrient broth	40	45	EDTA, 1,10- Phenathroline, Mg^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+}	9% (160 ℃, 20s)	Mu et al. 2009
Pseudomonas fluorescens F	8.5	synthetic medium	45	45	EDTA, EGTA, 1,10-phenanthroline, NaCl	NS	Dufour et al. 2008
Pseudomonas fluorescens SMD 31	6 in citrate buffer, 7 in PBS	minimal salt medium	NS	NS	EDTA	68.8% (121 ℃, 20 min)	Rajmohan, Dodd & Waites, 2002
Pseudomonas fluorescens RO98	5	minimal salts medium	35	52	EGTA, 1,10-phenanthroline, EDTA	NS	Koka & Weimer, 2000
Pseudomonas TOLAASII	7	Sterilized skim milk	40	45	EDTA, 1,10-phenanthroline	25% (140 ℃, 1.5 min)	Baral, Fox & O'connor, 1995
Pseudomonas fluorescens No. 33	8.0-9.8	skim milk and nutrient broth	30-35	48	1,10-phenanthroline, EDTA, Hg^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}	NS	Kumura, MiKawa, & Saito, 1993

NS: Not specified.

1.3.3   Low-temperature inactivation

Except for the thermal inactivation in the UHT region, many studies have reported another thermal labile zone for AprX at 50-60 ℃ (Barach, Adams & Speck, 1976; West, Adams & Speck, 1978; Diermayr, Kroll & Klostermeyer, 1987; Glück et al., 2016). The characteristic inactivation behavior at lower temperatures is termed low-temperature inactivation (LTI), which is attributed to self-digestion of the protease (Schokker & van Boekel, 1998a, b; Stoeckel et al., 2016b).

As sketched in **Fig. 4**, it is possible to divide the inactivation of AprX into three stages. The inactivation by LTI is more pronounced in buffer systems than in milk. These stages were termed as active enzyme stage (I), autolysis stage (II), and thermal-inactivation stage (III). During stage I, the enzyme is active and reaches its optimal activity at temperatures around 35-45 ℃, as also shown in **Fig. 3**. The tertiary structure is most stable at the optimal growth temperature of *Pseudomonas* species (around 25 ℃), and begins to unfold at the temperature of maximum enzyme activity (35-45 ℃). In stage II, autoproteolysis occurs, indicated by a decrease in the residual enzyme activity. In this stage, the enzyme molecules are present as a mixture of 1) folded, active, proteolytic, compact molecules with small surface area in relation to molecular weight; 2) (partially) unfolded, inactive molecules. The native state can protect the enzymes from autoproteolysis, while the (partially) unfolded tertiary structure renders the enzyme molecules susceptible to autolysis. In stage III, the unfolded enzymes are inactivated by heat, as explained in **Section 1.3.2**.
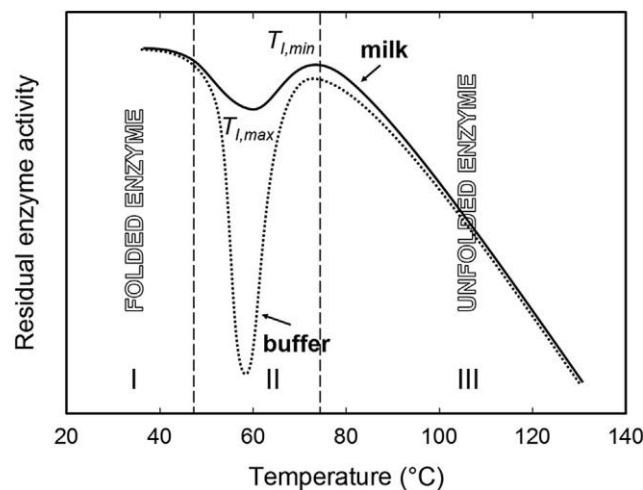
Fig. 4. Schematic illustration of the inactivation of proteases from *Pseudomonas* species as a function of temperature in milk (solid line) and in a buffer system (calcium- and casein-free; dotted line), reproduced with permission from (Stoeckel et al., 2016b).

In **Fig. 4** stage II, we can observe that milk components have a protective effect on AprX. The protective effect may be ascribed to the presence of caseins and Ca^{2+}. The caseins can either bind to AprX molecules and increase the steric hindrance, or can exist as alternative substrates competing with AprX (Stoeckel et al., 2016b). Ca^{2+}, on the other hand, can promote a more ordered partially unfolded protein structure that resists transition to a fully unfolded state, which means fewer AprX molecules are susceptible to autolysis, which may also reduce autolysis (Schokker & van Boekel, 1999b; Ertan et al., 2015).

Even though many studies have demonstrated that LTI can promote the inactivation of AprX, the results are conflicting about its effects on increasing the shelf life of UHT milk (West et al., 1978; Driessen, 1983; Kocak & Zadow, 1985b, 1989). To develop a feasible processing step from LTI, broadening of the hydrolytic specificity, and whether effective inactivation exists, must be determined.

2. Proteolytic potential of AprX

The biochemical characteristics are variable and strain-dependent for AprX produced by different *Pseudomonas* strains, and, similarly, also its proteolytic properties are variable (Baglinière et al., 2012). This variability in proteolytic activity of AprX is discussed here.

2.1 Variability in the proteolytic activity of AprX

The caseinolytic potential of AprX from different strains of *Pseudomonas* is variable. Even though the heterogeneity in proteolytic activity has been reported in many studies, it is important to bear in mind that there is more quantitative (degree of hydrolysis) than qualitative (cleavage sites) variability in the proteolytic potential among *Pseudomonas* strains.

Several studies investigated the heterogeneity of heat-resistant proteases from *Pseudomonas* species. Recently, Marchand et al. (2017) found large differences in the off-flavor-generating capacity, which were related to the generation of hydrophobic peptides by hydrolysis of caseins, in 6 major milk spoiling *Pseudomonas* strains in UHT milk. Caldera et al. (2015) examined the phenotypic features and enzymatic spoilage activities of 66 putative *Pseudomonas* strains isolated from different food matrices and found large variations in proteolytic activity as measured in UHT-milk. Baglinière et al. (2012) qualitatively analyzed the peptide composition in the non-casein nitrogen fraction of 9 strains of *Pseudomonas fluorescens*, which were classified into two groups according to their ability to destabilize UHT milk. They found that, even though the destabilization of UHT milk by *Pseudomonas fluorescens* was highly variable and strain-dependent, different caseins were hydrolyzed in a similar way. Similar quantitative

heterogeneity in the proteolytic activity has been reported for many other *Pseudomonas* strains (Dufour et al., 2008; Marchand, Coudijzer, Heyndrickx, Dewettinck & De Block, 2008).

Different hypotheses can be formulated to explain this variability in proteolytic activity, of which the heterogeneity in enzyme expression has been discussed most and probably is a main cause. Dufour et al. (2008) investigated the *aprX* gene and its expression level in 3 strains of *Pseudomonas fluorescens* with highly variable proteolytic activity. They found that the variation mainly seemed to result from the level of AprX expression, rather than the AprX sequence/structure. These results correspond to those of Marchand et al. (2008) who found no considerable differences in AprX sequences between strains with variation in proteolytic activity.

2.2   Hydrolysis patterns of milk proteins by AprX

Compared with the heterogeneity of AprX activity between strains, as discussed in **Section 2.1**, the similarities in the cleavage specificity on caseins can provide more insights into the influence of AprX on milk. In **Table 2**, the resulting AprX-induced peptides formed through casein hydrolysis are summarized from the studies that used liquid chromatography coupled to mass spectrometry (LC-MS) for peptide profiling.

The data in **Table 2** are based on the qualitative comparison of the numbers of identified unique peptides, in which the order of cleavage sites from each casein to the hydrolysis by AprX was found to be β->α_{s1}->κ->α_{s2}-caseins. However, studies which quantitatively characterized the decrease in intact caseins showed the order κ->α_{s}->β-casein when using electrophoresis (Adams et al., 1976) and κ->β->α_{s}-casein when using RP-HPLC (Zhang, Bijl & Hettinga, 2018). The different orders from these studies may be attributed to the different ways of the addition of AprX and the different analytical approaches. In spite of the differences, a consensus can be reached that AprX preferentially hydrolyzes κ-casein.

Table 2. A summary of the number and localization of the peptides release from β-, α_{S1}-, κ-, and α_{S2}- casein upon the action of AprX.

References	Sample treatments	The number and main localization of the identified unique peptides			
		β-casein	**α_{s1}-casein**	**κ-casein**	**α_{s2}-casein**
Stuknytė et al. 2016	Incubated single casein fractions with the cell-free supernatant of *P. fluo* PS19	103 (7 ℃, 6 days) and 110 (22 ℃, 4 days); no peptide from f(11-41), f(111-123) and f(157-160)	71 (7 ℃, 6 days) and 108 (22 ℃, 4 days); no peptide from f(37-80)	102 (7 ℃, 6 days) and 104 (22 ℃, 4 days); few peptides from f(1-13)	4 (7 ℃, 24h); f(117–125) and f(190–207)
Matéos et al. 2015	Added about 5×10^4 cfu/mL of *P*. LBSA1 to milk before UHT treatment	29 (after 24h); no peptide from f(79-94) and f(135-140), and only 1 cleavage site in the hydrophilic N terminal region f(1-50)	27 (after 24h); no peptide from f(37-79) and f(166-170)	6 (after 24h); no peptide from f(124-161)	NS
Baglinière, et al. 2013	Added purified AprX (0.2mg/L) to milk before UHT treatment	91 (after 90 days); no peptide from f(13-25)	23 (after 90 days); f(5-35) and f(105-125)	3 (after 90 days); f(105-125)	5 (after 90 days)
Gaucher et al. 2011	Incubated milk with 2.2×10^5 cfu/mLof *P. fluo* CNRZ 798 before UHT treatment	118 (after 92 days); f(29–69), f(84–110) and f(157–191)	22 (after 92 days); f(8–38)	9 (after 92 days); f(105–123)	4 (after 92 days); No preferential part

NS: Not specified.

Kappa-casein was found to be hydrolyzed readily in the region f(105–125) by AprX. The relative low number of peptides identified from κ-casein by LC-MS may be due to the specific region in which κ-casein is cleaved, and the difficulty of detecting the resulting glycosylated peptides when using mass spectrometry. Beta-casein is strongly hydrolyzed throughout its entire sequence, except for its N-terminal region. This is probably due to the presence of phosphoseryl residues in the positions 15, 17, 18, and 19, which are involved in the nanoclusters of micellar calcium phosphate. Consequently, this region of β-casein is not easily accessible to proteases (Baglinière et al., 2012). The sequence of α_{s1}-casein was almost entirely recovered, except f(40-79) which contains three phosphoserine clusters, and f(144-170) which is hydrophobic. For α_{s2}-casein, fewer peptides were detected compared to the other caseins. Most peptides were found in the C-terminal region, which has previously been shown to be exposed and easily accessible for hydrolysis (Farrell, Malin, Brown & Mora-Gutierrez, 2009).

By studying the cleavage sites, based on the identified peptides in milk proteins, it can be concluded that AprX does not have a strong specificity to certain amino acids. Matéos et al. (2015) showed that the presence of basic (Arg, Lys, His) or aromatic (Tyr, Phe, Trp) amino acid residues in the P1 position led to a strong cleavage of the peptide bond, and that the presence of Val, Met, Phe, Tyr, His, and Gln in the P1' position also seemed favorable for cleavage of the peptide bond, while Pro, Trp, Asp, SerP, Lys, and Arg appeared unfavorable. In view of the broad specificity of AprX to amino acids, the preference of cleaving the Phe105-Met106 of κ-casein may be mainly due to the special conformation with charged residues of κ-casein, which can play an important role in binding and interacting with enzymes like chymosin (Palmer et al., 2010).

3.   Relationship between AprX and the instability of UHT milk

UHT processing can effectively sterilize the milk and destroy microorganisms. However, the attained commercial sterility does not guarantee constant stability during the long shelf life. Residual AprX activity in UHT milk can lead to age gelation, fat separation, and bitterness, which will reduce consumer acceptability of the milk. In this chapter, we will discuss how AprX is associated with this instability and compare the effects of AprX on instability with plasmin and Ser2, two other heat-resistant proteases that can occur in milk.

3.1   Role of AprX in the age gelation of UHT milk

Age gelation is an irreversible condition, described as coagulation, sweet-curd formation, thixotropic thickening, or lumpiness, after the product has been stored for weeks to months.

AprX has been reported to promote the occurrence of age gelation by mediating the hydrolysis of caseins (Baglinière et al., 2013; Andreani et al., 2016; Stoeckel et al., 2016a).

As described in **Section 2.2**, AprX can not only cleave the peptide bond Phe_{105}-Met_{106} of κ-casein, like chymosin (Recio, García-risco, Ramos & López-fandiño, 2000), but also non-specifically cleave in the region of the soluble hydrophilic caseino-macropeptide (CMP) which is normally present as the "hairy layer" or "polyelectrolyte brush" on casein micelles (Gaucher et al., 2011). Such cleavage of the hydrophilic tails on the surface of the micelles reduces both the steric and electrostatic repulsion between casein micelles. On the other hand, the insoluble, hydrophobic para-κ-casein and other peptides may also directly promote the formation of a gel network (Matéos et al., 2015). Additionally, the ability of AprX to hydrolyze β- and $α_s$-casein can destabilize the interior of casein micelles, further stimulating gelation. The hydrolysis patterns and physical state of the resulting gels will be discussed in more detail in **Section 3.4**.

3.2   Comparison between plasmin and AprX

Next to the *Pseudomonas* proteases, proteolysis in UHT milk may also be attributed to native milk enzymes, for example plasmin, elastase, cathepsin B, and cathepsin D (Datta & Deeth, 2001; Kelly & Fox, 2006). Of these, plasmin-induced proteolysis has been closely linked to the age gelation of UHT milk, because it may partially survive mild UHT-processing conditions (Kohlmann, Nielsen & Ladisch, 1991; Newstead et al., 2006). In order to address the problem of proteolysis in UHT milk, it is necessary to determine the origin of the responsible enzyme(s). Therefore, the effects of plasmin and AprX on the destabilization of UHT milk is compared in this part.

Different gelation behaviors caused by AprX and plasmin during the storage of UHT milk have been observed and reported in many studies (Datta & Deeth, 2003; Baglinière et al., 2013; Rauh et al., 2014b; Matéos et al., 2015), and directly compared by Datta & Deeth (2003) and Zhang et al. (2018). The non-specific protease AprX and the lysine/arginine-specific protease plasmin differ in their preferences and cleavage patterns towards caseins, and therefore lead to different hydrolysis products and gelation behaviors. The differences in gelation properties in UHT milk are summarized in **Table 3**.

Plasmin mainly hydrolyzes β-, $α_{s1}$- and $α_{s2}$-casein, but hardly hydrolyzes κ-casein (Zhang et al., 2018). AprX, on the other hand, as described in **Section 3.1**, readily hydrolyzes κ-casein besides being able to hydrolyze β- and $α_s$-casein. In plasmin-containing samples, the breakdown of many casein-casein and casein-calcium phosphate interaction sites inhibits the formation of a

strong gel (Rauh et al., 2014a), whereas the cleavage of κ-casein by AprX does lead to a strong, compact gel.

Table 3. A summary of differences between AprX- and plasmin-induced changes in UHT milk, mainly based on Datta & Deeth (2003) and Zhang et al. (2018).

Enzyme	Specificity	State of milk	Change in particle size	Hydrolysis patterns				Hysrolysis products
				κ-casein	α$_{s2}$-casein	Preference towards A1 β-casein	Preference towards α$_{s1}$-casein 9P	
AprX	None	Compact, curd-like gel	Only increase, but no decrease	✓	Hardly	✗	✗	Small, soluble in 12% TCA
Plasmin	Lysine and Arginine	A soft gel that can be physically resuspended, or a translucent appearance	Both increase and decrease	✗	✓	✓	✓	Large, hardly soluble in 12% TCA

α$_{s1}$-casein 9P: α$_{s1}$-casein with 9 phosphorylations.

In dairy manufacturing, plasmin was mostly reported to be responsible for proteolysis of directly heated UHT milk products, because of a lower heat load than that is achieved with indirect heating. This plasmin survival may be solved by inclusion of a preheating step at around 85-95 ℃ for around 3 minutes or by increasing the temperature during UHT treatment (Topcu, Numanoglu & Saldaml, 2006). AprX, which exhibits a higher heat resistance than plasmin is, on the other hand, implicated in the spoilage of both directly and indirectly heated UHT milk products (Datta & Deeth, 2003; Stoeckel et al., 2016b). In addition, AprX may also influence plasmin activity by affecting plasmin localization due to disrupting the casein micelles (Fajardo-Lira & Nielsen, 1998; Fajardo-Lira, Oria, Hayes & Nielsen, 2000), and by enhancing the catalytic activity of plasminogen activator (Frohbieter, Ismail, Nielsen & Hayes, 2005).

3.3   Comparison between Ser2 and AprX

Recent studies have shown that *Pseudomonas* is not the only psychrotrophic genus frequently encountered in raw milk with the capacity to secrete a heat-resistant protease. The genus *Serratia*, especially the species *Serratia liquefaciens*, is also frequently found as a psychrotrophic contaminant in raw milk (Machado et al., 2015; Baglinière et al., 2017a, b), and it has been shown to secrete a heat-resistant spoilage-inducing metalloprotease of approximately 52 kDa encoded by the *Ser2* gene. The protease was therefore named Ser2. Because both AprX and Ser2 are from the serralysin subfamily of bacterial zinc-

metalloproteases, they share a high degree of homology in protein structure, and biochemical properties (Machado et al., 2016).

To assess the heterogeneity among Ser2, Machado et al. (2016) sequenced the *ser2* gene in 23 *Serratia liquefaciens* isolates from milk and the *aprX* gene in 8 *Pseudomonas* isolates in milk. One hundred percent similarity was found among the Ser2 sequences, whereas a large heterogeneity was found for the AprX sequences. Despite the 100% sequence similarity of Ser2, high variability in milk spoilage potential was found, suggesting that the differences in proteolytic ability for *Serratia liquefaciens* can be explained by differences in enzyme expression levels or post-translational modifications.

Baglinière et al. (2017a, b) compared the hydrolysis patterns of caseins by Ser2 and AprX. They found Ser2, like AprX, could hydrolyze β-, $α_s$-, and κ-caseins in UHT milk, thereby destabilizing casein micelles. Comparison of the peptide patterns of UHT milk samples destabilized by AprX and Ser2 showed similarities, suggesting that both do not have a strong specificity to certain amino acids. These findings further confirm the many similarities among these two members of the serralysin subfamily of bacterial zinc-metalloproteases.

4. Detection of AprX

Currently, the detection of AprX is mainly based on either of four principles: 1) the detection of the *aprX* gene; 2) the detection of the AprX protein; 3) the direct detection of AprX enzymatic activity; and 4) the indirect detection of AprX enzymatic activity (by detecting the peptides formed). The principle, reaction condition, detection limit, and application potential of these four detection approaches are explained in detail in this section and summarized in **Table 4**. Detection strategies that could be used for AprX detection in UHT milk are also proposed.

4.1   AprX detection approaches

4.1.1   Genetic detection of the *aprX* gene

Genetic methods, especially PCR, have been considered as the 'gold standard' method for bacterial identification (Vithanage, 2017). Recently, many researchers also have tried to develop a screening test for detecting the *aprX* gene (Bach, Hartmann, Schloter & Munch, 2001; Martins et al., 2005; Marchand et al., 2009a; Machado, Bazzolli, &Vanetti, 2013). The principle of this method is determining the presence of the *aprX* gene in bacterial cells as an indirect indicator of the spoilage potential using specific primers.

Bach et al. (2001) first indicated that PCR primers targeting the genes *apr, npr*, and *sub* could be used for detection of a wide range of proteolytic bacteria, as these genes are all part of the same genomic region as explained in **Section 1.1**. Later on, Martins et al. (2005) found that the *apr* gene could be directly amplified from pasteurized milk inoculated with 10^8 cfu/mL of *Pseudomonas fluorescens*. The detection limit could be improved to 10^5 cfu/ml in reconstituted skim milk powder if cells were recovered for DNA extraction before amplification. An even lower detection limit (10^3 cfu/mL in reconstituted skim milk) was reported by Marchand et al. (2009a), when amplifying a larger fragment from the *aprX* gene. In 2013, Machado et al. (2013) modified the filtration method for total DNA extraction and developed a multiplex PCR assay to detect multiple target protease genes such as *aprX, apr,* and *ser* simultaneously. This method allowed a detection limit of 10^2 cfu/mL of *Pseudomonas fluorescens* inoculated into sterilized whole milk. However, in raw milk, this *Pseudomonas fluorescens*-specific fragment could only be detected when the count of *Pseudomonas* was 10^7 cfu/mL, which was explained by the wider range of microflora present in raw milk.

Despite the PCR technique being regarded as a powerful tool for the analysis of large numbers of samples (Martins et al., 2005), practical applications of PCR for AprX detection remain difficult. First, the detection limit of currently available methods is higher than the maximum total bacterial count (10^4-10^5 cfu/mL) in many industrial standards; the sensitivity should thus be improved, for example by choosing the right target gene fragment and primer sets. Second, DNA extraction from milk could introduce inhibitory substances (i.e. Ca^{2+}, fat, proteases), which may affect the PCR amplification (Ramesh, Padmapriya, Chrashekar & Varadaraj, 2002; Martins et al., 2005; Dufour et al., 2008); therefore, the extraction of total DNA from milk samples should be optimized;

Evaluating the proteolytic potential by solely detecting the genes encoding extracellular proteases might be inadequate, because the presence of the gene per se does not indicate its expression or activity (Dufour et al., 2008); therefore the PCR method should ideally be combined with other detection methods.

4.1.2   The detection of oxygen tension

As mentioned in **Section 1.2.1.2**, the oxygen tension in the medium drops sharply prior to the onset of AprX production. Therefore, measurement of oxygen tension may have predictive value in determining the imminent production of AprX in milk. Rowe and Gilmour (1986) used a dissolved oxygen meter to monitor the oxygen tension of raw milk in both a fermenter with

constant aeration and agitation, and an orbital incubator without aeration. The same large decrease of oxygen tension was observed in both systems. The method of measuring oxygen tension is as fast and inexpensive as pH value determination, and may also allow continuous monitoring. In addition, the detection of oxygen tension has the advantage of early detection of AprX production, because the decrease of oxygen tension occurs before detectable AprX production.

4.1.3   Detection of the AprX protein

The method of direct detecting the AprX protein has been developed mainly based on enzyme-linked immunosorbent assay (ELISA). Monoclonal antibodies were designed on the basis of the protease structure, and the signal can be detected in a test solution in proportion to the amount of AprX (Birkeland, Stepaniak & Sørhaug, 1985; Clements RS, Wyatt, Symons & Ewings 1990; Matta, Punj & Kanwar, 1997). These ELISA assays have been shown to be rapid, sensitive, specific and have the ability to test a large number of samples without interference of milk proteins. A broadening of the specificity can be accomplished by designing a mixture of defined antibodies, whereas an increased sensitivity may be achieved with more concentrated conjugate preparations, extended substrate incubation, and the introduction of fluorescent substrates (Birkeland et al., 1985).

4.1.4   Direct detection of AprX enzymatic activity

Measuring AprX activity directly can be achieved by means of skim milk agar diffusion, casein zymography, and spectrophotometric, fluorometric, luminometric assays.

The skim milk agar diffusion method is more suitable for the screening of presence of caseinolytic proteases than quantitatively measuring the proteolytic activity. Casein zymography can visualize AprX activity by appearing as clear bands on a blue background on SDS-PAGE gels (Nicodème et al., 2005; Stuknytė et al., 2016). Despite the low cost and simplicity of these two methods, the low sensitivity and the long detection time make these techniques unsuitable for routine analysis (Vandooren, Geurts, Martens, Van den Steen & Opdenakker, 2013). Radiometric and bioluminescence-based assays have also been tested for measuring protease activity, but utilizing radioactive materials requires careful handling and specialist instrumentation (Christen, 1987), and bioluminescent assays are tedious and time-consuming (Sutherland, 1993), so these methods may not be easily performed in a dairy factory so far.

The spectrophotometric and fluorometric assays used for AprX activity measurement are based on the detection of either the hydrolysis of milk proteins, or the hydrolysis of externally added substrates. TNBS and OPA methods have been widely used for determining the degree of hydrolysis of milk protein (Marchand et al., 2009b; Chove, Abdulsudi & Lewis 2013; Zhang et al., 2018), by determining the number of free amino groups. Compared with the TNBS assay, the reagents used in the OPA method are more stable, and less toxic (Nielsen, Petersen & Dambmann, 2001). Therefore, the OPA method has been more frequently used in detecting AprX activity (Baur et al., 2015; Stoeckel et al., 2016a). Another approach is determining breakdown of an external substrate. Azocasein and FITC-casein assays are sensitive to the action of AprX in milk, because of the involvement of casein derivatives, rather than alternative external substrates. Hydrolysis of azocasein or FITC-casein by AprX results in the formation of chromogenic or fluorescent components whose intensity is a function of AprX activity (Charney & Tomarelli, 1947). The main advantages of spectrophotometric and fluorometric methods are the easily accessible equipment and relatively simple experimental operation. However, these methods are not sensitive enough to detect trace levels of AprX, such as present in UHT milk, although the incubation time and temperature can be optimized to improve the sensitivity.

4.1.5   Indirect detection of AprX enzymatic activity

Instead of measuring the enzymatic activity directly, characterizing the degradation of milk proteins by approaches like ELISA and RP-HPLC is an indirect strategy to detect AprX activity.

AprX has been shown to cleave κ-casein at the Phe_{105}-Met_{106} bond and produce CMP. Antibodies against the specific peptide bond or hydrolytic product have been developed and may be used for detecting this specific cleavage (Picard, Plard, Rongdaux-Gaida & Collin, 1994; Dupont, Lugand, Rolet-Repecaud & Degelaen, 2007), although AprX is known to further cleave CMP into smaller peptides.

The specific breakdown products generated during the milk protein hydrolysis by AprX can be identified using RP-HPLC or LC/MS. Different protein and peptide profiles were derived from whole milk with AprX (Matéos et al., 2015; Zhang et al., 2018) or its pH 4.6 and TCA-soluble fraction (Datta & Deeth, 2003). Even though such data can be used to diagnose the cause of proteolysis (Datta & Deeth, 2003), RP-HPLC and other chromatographic methods are too complex for routine analysis, and no suitable standards have been found so far to quantify protease activity.

4.2   AprX detection strategies for UHT milk

The previous section dealt with several detection approaches for AprX. One of the most important applications of AprX detection is to predict the stability of UHT milk. There is a great need for developing reliable methods for its detection, at both raw milk and final product level, to predict the stability of UHT milk. A fast detection of the initial proteolytic activity and/or AprX level should be implemented as soon as the milk is received at the processing plant. Knowing the total proteolytic activity in raw milk can provide information for choosing the specific heating method to apply. Meanwhile, considering the variation in heat resistance of among AprX from different *Pseudomonas* strains, determination of the AprX level in the final product right after processing is also needed, in order to determine if the products can be destined for export, for which a shelf life of 1 year at temperatures of 30-50 ℃ is desired (Stoeckel et al., 2016b).

Comparing the above-mentioned detection methods, the multiplex PCR assay and ELISA have the greatest potential for the detection of a broad range of psychrotrophs and potential proteolytic enzymes in both initial or final product by using multiple primers and antibodies. These two methods can also test a large number of samples concurrently. However, the current PCR and ELISA methods need to be improved in the aspects of shortening the analysis time, increasing the sensitivity, and reducing the interference from milk components to meet the requirement of practical applications. During storage, more time-consuming, but more informative, methods like RP-HPLC can be adopted to monitor the changes occurring in UHT milk.

Table 4. Summary of methods of AprX detection in milk.

Detection assay	Detection target		Reaction condition	Detection limit	Reference
Molecular methods	Using PCR assay to determine target genes		Predicted size of PCR product:194bp; Primer designed by Bach (2001); 30 cycles (94 ℃ for 1 min, 55 ℃ for 30s; 72 ℃ for 30s), 72 ℃ for 10 min	10^8 cfu/mL in pasteurized milk, and 10^5 cfu/mL in 12% (w/v) reconstituted skim milk when cells were recovered for DNA extraction before amplification	Martins et al. 2005
			Predicted size of PCR product:800bp; Primer set: SM2F/SM3R; 95 ℃ for 5 min, 30 cycles (95 ℃ for 30 s, 60 ℃ for 30 s and 72 ℃ for 1 min), 72 ℃ for 8 min	10^3 cfu/mL in 10% reconstituted skim milk; 10^4 cfu/mL in milk with an excess of bacterial contaminants (>10^6 cfu/mL)	Marchand et al. 2009a
			Predicted size of PCR product:850bp; Primer set: 16SPSEfluF/16SPSER; 94 ℃ for 2 min, 35 cycles (94 ℃ for 1min, 55 ℃ for 40 s and 72 ℃ for 1 min), 72 ℃ for 7 min	10^2 cfu/mL in sterilised whole milk; 10^7 cfu/mL in raw milk	Machado, 2015
ELISA	The detection of AprX protein	Antibody of *Pseudomonas fluorescens* P1 protease	37 ℃, 1 h (sandwich ELISA)	0.25 ng/mL or 10^6-10^7 cfu/mL in diluted skim milk	Birkeland et al. 1985
		Antibody of proteases of *Pseudomonas fluorescens* OM82, N73A, M143A, and OM186	RT, 6 h (inhibition ELISA)	0.24-7.8 ng/mL milk	Clements et al. 1990
		Antibody of protease of *Pseudomonas spp.* AFT-36	RT, 2.5 h (dot-ELISA)	1.01 ng/mL in buffer or milk	Matta et al. 1997
	The detection of hydrolyzed products	Detection of Phe_{105}-Met_{106} peptide bond of the κ-casein	37 ℃, 4.17 h	15.3 ng κ-casein/mL milk	Dupont et al. 2007
		Detection of CMP	37 ℃, 7.5 h	0.1 μg CMP/mL	Picard et al. 1994
Dissolved oxygen meter	Oxygen tension		7℃，real-time	Not specified	Rowe & Gilmour, 1986
Skim milk agar diffusion	The clear zones on skim milk agar		28 ℃, 72 h	Not specified	Zhang et al. 2015

Detection assay		Detection target	Reaction condition	Detection limit	Reference
Casein zymography		Active protease appears as clear bands on a blue background on SDS-PAGE	Not specified	3 μg	Nicodème et al. 2005
			37 ℃, overnight	Not specified	Stuknytė et al. 2016
Spectrophotometric	TNBS	The release of α-amino groups reacting with the TNBS reagent (a yellow-orange colour)	25 ℃, 30 min (420 nm)	Not specified	Chove et al. 2013
			37 ℃, two weeks (420 nm)	Not specified	Marchand et al. 2009b
	azocasein	The released azo dye	37 ℃, 1 h (366 nm)	Not specified	Dufour et al. 2008
			37 ℃, 8 h (440 nm)	Not specified	Alves et al. 2016
	Casein/OPA assay	The results obtained with the azocasein assay ($\Delta A\ h^{-1}\ mg^{-1}$ AprX) and OPA assay (the newly-formed amino groups from Na-caseinate and β-casein) are correlated	Azocasein: 40 ℃, 2 h (450 nm); OPA: preheat 37 ℃ for 5 min, 4 ℃ for 10 min, 37 ℃, 1min (340 nm)	Not specified	Stoeckel et al. 2016a; Baur et al. 2015
Fluorometric		Hydrolysis of the casein releases the FITC group, which can be detected by fluorescence spectrophotometer	37 ℃, 1 h (excitation 485 nm, emission 530 nm)	0.001-0.25 units/mL in buffer and 0.001-0.2 units/mL in raw milk	Sutherland, 1993
Luminometric		The bioluminescence emitted by the luciferase-luciferin system	25 ℃, 5 min	0.03-0.25 unit/mL in buffer and 0.125-0.5 units/mL in diluted raw milk	
RP-HPLC		Detection of the protein or peptides profile	41 min (214 nm)	Not specified	Zhang et al. 2018
			90 min (215 nm)	Not specified	Matéos et al. 2015
			30 min (210 nm)	Not specified	Datta & Deeth, 2003

5. Ways to control AprX in UHT milk

Minimizing the undesirable effects of AprX and other heat-resistant enzymes produced by psychrotrophs can be carried out at various stages of the UHT milk production chain, mainly using these three strategies: hygiene, cooling, and thermization. These management strategies are discussed in this section.

5.1   Hygiene

High bacterial counts and improper temperature are prerequisites for the mass production of bacterial proteases (Vithanage, 2017). Therefore, keeping psychrotroph populations low is of utmost importance for controlling enzyme production (Law et al., 1977). To achieve a superior microbiological quality, it is a must to implement good hygiene practices at both farm and industry levels and to reduce post-processing contamination (Martin, Boor & Wiedmann, 2018).

Biofilms are particularly blamed for enhancing thermo-resistant enzyme-producing psychrotrophs, because biofilms can act as a reservoir of these bacteria, protecting them from the activity of detergents and sanitizers (Teh et al., 2011). High densities of bacterial cells can be trapped in such biofilms, as discussed in **Section 1.2.1.1**, the high cell density will provoke quorum sensing and induce the production of proteases, including AprX. For this reason, uncontaminated raw milk is not synonymous with unspoiled final product, and all of the equipment coming in contact with the raw milk should be adequately cleaned and disinfected to prevent the formation of biofilms.

5.2   Cooling

According to European microbiological requirements described in Council Directive 92/46/EEC (Council Directives 1992) and Regulation (EC) No 853/2004 (Regulation 2004), raw milk intended for heat-treated drinking milk production must not exceed 1.0×10^5 cfu/mL of total plate count. Following this technological requirement can effectively reduce the secretion of proteases in bulk tank milk, but the final product quality may still be compromised if the proteases have been produced in part of the raw milk which was not collected and/or cooled under proper conditions.

A rapid precooling of milk is necessary because milk leaves the udder at approximately 35 ℃, which is a favorable temperature for bacterial growth. If the milk is not precooled before it enters the bulk tank, the warm milk will cause an increased temperature in the tank and thereby

induce bacterial growth and AprX production. AprX produced in the tank can then lead to protein hydrolysis of the raw milk.

Besides precooling, efforts in refrigerated storage and short collection intervals should be made. As discussed in **Section 1.2.1.1**, at refrigeration temperatures AprX may be produced at lower bacterial counts and at an earlier stage of bacterial growth, so it is possible that AprX can still be produced in the milk tank, even if cooling is applied. Alves et al. (2018) found that the refrigeration temperatures at which raw milk is usually stored, are not favorable for AprX production, but still not sufficient to completely prevent enzyme production if the initial psychrotrophic bacterial count is above 10^3 cfu/mL. Therefore, if bacterial quality is low, shorter collection intervals may be needed to prevent AprX synthesis (Lafarge et al., 2004).

5.3   Thermization

Large dairy companies always need to store raw milk in silos under refrigerated conditions for several hours or days prior to processing into dairy products. Raw milk is therefore subjected to thermization. Since most psychrotrophic bacteria are heat-labile (Senyk, Zall & Shipe, 1982), the thermization process (57-68 ℃ for 15-30 s or 63-65 ℃ for 15 s is most common) can be expected to kill most of the psychrotrophs in raw milk (Fairbairn & Law, 1986; Matselis & Roussis, 1998), and thereby inhibit the proliferation of psychrotrophs as well as the production of heat-stable enzymes. Rapid cooling to $\leq$ 4 ℃ should follow thermization. It has been observed that the combination of thermization, rapid cooling and storing at 2 ℃ was very effective in maintaining the quality of stored milk prior to further processing for several days (Griffiths, Phillips & Muir, 1987; Vithanage, 2017).

6. Future perspectives

This review summarizes the pioneering work on the extracellular protease AprX produced by pseudomonads. A better understanding and control of AprX may contribute to producing superior-quality fluid milk with longer shelf life, and it may bring overall benefits to the dairy industry. The key future challenges and research needs are outlined below.

Biological properties of the protease AprX: More insights into the crystalline structure of AprX must be gained in the future in order to study structure-function relationships. Knowledge thereof with clarify the molecular basis of its activity and relationships to substrates, and help to design specific antibodies for AprX detection using ELISA.

Additionally, the growth of other psychrotrophs and their production of enzymes at low temperature, as well as the resistance of these enzymes at different UHT intensities, should be studied more closely, to be able to assess the extent of the problems that may be induced by these other bacteria regarding protein destabilization.

Proteolytic potential of AprX: Given the inconsistent conclusions drawn from different approaches, to better quantitatively discriminate the AprX preference towards milk proteins, a combined use of mass-spectrometric, chromatographic, and electrophoretic techniques is suggested. Protein degradation kinetics, and both the sequences as well as abundances of peptides formed, can be exploited in further investigation. In addition, limited research has been done about the cleavage specificity of AprX towards amino acids; a more systematic analysis would thus need to be done with more up-to-date mass-spectrometric detection methods.

Beyond this, from a practical point of view, kinetic models depending on the AprX level, inactivation during processing, and temperature (fluctuations) during storage could be developed. Using such modeling, milk protein degradation can be predicted and eliminated by selecting processes for specific contamination levels as well as storage requirements.

Relationships between AprX and instability of UHT milk: Besides age gelation, for non-skimmed UHT milk as an emulsion, its shelf life can also be compromised by fat separation. Theoretically, it can be assumed that in the presence of residual activity of AprX, not only the caseins in milk plasma, but also the caseins on the milk fat globules will be hydrolyzed during storage of UHT milk. The proteolysis of the fat surface-adsorbed proteins may subsequently destabilize the fat globules, although such effects have not been studied yet in UHT milk. Better insights in the linkages between protein and fat stabilization of UHT milk may help in better understanding these two phenomena that are the two main factors limiting UHT milk shelf life quality.

Detection of AprX: Even though many approaches have been tested for the detection of AprX, none of them is suitable for application in practice yet. In this respect, developing more rapid and sensitive methods to monitor microorganisms with proteolytic spoilage potential is a pressing need. Except for the methods discussed in **Section 4**, some other methods have potential for assaying either the activity or the concentration of AprX in milk.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS), as a rapid, reliable, and cost-effective identification method, was recently developed for the detection of protease activity by using a peptide-encoded microplate to measure the

cleavage products. Even though AprX has a broader specificity than trypsin and chymotrypsin, peptide profiling using MALDI-TOF-MS, in combination with multivariate statistical techniques, has potential for future use in AprX activity detection.

FTIR spectroscopy has been widely used in the food industry as a simple and rapid detection method with minimum sample preparation. It has been proved to be able to indicate physicochemical changes during the shelf life of both skim and full-fat milk (Grewal et al., 2017a, b), because FTIR is sensitive to the changes in covalent bonding and non-covalent electrostatic and hydrophobic interactions, which are believed to be involved in protease-induced aggregation and gelation. Even though a published report is currently lacking, there is potential that AprX activity can also give apparent spectral changes in FTIR signal, like chymosin and metalloprotease Prt1 from *Pectobacterium carotovorum* (Baum, Hansen, Nørgaard, Sørensen & Mikkelsen, 2016).

Furthermore, besides AprX, there are more proteases which can remain active after heat treatment. Thus, the dairy industry needs to develop a method, on the basis of AprX detection, to estimate the overall proteolytic activity covering as many origins of proteases as possible.

Ways to control AprX in UHT milk: For the control of psychrotrophs, even though biofilms are one of the main recontamination sources of milk, there is little knowledge on avoiding contamination sources. Innovative cleaning and disinfection techniques are needed.

In addition to control bacteria in the first place, specific emphasis on developing innovative strategies for inactivating bacterial proteases is recommended. As soon as more knowledge is available on the sensitivity of AprX to different treatments, the use of new time/temperature conditions, such as LTI and innovative steam injection, or high-pressure homogenization, pulsed electric field, and ultrasound, may have potential for enzyme inactivation. Further research can be done to explore the feasibility of applying these methods for commercial-scale dairy processing.

7. Summary

Overall, raw milk is a favorable medium for the growth of pseudomonads and the production of the protease AprX by these bacteria. AprX is remarkably physiologically and genetically adaptable, which, together with its flexible structure, make it resistant to various conditions, including UHT processing. Despite some quantitative variations in the AprX proteolytic potential among the *Pseudomonas* species, all AprX molecules from proteolytic strains can

hydrolyze κ-, β- and α-caseins. AprX is particularly blamed for inducing solid and compact gels in UHT milk by specifically hydrolyzing κ-casein. To reduce the risks of destabilization of UHT milk during storage, many approaches have been tried for detecting AprX. Among all the methods, multiplex PCR assay and ELISA have the greatest potential, although both have not been implemented in practice yet as improvements are still needed. In addition, at this time, AprX spoilage is mainly controlled by implementing good hygiene practices, low storage temperature (at farm/ transport/ factory levels), and thermization.

Chapter 3

Destabilization of UHT Milk by protease AprX from *Pseudomonas fluorescens* and plasmin

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Abstract

Destabilization of UHT milk during its shelf life is mainly promoted by the residual proteolytic activity attributed to the psychrotrophic bacterial proteases and native milk proteases. In this study, we built skim UHT milk-based model systems to which either the major bacterial protease (AprX from *Pseudomonas fluorescens*), or the major native milk protease (plasmin) was added, to allow a direct comparison between the destabilization of skim UHT milk by both categories of enzymes. The physical and chemical properties were studied during 6 weeks. Our results showed AprX induced compact gels when almost all the κ-casein was hydrolyzed and the degree of hydrolysis (DH) exceeded 1.3%. Plasmin induced soft gels when around 60% of both β- and α_{s1}-casein were hydrolyzed and the DH reached 2.1%. The knowledge gained from this study may be used for developing diagnostic tests for determining the protease responsible for UHT milk destabilisation.

1. Introduction

The demand for ultra-high-temperature (UHT) processed and aseptically packaged milk is increasing worldwide. UHT milk is the best choice of liquid dairy products for many developing and tropical countries because it does not required cooled logistics and storage, and has a relatively long shelf life (≥ 6 months). These features also well facilitate its compatibility with the commercial exploitation in international trade for dairy exporting countries.

In spite of the broad market for UHT milk, it can be subject to a range of undesirable changes, such as age gelation and fat separation during its shelf life (Datta & Deeth, 2001). The onset of sedimentation, age gelation and, sometimes, a bitter taste is promoted by the proteolytic activity due to residual enzymes, which can survive the UHT treatment and remain active during storage (Manji & Kakuda, 1988; Datta et al., 2001; Rauh et al., 2014b).

Milk contains a large number of native enzymes with differing specificity, stability and impact on product quality (Kelly & Fox, 2006). Plasmin (EC 3.4.21.7), with its zymogen plasminogen and other parts of the complex enzyme system, constitute the major native protease system in milk, and has been reported to be correlated with udder health, as indicated by the somatic cell count (SCC) (Ramos, Costa, Pinto, Pinto & Abreu, 2015; Musayeva, Sederevičius, Želvytė, Monkevičienė, Beliavska-aleksiejūnė & Stankevičius, 2016). This protease system exhibits a high thermal stability and can remain partially active after the UHT treatment; therefore, the plasmin system has been closely linked to physicochemical deterioration of UHT milk (Kohlmann, Nielsen & Ladisch, 1991; Kelly & Foley, 1997; Rauh et al., 2014b).

In addition to the well-known detrimental effects of plasmin, enzymes originating from psychrotrophic bacteria can also be a serious problem in UHT milk because these bacteria are inevitable in raw milk and some of them can produce heat-resistant proteases and lipases during cold storage, that can withstand the UHT process. Among all the psychrotrophic bacteria, *Pseudomonas* species are particularly incriminated in the destabilisation of UHT milk (Vithanage, Yeager, Jadhav, Palombo & Datta, 2014). A single specific extracellular alkaline metallo-protease belonging to the AprX enzyme family has been discovered in genus *Pseudomonas*, which is responsible for milk spoilage (Vithanage et al., 2014; Matéos et al., 2015). The heat-stable proteases have been reported to be produced by pseudomonads during the late exponential/early stationary growth phase of the bacteria, generally at bacterial cell counts of 10^7-10^8 cfu/mL (Stoeckel, Lidolt, Stressler, Fischer, Wenning & Hinrichs, 2016b). This means that the production of AprX is determined by the storage time, temperature, and the

count of pseudomonads. Therefore, the AprX level indirectly reflects the hygienic management of the farm and the storage history of the milk.

Cow health, hygiene management and storage history of milk are all crucial links in the dairy chain that can influence the stability of UHT milk. To better trace back which links needs to be improved to prevent instability, we need knowledge of the responsible enzymes, especially with regard to the differences between them in their proteolytic activities on milk proteins, and the changes they induce in milk. However, to date, such systematic understanding of these enzymes systems is still lacking.

The mechanisms for age gelation of UHT milk have been mainly described by two theories (Kocak & Zadow, 1985a; McMahon, 1996; Datta et al., 2001). The first involves the enzymatic degradation of the milk proteins, and the promotion of age gelation by the formation of peptides. The second mechanism is often referred to as "non-enzymatic" or "physicochemical" age gelation in which no protein degradation is observed (McMahon, 1996; Anema, 2017). Age gelation through the physicochemical mechanism is slow for unconcentrated milk samples, in which it usually takes longer than 12 months (Anema, 2017). Therefore, the focus of this study is the enzyme-induced destabilization. To reduce the interference from the non-enzymatic physicochemical changes, visible destabilization was induced within a relatively short time by the addition of high concentrations of enzymes.

This study aims to provide insights in the differences of the hydrolytic process on milk proteins between AprX and plasmin. To assess this, skim UHT milk samples to which different concentrations of AprX or plasmin were added, and were stored for 6 weeks at both room temperature and the optimal temperatures for both proteases. During this period, the differences in the visual deterioration of milk, physico-chemical modifications and hydrolysis patterns of caseins were studied.

2. Materials and Methods

2.1 Enzymes

The AprX-producing bacterial strain *Pseudomonas fluorescens* Migula 1895 (DSM 50120) was obtained from Deutsche Sammlung von Mikroorganismen (DSM). This strain has been reported to be able to grow at 4-37 ℃ and have proteolytic, lipolytic and pectinolytic activities. The AprX sequence was found in the gene with an 15 bp insertion between bp position 395 and 410 on the sequence, proving the dairy origin of this isolate (Caldera et al., 2015). For cultivation, the strain was cultured in a nutrient broth (VWR International B.V.) to the end of

its log phase (around 26 h) at 25 ℃. The bacteria were then harvested by centrifugation (4,000 g, 10 min, 20 ℃). To purify the extracellular enzymes, we inoculated the bacteria in minimal medium (7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 1 g/L $(NH_4)_2SO_4$, 4 g/L glycerol, pH 7.0) containing 2% (v/v) UHT milk as protease inducer for 24 h at 25 ℃ with stirring at 160 rpm (Matéos et al., 2015). After 24 h of culturing, cells were removed by centrifugation at 10,800 g for 30 min at 4 ℃ (rotor JA 16.250, Avanti Centrifuge J-26 XP, Beckman Coulter, USA). The supernatant was first concentrated by centrifuging at 4,000 g for 20 min at 4 ℃ in Amicon Ultra filters (10 kDa cut-off, Millipore), after which the concentrate obtained was dialysed against sterile 10 mM potassium phosphate buffer (pH 7.0) at 4 ℃ for 48 h (3.5 K, Thermo Scientific Slide-A-Lyzer™ Dialysis Cassette) and lyophilised. No further purification of the extracellular bacterial enzymes was performed, because the crude AprX extract is more similar to the real situation in milk, which facilitates our simulation of the real destabilization caused by pseudomonads.

Plasmin from bovine plasma was purchased from Roche, 5 units of plasmin were suspended in 3.2 mol/L ammonium sulfate solution, where the unit is defined at 25 ℃ with Chromozym PL as the substrate.

2.2   Milk sample preparation

Commercial skim UHT milk was purchased from a local supermarket, the milk was subjected to direct sterilization by steam infusion heat treatment. The protein and fat content was 3.76% and 0.07% (w/v) respectively, analyzed by MilkoScan 134A/B (Foss Electric, Hillerød, Denmark). To keep a low starting degree of hydrolysis, we used milk samples that were not older than 1 month after manufacture. To prevent spoilage during storage, 0.02% sodium azide and 0.0005% bronopol were added as preservatives.

In a preliminary study, the enzyme concentrations were determined, to allow both model systems to gel in around 1 month at room temperature. In the main experiment, the AprX extract was accurately weighed into skim UHT milk samples at the concentrations of 0, 10, 20, 50 μg/mL, the samples were aliquoted in flat-bottomed screw-top Turbiscan tubes and centrifugation tubes in an upright position, and incubated in dark without agitation at room temperature and 42 ℃, respectively. Likewise, plasmin was added at the concentrations of 0, 0.8, 1.6, 2.4 μL/mL, and the samples were incubated at room temperature and 37 ℃, respectively. All the samples were analysed at 1 week intervals over 6 weeks of storage.

2.3   Dynamic light scattering (DLS)

The average hydrodynamic particle size was determined using a Zeta-sizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) and the associated DST1070 disposable folded capillary cell. Samples were diluted 1/50 in milliQ water prior to the measurement. All the measurements were performed in triplicate at 25 ℃. The refractive index was set to 1.45 for particles and to 1.354 for the dispersant. Since the AprX-induced gelled samples could not be mixed homogeneously by vortexing, the size distribution of AprX-containing samples upon and after gelation was not determined.

2.4   Characterization of stability by Turbiscan

Sample stability was monitored using the optical analyzer Turbiscan MA 2000 (Formulaction, Ramonville St. Agne, France) under gravity force. The back scattering intensity were measured as a function of height under a near-infrared light source at 880 nm. It was carried out in 100 mm tall borosilicate glass tubes with 12 mm inner diameter. The apparatus scanned at 2000 acquisitions/scan in 3 s (i.e. 1 acquisition each 40 μm) at room temperature. The thickness of sediment (mm) is the length where the sample's backscattering intensity is higher than the blank.

2.5   Determination of protein degree of hydrolysis

DH values were determined in triplicate using o-phthaldialdehyde (OPA, Sigma) assay. The OPA reagent was prepared as described by Wierenga, Meinders, Egmond, Voragen & de Jongh (2003). Samples were diluted 2 times in a 2% (w/v) SDS solution, stirred for 20 min, and stored at 4 ℃ overnight to fully solubilise the peptides and the possibly present intact protein. The samples were then diluted 5 times in milliQ water aliquots (50 μL) for adjusting the concentration within the linear range of the standard curve, and were added to 1500 μL of the reagent solution in a cuvette and equilibrated for 10 min at room temperature. The presence of alkylisoindoles formed by the reaction of free amino groups with OPA was measured by the absorbance of the sample at 340 nm. To calculate the amount of free NH_2 groups, a calibration curve was made using leucine as a reference compound, the number of free NH_2 groups per gram samples was expressed as h. The total number of peptide bonds per gram of protein substrate was obtained by complete hydrolysis of the blank skim UHT sample in 6 mol/L HCl, 110 ℃ for 24h; h_{tot} was 8.68 mmol/g in this case. The detected DH value of the blank UHT milk was 7.0%, this value originated from native lysines and the N-termini of the milk proteins, as well as the hydrolysis that had happened in the milk prior to our experiment. This value was considered as the "blank" DH in milk and was subtracted from all the other data. The additional

amino groups from the added AprX and plasmin were also subtracted accordingly, but the number of peptide bonds originating from the autolysis of proteases were neglected because the level of autolysis differs in a system without caseins as substrates, and can thus not be properly assessed. In this way, the DH discussed here refers to the hydrolysis caused merely by the added proteases, and was calculated as DH = (h/h$_{tot}$*100%)-7.0%. The OPA reagent in the presence of SDS could sufficiently dissolve plasmin-induced gelled samples, but not the AprX-induced gelled samples. Therefore the AprX-containing samples were only analysed until 1 week before gelation happened.

2.6   Protein Profile Analysis

2.6.1   RP-HPLC

The intact protein composition was measured by Reversed Phase High Pressure Liquid Chromatography (RP-HPLC, Thermo Scientific™ UltiMate 3000) equipped with an Aeris Widepore 3.6 μm XB-C18 column, 250×4.6 mm (Phenomenex, Utrecht, the Netherlands), according to the method described by de Vries et al. (2015). Protein standards (β-casein, α$_S$-casein, κ-casein, α-lactalbumin and β-lactoglobulin; purities 70–85%, all from Sigma-Aldrich) were used to validate the elution times of milk proteins. The resulting chromatograms were analysed through the software Chromeleon 7.1.2. We determined the total peak areas of β-, α$_{s1}$ and κ-casein, and assume the peaks with same retention time are intact caseins. However, we could not quantitatively describe the development in α$_{s2}$-casein due to the co-elution of the peaks of this protein with other breakdown products, as also explained by Rauh et al. (2014b). The buffers in the presence of DL-dithiothreitol and urea could sufficiently dissolve plasmin-induced gelled samples, but not the AprX-induced gelled samples, so again the AprX-containing samples were analysed until 1 week before gelation happened.

2.6.2   Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed to complement RP-HPLC results. The sample buffer, reducing agent, gels, running buffer and antioxidant agent were all purchased from Invitrogen (Carlsbad, USA). Two μL sample was diluted in 5 μL 4× concentrated NuPAGE® LDS sample buffer, 2 μL NuPAGE® sample reducing agent and 15 μL MilliQ water. Then the mixture was centrifuged at 425 *g* for 1 min and heated at 70 ℃ in a heating block (Labtherm Graphit, Liebisch, Germany) for 10 min. Samples were then loaded onto NuPAGE® Novex® 4–12% Bis–Tris Gels. The BlueRay Prestained Protein Marker 10-180 kDa (Jena, Germany) was

applied as a reference. The running buffer was NuPAGE® MES running buffer, 0.5 mL NuPage® antioxidant was added to the running buffer. Electrophoresis was performed using an XCell SureLock™ unit (Invitrogen, Paisley, UK) at constant voltage (120 V). The gels were then Coomassie-stained.

3. Results

3.1 Physical changes during storage

3.1.1 Appearance and stability

The stability of the samples was monitored both visually and with a Turbiscan analysis. During 6 weeks of storage, the blank UHT milk remained liquid and homogeneous, and no sign of destabilization was observed (**Fig. 1a**). Visible differences in the structure of gels were observed between AprX and plasmin containing UHT milk samples, the gelled samples containing 20 µg/mL AprX and 1.6 µL/mL plasmin are shown as examples (**Fig. 1a, 1b**).

In AprX containing gelled samples, the gel-like sediment became more solid along with the liquid phase becoming less opaque (**Fig. 1a**). The formed coagulation would remain intact at the bottom when inverting the tube (**Fig. 1b**), showing that the structure of the gel was compact and firm.

In plasmin containing samples, the destabilization appeared as a floating white soft gel (**Fig. 1a**). Unlike the firm gel induced by AprX, the plasmin-induced gel was soft and fragile, it would not stay at the bottom when inverting the tube (**Fig. 1b**), and the flowy gel can be easily dispersed in the samples therefore allowed the subsequent physical and chemical measurements after gelation. In addition, the clarification was more obvious in plasmin-containing gelled samples, as revealed by the lower backscattering intensity by Turbiscan (**Fig. 1c**).

Gelation could be visually distinguished when the thickness of the sediment was higher than 8 mm in the backscattering profile by Turbiscan, and the onset of gelation was thus determined accordingly (**Fig. S1**). The samples with the addition of 20 µg/mL AprX and 1.6 µL/mL plasmin both gelled in the fourth week, when stored at room temperature. With increasing enzyme concentration, and increased storage temperature, the time necessary to observe visible milk gelation shortened.
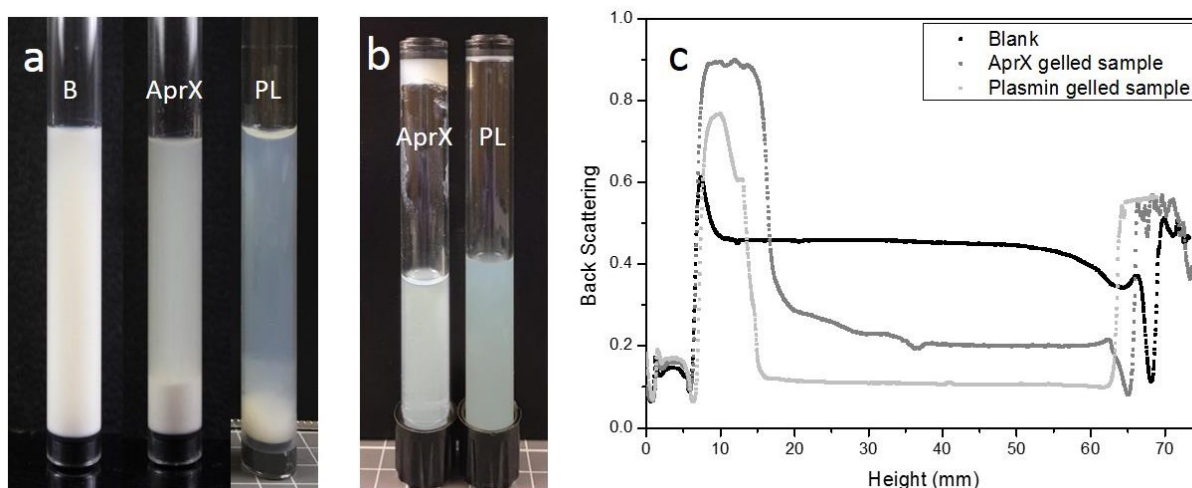
Fig. 1. Images of the blank sample, the gelled samples containing 20 µg/mL AprX and 1.6 µL/mL plasmin (a); the image of the inverted tubes of gelled samples containing 20 µg/mL AprX and 1.6 µL/mL plasmin (b); the corresponding backscattering intensity profiles of samples in (a), the horizontal axis represents the position along the tube (c).

3.1.2   Size distribution

Along with data from the Turbiscan, the physical stability of protease containing UHT milk samples was also characterised by the size distribution of the casein micelles and the agglomerates that may be generated. The change in size distribution was quicker with higher concentrations and temperatures, but the trend was the same for the individual enzymes. The results of samples with the addition of 20 µg/mL AprX and 1.6 µL/mL plasmin at room temperature are shown as examples (**Fig. 2a, 2b**).

The skim UHT milks (week 0) without incubation were determined to have a monomodal particles size distribution with an average particle size of around 200 nm, which corresponds to the normal casein micelle size (McMahon & Oommen, 2013) (**Fig. 2a, 2b**). For the UHT milks containing AprX (**Fig. 2a**), the peak corresponding to regular casein micelles diminished gradually until disappearance during storage at room temperature. After 1 week of storage, the distribution became bimodal. The size distribution of the second peak ranged from 500 nm to microns, and the size distribution further broadened to larger sizes with increased storage time. Similar trends in size distribution were found in all the one-week-before-gelled AprX containing samples, as shown in **Fig. S2a**.
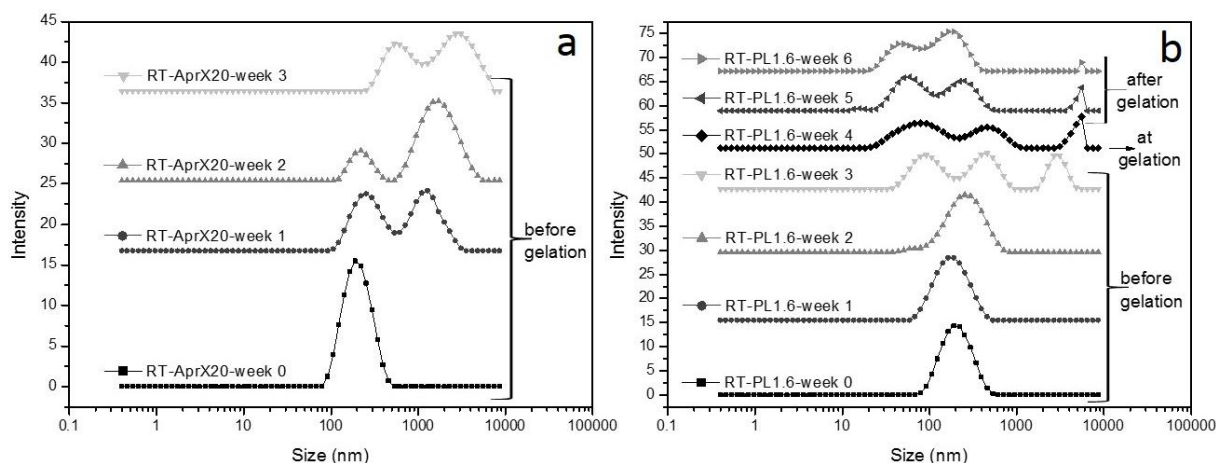
Fig. 2. Intensity based particle size distribution of samples containing 20 µg/mL AprX stored from 0 to 3 weeks at room temperature (a); and samples containing 1.6 µL/mL plasmin from 0 to 6 weeks at room temperature (b).

The example of changes in size distribution in plasmin containing samples is shown in **Fig. 2b**. After a slight shift to a larger mean size in week 2, the main peak below 1,000 nm split into two parts in week 3, which is one week before gelation, and a new peak at micron size arose. Soon after this, the size distribution became even wider at gelation, with the peaks of small particles moving to smaller sizes, and at the same time peaks of particles at micron size further developing to larger sizes. The particles larger than 10 µm in size might also exist, but were outside the detection range of the equipment. Gelation and clarification could be observed along with these changes of size distribution. Similar trends were found in all the other gelled plasmin containing samples; the comparisons of size distribution before and at the onset of visible gelation are shown in **Fig. S2b, S2c**. After gelation (week 5 and 6 in **Fig. 2b**), the peaks of small particles below 1,000 nm shifted to smaller size over time and the peak at microns lowered.

3.2   Chemical changes during storage

3.2.1   Changes in degree of hydrolysis
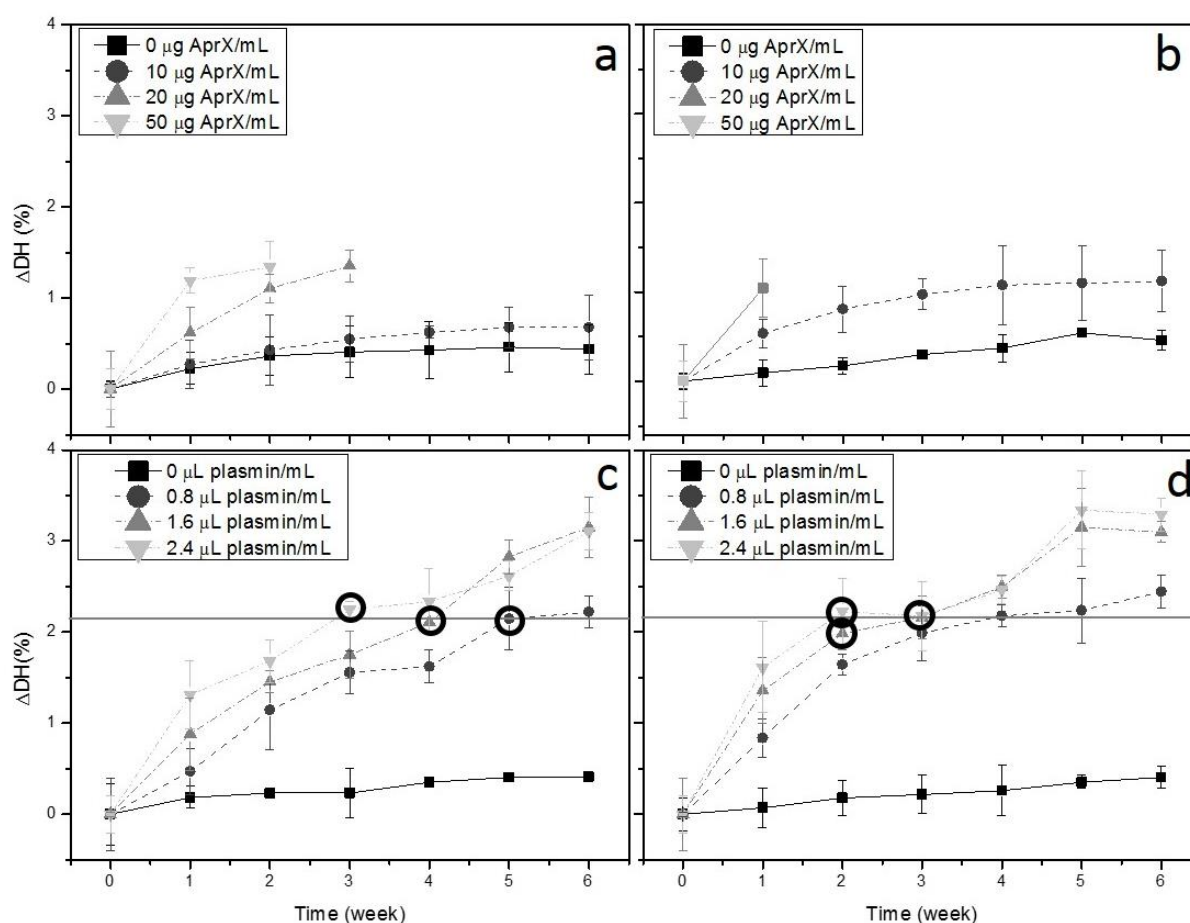
Fig. 3. Degree of hydrolysis values of skim UHT milk hydrolyzed by 0, 10, 20, 50 μg/mL AprX at room temperature (a) and 42 ˚C (b); and by 0, 0.8, 1.6, 2.4 μL/mL plasmin at room temperature (c) and 37 ˚C (d). The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH values. Error bars represent the standard deviations.

To provide an overall description of the activity of these two proteases on milk proteins, we studied the DH by OPA. Upon incubation, DH increased in the presence of AprX and plasmin. For both proteases, a specific DH was reached at the onset of gelation. When the DH in AprX containing samples increased beyond 1.3%, the samples gelled, irrespective of storage time or temperature (**Fig. 3a, 3b**). Likewise, in plasmin containing samples, there is also a "limit DH" of 2.1%, again irrespective of storage time or temperature (**Fig. 3c, 3d**).

3.2.2   Correlation of casein hydrolysis and UHT instability
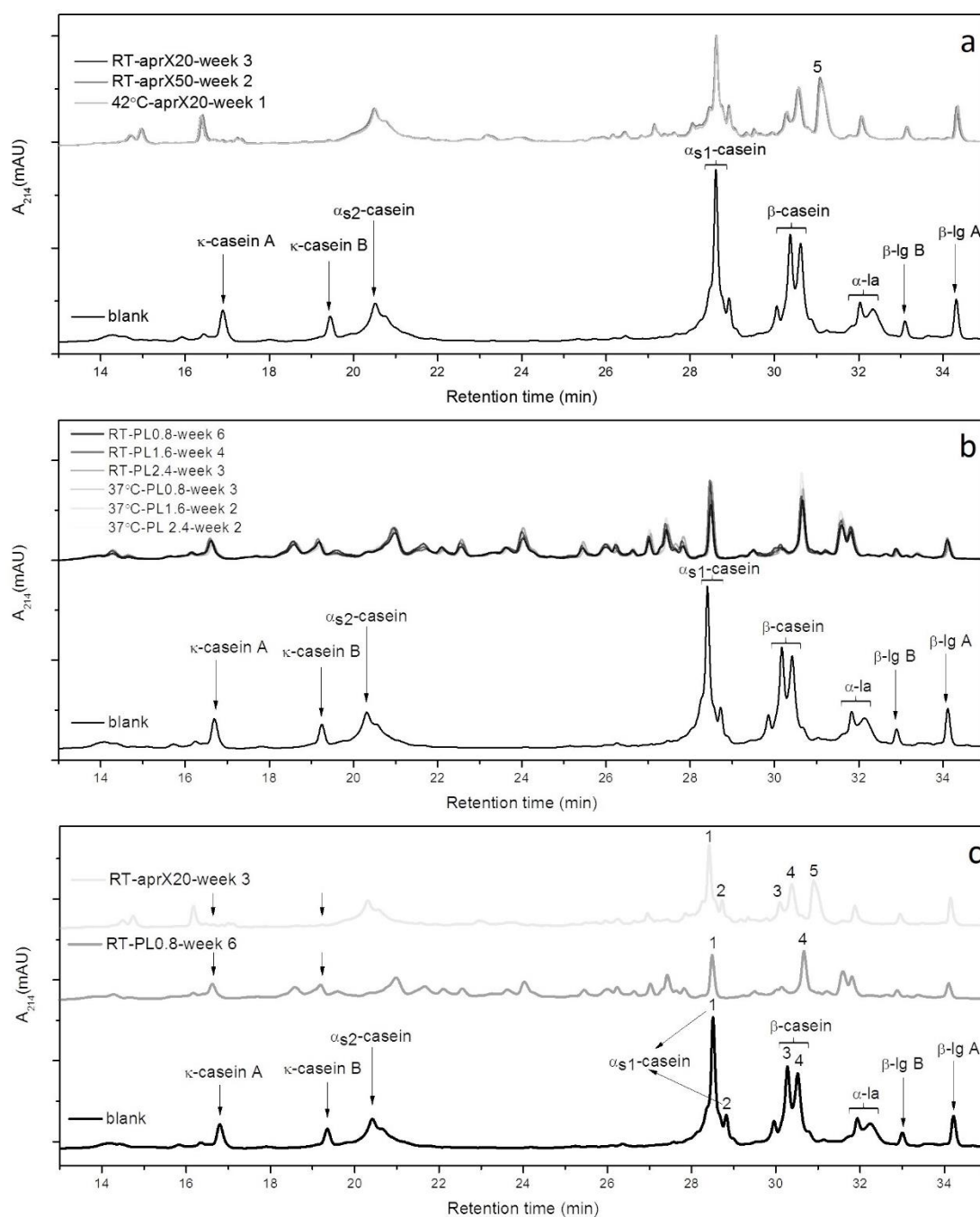
Fig. 4. The comparison of RP-HPLC chromatograms between the AprX-induced gelation samples and plasmin-induced gelation samples. Peak 1, 2 stands for α_{s1}-casein with 8 or 9 phosphorylations (8P & 9P), respectively; peak 3, 4 stands for β-casein A1 & A2, respectively; peak 5 stands for the unique peak in AprX-hydrolyzed samples.

Fig. 4 illustrates the comparison of protein profiles by RP-HPLC in both the blank UHT milk and the samples showing gelation due to the action of AprX or plasmin. As shown in the chromatograms, the gelled samples in both model systems went through extensive proteolysis,

and many breakdown products were generated, as reflected by the increase of various unidentified peaks in the HPLC profiles during incubation. What also stands out in **Fig. 4** is that for the respective model system with the addition of the same enzyme, the chromatograms of the gelled samples looked identical independent of temperature and protease concentration. Comparing the hydrolysis patterns between AprX and plasmin, the most apparent differences are in κ-casein, α_{s2}-casein, and differences in peaks 1-5. The two κ-casein peaks almost completely disappeared during the incubation with AprX, whereas these peaks did not decrease markedly in the samples gelled by plasmin. Besides, plasmin led to more peaks of breakdown products of α_{s2}-casein than AprX. In addition, we observed significant differences in the peak development of α_{s1}-casein with 8 and 9 phosphorylations (8P & 9P, peak 1 and 2 in **Fig. 4**) as well as for A^1 and A^2 β-casein (peak 3 and 4 in **Fig. 4**). In plasmin containing samples, peaks of A^1β -casein and α_{s1}-casein 9P disappeared at the onset of gelation, indicating that these had been almost completely hydrolyzed when gelation occurred. AprX, on the other hand, doesn't show such preference towards α_{s1}-casein 8P & 9P, and A^1 & A^2 β-casein. Moreover, a unique peak at a retention time of 31 min was found only in the AprX-hydrolyzed samples, the nature of this peak will be studied in future studies.

To understand how the hydrolysis of certain caseins can quantitatively affect the stability of UHT milk during storage, we determined the changes of the peak areas of β-, α_{s1} and κ-casein in AprX- and plasmin-containing samples during storage at room temperature (**Fig. 5**). As shown in **Fig. 5a-c**, AprX hydrolyzes κ-> β-> α_{s1}-casein, gelation occurred after more than 45% of β-casein, 35% of α_{s1}-casein and 95% of κ-casein was hydrolyzed, respectively. While, as shown in **Fig. 5d-f**, plasmin mainly hydrolyzes β-, α_{s1}-caseins, but hardly κ-casein. The intact β-casein and α_{s1}-casein fraction decreased in an exponential manner during storage. At the onset of gelation, more than 60% of both β-and α_{s1}-casein were hydrolyzed. The protein degradation at the optimal temperature of the enzymes was similar with that at room temperature (**Fig. S3**).

Hydrolysis of caseins was also analysed by SDS-PAGE (**Fig. S4**). The band intensities of β- and κ-casein diminished faster than the band of α_s–casein, indicating κ-casein and β-casein were more rapidly hydrolyzed. These results are in line with our RP-HPLC results. At the same time, many new bands at lower molecular weight appeared, as can be expected during proteolysis.
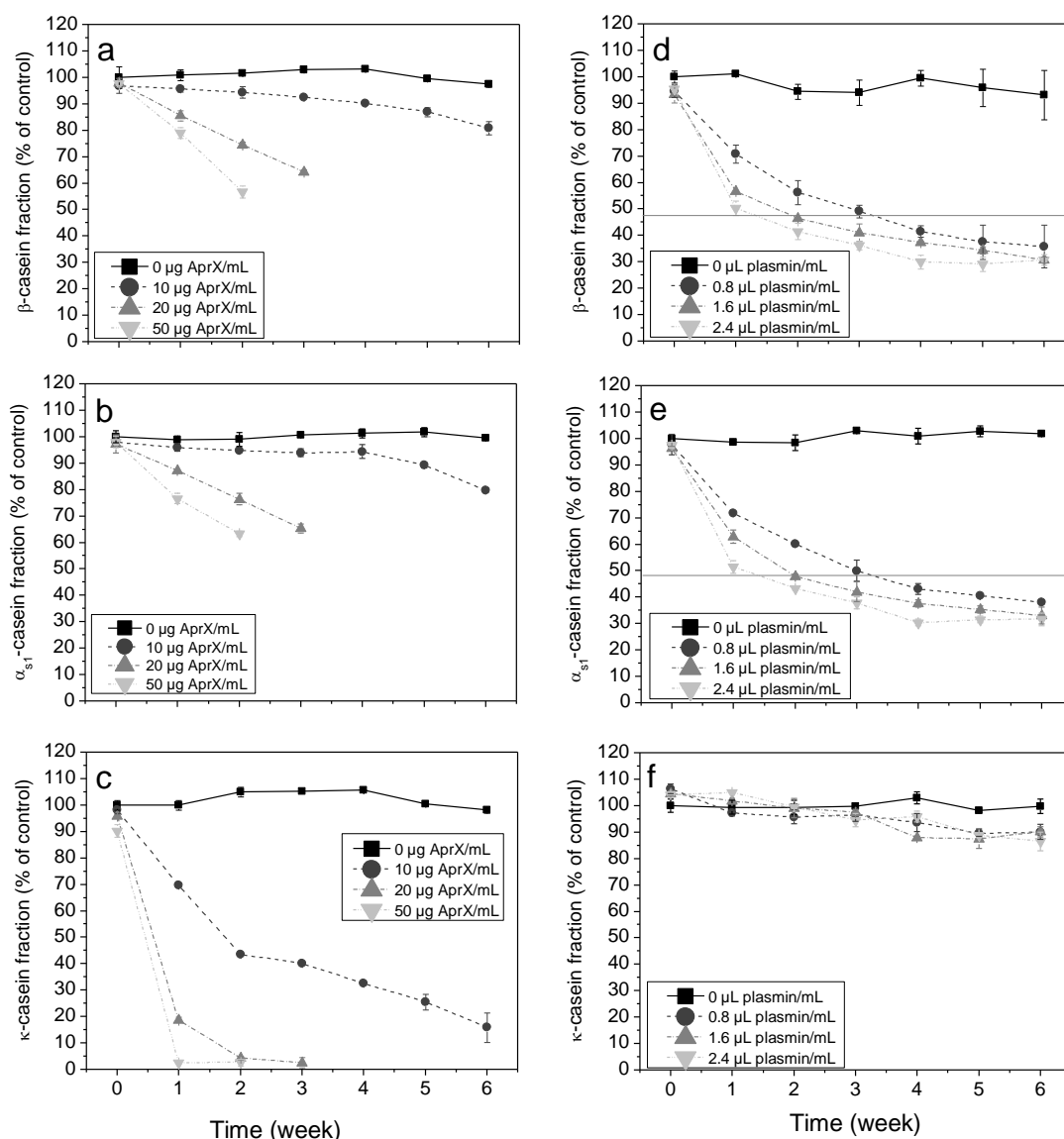
Fig. 5. Hydrolysis of β-casein (a), α_{s1}-casein (b) and κ-casein (c) in samples containing 0, 10, 20, 50 µg/mL AprX during storage at room temperature; and in samples containing 0, 0.8, 1.6, 2.4 µL/mL plasmin at room temperature (d, e, f), expressed as the relative change in peak area of UV absorption at 214 nm. The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH relative peak area values. Error bars represent the standard deviations.

4. Discussion

This study compared the destabilization of skim UHT milk by AprX and plasmin during 6-weeks storage at two temperatures. The findings showed that different specificities towards cleaving the caseins of AprX and plasmin on milk proteins are the main cause of different gelation behaviours during the storage of UHT milk.

4.1 Proteolysis and gelation

4.1.1 AprX-induced gelation

AprX can not only cleave the peptide bond Phe_{105}-Met_{106} of κ-casein like chymosin (Recio, García-risco, Ramos & López-fandiño, 2000), but also non-specifically cleave around the region of soluble hydrophilic glycomacropeptide that is normally present as the "hairy layer" (Gaucher et al., 2011). As a result, the cleavage of the hydrophilic tails on the surface of the micelles reduces both the steric and electrostatic repulsion, which may promote the formation of a gel network (Matéos et al., 2015). Additionally, the ability of hydrolyzing β- and $α_s$-casein, even though to a lower extent than plasmin (**Fig. 4, 5, S3, S4**), may also help destabilize the internal part of casein micelles, further stimulating gelation.

Comparing the changes in size distribution in AprX- and plasmin-containing UHT milk samples, we can see that, different from plasmin, under the action of AprX the size distribution immediately started to shift to larger sizes (**Fig. 2a, S2a**). This is probably because AprX can easily access and breakdown κ-casein. Once a sufficient amount of κ-casein has been hydrolyzed, the collisions between the particles will lead to adhesion and the formation of casein aggregates (Sandra, Alexander & Dalgleish, 2007). The level of hydrolysis of κ-casein at the onset of gelation is found to be more than 95% in our study (**Fig. 5c, S3c, S4a**), which is similar to cheese making, where extensive (80–90%) hydrolysis of κ-casein needs to occur before visible coagulation (Sandra et al., 2007). The AprX-induced gels could not be physically resuspended in the samples, which made analysis of these samples difficult. Further studies on the gel itself may aid in further understanding the mechanism of AprX-induced gelation.

4.1.2 Plasmin-induced gelation

Plasmin-induced gelation in UHT milk is more complicated compared with the AprX-induced gelation. According to our results, β-casein is most susceptible to plasmin action, followed by $α_{s1}$-casein and $α_{s2}$-casein (**Fig. 4**). Although κ-casein contains several lysine and arginine residues, it appears to be resistant to plasmin as shown before (Rauh, Johansen, Ipsen, Paulsson, Larsen & Hammershoj, 2014a; Fox, Uniacke-Lowe, McSweeney & O'Mahony, 2015). The final effect of plasmin depends on both the preference towards specific caseins as well as the dynamics of proteolysis. Combining our results with previous studies, the process of plasmin-induced gelation can be inferred as the following steps:

1) Penetrating phase: in our study, this phase corresponded to the first week for samples with 1.6 μL plasmin/mL at room temperature. During this phase, the size distribution

hardly changed (**Fig. 2b**), but the β- and α_s-caseins were rapidly hydrolyzed (**Fig. 5d, 5e**), along with an increase in DH (**Fig. 3c**). Plasmin will first hydrolyze the easily accessible β- and α_s-caseins, after which plasmin needs some time to penetrate the casein micelles for further hydrolyzing β- and α_s-caseins.

2) Loosening phase: this phase is characterised by a slight increase in the mean particle size (week 2 in **Fig. 2b**), due to loosening of the micellar structure (Rauh et al., 2014a). Rauh et al. (2014a) found that plasmin could hydrolyze around the regions that are essential for the internal integrity and stabilization of the casein micelle, thereby weakening the hydrophobic interactions between caseins, the interactions of caseins with calcium phosphate, and the ionic or salt interactions between caseins.

3) Disassembling, rearranging and aggregation phase: in this phase, sample becomes polydisperse; some particles increased in size, whereas others dropped, and at the same time a new peak at micron size appeared (week 3-4 in **Fig. 2b**). The larger particles might be underestimated due to their settling in the sample cell during DLS measurement. The increasing size corresponds to further loosening of the casein micelles, while the decrease is due to hydrolysis causing the interactions to be insufficient to maintain an intact micellar arrangement. During the disassembly, some generated amphiphilic and charged polypeptides tend to rearrange and aggregate into a gel network (Rauh et al., 2014a). Visible gelation can be observed when enough aggregates are formed.

4) Clarification phase: in this period, the peaks of aggregates decreased (week 5-6 in **Fig. 2b**) and the particles smaller than 1,000 nm further reduced to smaller sizes. Both the gel and the caseins in micellar structures will be further hydrolyzed by plasmin until a translucent peptide solution is obtained.

Upon gelation, A[1] β-casein and α_{s1}-casein 9P had been almost completely hydrolyzed by plasmin (**Fig. 5c**). Our finding is in accordance with Rauh et al. (2014b), who showed that more than 95% of A[1] β-casein and α_{s1}-casein 9P had been hydrolyzed upon gelation. Besides, Kelly et al. (1997), Kohlmann et al. (1991) and Newstead et al. (2006) also observed that β-casein was almost completely hydrolyzed when gelation happened, based on gel electrophoresis. Likewise, we also found that all the bands of β- and α_s-caseins disappeared upon gelation (**Fig. S4b, S4c**).

The structure of the plasmin-induced gel was soft and fragile. Similar physical properties of plasmin induced gels have been reported by Kohlmann, Nielsen & Ladisch (1988), Kelly et al.

(1997), Newstead et al. (2006) and Rauh et al. (2014b), but different from the re-dissolving of gel and the final translucent peptide solution found in our study.

4.1.3   Relationships between DH and the onset of gelation

Regarding the critical level of protein breakdown at which gelation occurs, we found the DH for AprX- and plasmin-containing samples to be ~1.3% and ~2.1%, respectively, irrespective of enzyme concentration or storage temperature. This means that there is a direct correlation between the time of onset of gelation and the level of proteolysis expressed as DH. A strong correlation between the extent of proteolysis and gelation time was also found by Keogh & Pettingill (1984), Newstead et al. (2006) and Rauh et al. (2014b), i.e. the onset of sedimentation and gelation occurred earlier in milk with greater enzyme activity. However, Kohlmann, Nielsen & Ladisch (1988), Manji et al. (1988) and Auldist et al. (1996) reported that the extent of proteolysis in UHT milk, especially long-term stored UHT milk, was not always related to the onset of gelation. In addition to the differences in protein matrix resulting from different processing conditions, these inconsistent findings may be the result of different dominating reactions in each study, i.e. the enzymatic proteolysis or the non-enzymatic physicochemical reactions. Glycation (or Maillard reaction) is the most important non-enzymatic reaction that can retard gelation. On the one hand, the crosslinking between proteins and reducing sugars can prevent the release of gel-forming peptides (McMahon, 1996); on the other hand, the enzymatic cleavage involving lysine residues is inhibited by the casein–lactose interactions. Therefore, glycation can influence the enzyme-induced gelation in UHT milk during long storage.

In the cases with high enzyme activities, like ours, and the study of Rauh et al. (2014a), gelation was observed within a relatively short period. During this period, the enzymatic hydrolysis is the leading reaction, and the slow non-enzymatic physicochemical changes would be insignificant; no obvious browning within 6 weeks indicated there was no advanced Maillard reaction (**Fig. 1**). In such a situation, the level of enzymatic hydrolysis is crucial for the critical point of gelation. In long-term stored UHT milk with low proteolytic activity, on the other hand, the non-enzymatic physicochemical changes, which are mainly influenced by the storage temperature (Manji et al., 1988), would become more important.

4.1.4   Comparison between AprX- and plasmin-induced gelation

The DH for the control samples also increased slightly with storage, although less than those with added AprX or plasmin (**Fig. 3**), indicating that the blank samples presumably contained a low level of indigenous plasmin activity that survived the direct UHT heating. The residual

enzymatic activity should not be from AprX because no κ-casein cleavage is seen in the control sample (**Fig. 5f, S3f**). Comparing the destabilization of UHT milk by AprX and plasmin, a lower critical DH (~1.3%) was found in AprX containing samples than plasmin containing samples (~2.1%) (**Fig. 3**). With AprX, the combination of hydrolyzing κ-casein together with β-casein can dissociate the hairy layer and allow AprX to penetrate micelles, thereby quickly destabilizing the micelles leading to a compact gel. By contrast, β- and α_s-casein are less responsible for the micellar stability than κ-casein. Therefore, a higher DH is required for plasmin to induce gelation by hydrolyzing β- and α_s-caseins, and the breakdown of many interaction sites prevent the formation of a strong gel.

4.2   Identification of the enzyme causing age gelation

To identify the responsible enzyme for age gelation, some approaches can be proposed based on the above results. The action of AprX is indicated by:

1) A compact and strong gel that cannot be physically resuspended;
2) Only increase, but no decrease in particle size distribution compared to normal milk;
3) A fast specific hydrolysis of κ-casein detected by chromatographic or electrophoretic approaches;

On the other hand, the action of the native milk protease, plasmin, is indicated by:

1) A soft gel that can be physically resuspended, or a translucent appearance;
2) Both increase and decrease in particle size distribution compared to normal milk;
3) The selective hydrolysis of $A^1\beta$ -casein and α_{s1}-casein 9P, as detected by HPLC.

The work of Datta & Deeth (2003) on diagnosing the cause of proteolysis in UHT milk showed the non-specific cleavage of caseins by AprX can produce smaller peptides, that are soluble in 12% TCA, compared to the lysine/ arginine specific protease plasmin (Datta & Deeth, 2003).

In addition to identification of the responsible enzyme, the stability of UHT milk can be predicted by measuring the enzyme activity after heat treatment. Previous research recommended limits for protease activity (Adams, Barach & Speck, 1975; Ewings, O'Connor & Mitchell, 1984) in differently defined units, or in enzyme amount in weight (Mitchell & Ewings, 1985). But differences in assay techniques make comparisons inaccurate, and it is difficult to detect low amounts of enzymes in milk using these approaches. Therefore, developing an easy-to-use immunological assay that uses a combination of antibody affinity ELISA plates and spectrophotometric quantification of the enzyme activity may be a more

suitable indicator for the stability of UHT milk. The shelf life can also be adjusted depending on the enzyme activity and environmental conditions, where shorter shelf life should be set for the products with higher enzyme activity and products stored at increased ambient temperature.

5. Conclusion

This study has shown that different forms of destabilization in skim UHT milk were caused by the protease AprX from *Pseudomonas fluorescens* and plasmin during storage. Higher concentrations of proteases and higher storage temperature lead to faster gelation, although the critical degrees of hydrolysis of specific caseins determines the onset of gelation, irrespective of protease concentration or storage temperature. The strong AprX-induced gelation in UHT milk is mainly caused by the hydrolysis of κ-casein, whereas the soft plasmin-induced gelation in UHT milk is mainly caused by the hydrolysis of β- and α-casein; AprX can thereby induce gelation at lower DH than plasmin.

Supplementary materials
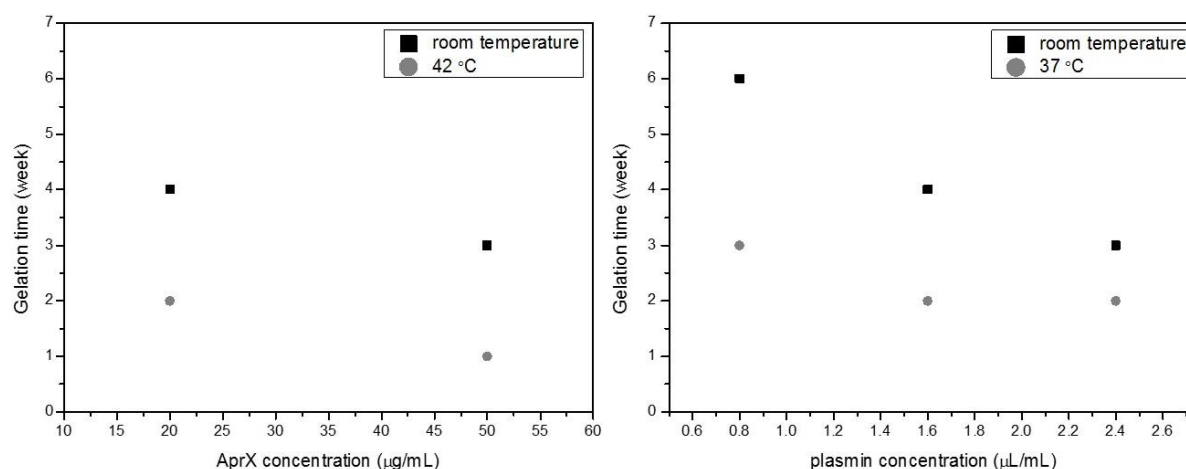
Fig. S1. The correlation between the AprX (a) and plasmin (b) concentration in UHT milk and the gelation time.
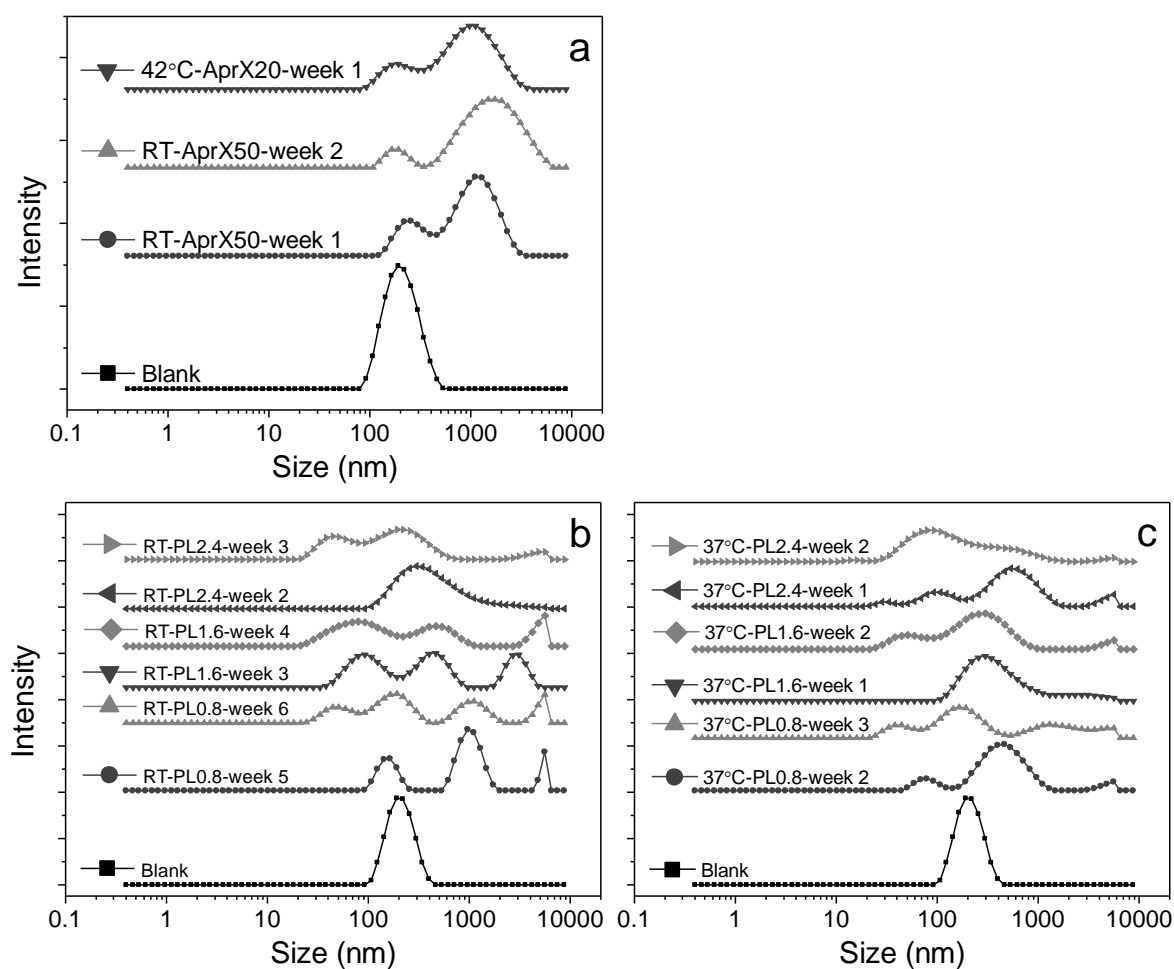
Fig. S2. Intensity based particle size distribution of samples containing 50 µg/mL AprX stored at room temperature and samples containing 20 µg/mL AprX stored at 42 ˚C before gelation (a); and samples containing 0, 0.8, 1.6, 2.4 µL/mL plasmin in 1 week before gelation and at the onset of gelation at room temperature (b) and 37 ˚C (c).
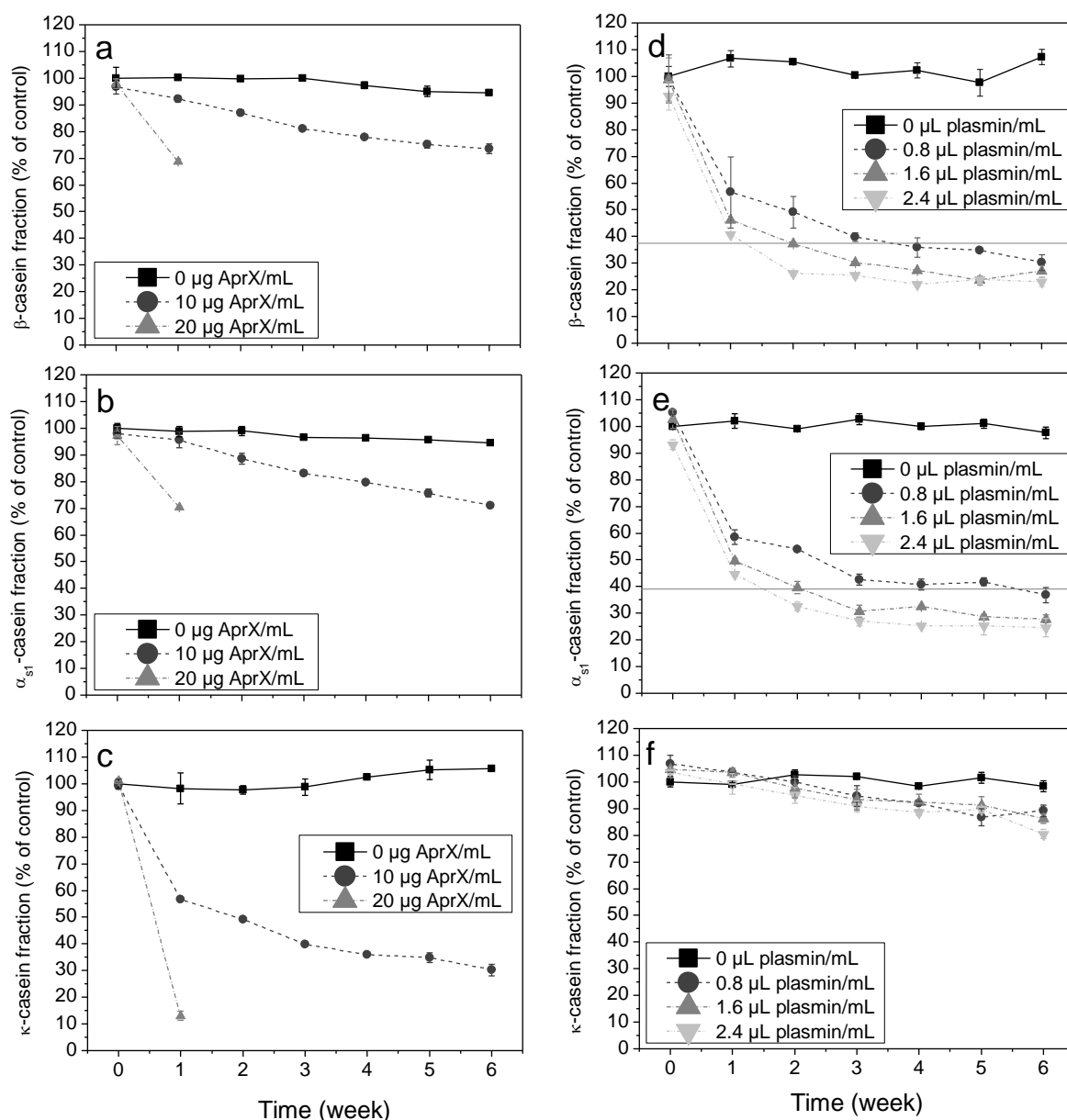
Fig. S3. Hydrolysis of β-casein (a), α_{s1}-casein (b) and κ-casein (c) in samples containing 0, 10, 20, 50 µg/mL AprX during storage at 42 ˚C; and in samples containing 0, 0.8, 1.6, 2.4 µL/mL plasmin at 37 ˚C (d, e, f), expressed as the relative change in peak area of UV absorption at 214 nm. The circled spots indicate the samples at the onset of gelation, the sublines crossing the circled spots are for guiding the eyes to the corresponding DH relative peak area values. Error bars represent the standard deviations.
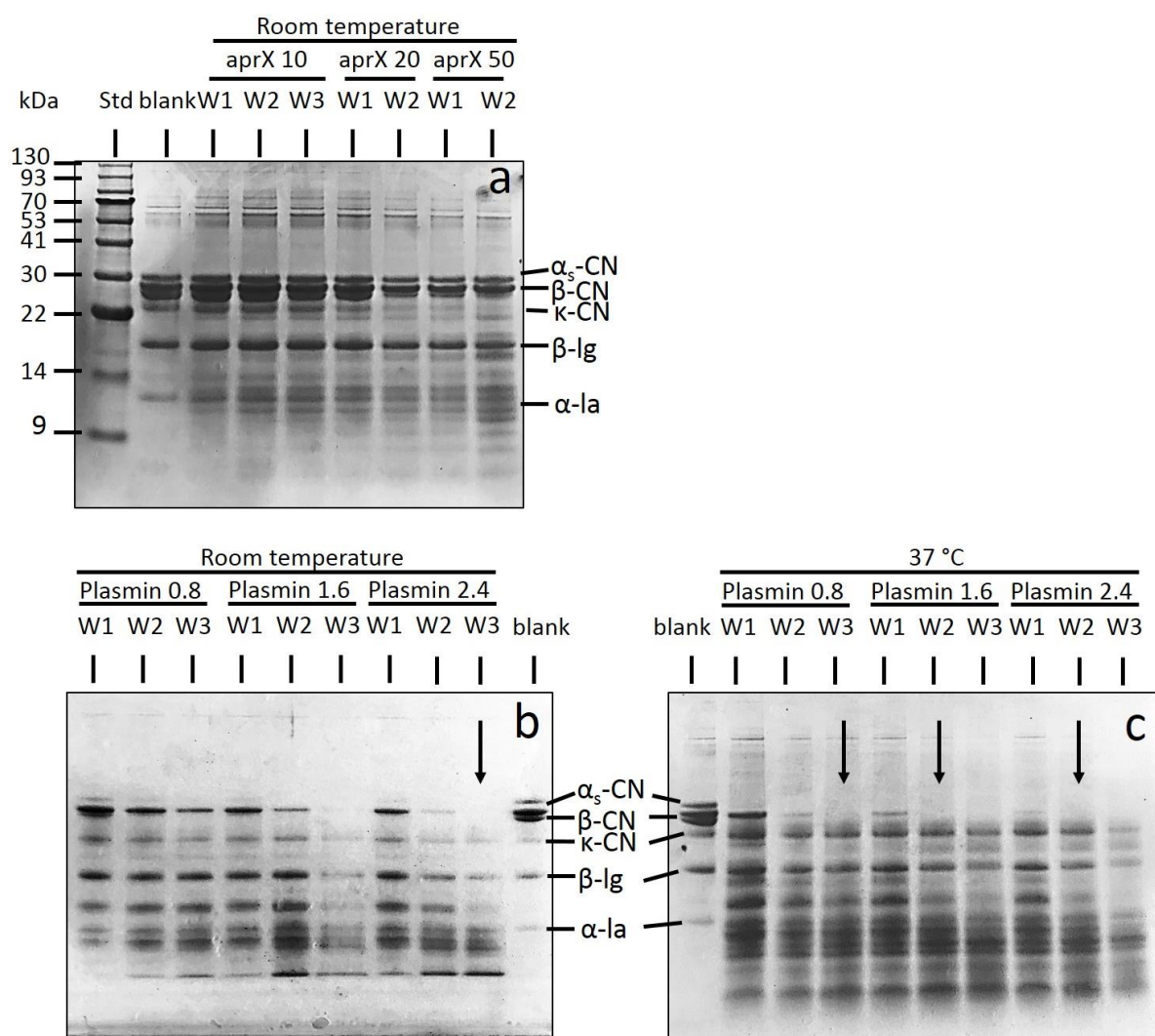
Fig. S4. SDS-PAGE gels of UHT milk hydrolyzed by AprX at room temperature (a); by plasmin at room temperature (b) and 37 ˚C (c). α_s-casein: α_{s1}+ α_{s2} casein; Std: molecular mass standards; blank: skim UHT milk without addition of protease; W: storage time (week); AprX 10, 20, 50: UHT milk containing 0, 10, 20, 50 µg/mL AprX; plasmin 0.8, 1.6, 2.4: UHT milk containing 0, 0.8, 1.6, 2.4 µL/mL plasmin. The arrows indicate the gelled samples.

Chapter 4

Stability of fat globules in UHT milk during proteolysis by protease AprX from *Pseudomonas fluorescens* and plasmin

Abstract

Fat separation is a limiting factor for the shelf life of UHT milk. It may be promoted by the proteolysis of the fat surface-adsorbed proteins (FSAP) by proteases that remain active after UHT treatment. The aim of this research was to explore the relationship between the proteolysis of FSAP and fat destabilization. In this study, we developed a full fat UHT milk-based model system to which either the major bacterial protease AprX from *Pseudomonas fluorescens* or the major native milk protease plasmin was added at high levels to induce fast destabilisation of the milk fat globules. The changes in physical properties, FSAP composition and the structural changes of the fat globules were monitored over 24 hours. Our results show that AprX induced sedimentation due to flocculation of fat globules, while plasmin induced cream to float due to coalescence of fat globules. This study confirmed that AprX and plasmin can indeed both lead to fat destabilization in full fat UHT milk, and provides insights in the underlying mechanisms.

1. Introduction

The consumption of ultra-high temperature (UHT)-processed milk is increasing worldwide because of its long shelf life and the possibility of long distance transport and storage at room temperature. However, its shelf life can be compromised by unwanted changes like age gelation and sedimentation. For full-fat and semi-skimmed UHT milk, another possible detrimental change during its storage is fat separation; the agglomeration of the fat globules with the formation of a floating fat layer , or a fat-rich protein aggregate that is dense enough to sediment (Hardham, Imison, & French, 2000; Chavan, Chavan, Khedkar, & Jana, 2011).

Fat globules in UHT milk are heated and homogenized. Homogenization causes the native milk fat globules to mechanically disrupt into smaller particles, after which the natural milk fat globule membrane material is insufficient to cover the larger fat globule surface area. Hence, the surface of fat globules is covered and stabilized by adsorbed milk proteins, primarily caseins (Cano-Ruiz & Richter, 1997), which we will call fat-surface adsorbed proteins (FSAP). Fat separation during shelf life of UHT milk has been commonly recognized to be caused by insufficient homogenisation (Ramsey & Swartzel, 1984; Hardham et al., 2000). If fat globules do not achieve sufficient homogenization, a small number of fat globules will remain large and represent a significant volume of the total fat; these large globules can rise rapidly and cause a significant cream layer (Wilbey, 2011).

However, it is assumed that nowadays, adequate homogenization is used in most commercial UHT production processes, thus insufficient homogenisation should not be a major issue. In UHT milk that has been adequately homogenized, proteolysis of FSAP could be the main cause of fat instability during storage. Such proteolysis of UHT milk may be due to the residual activity of heat-stable proteases, which are mainly bacterial proteases, represented by AprX, and native milk proteases, represented by the plasmin system. AprX is a caseinolytic extracellular alkaline metalloprotease produced by *Pseudomonas* species (Vithanage, Yeager, Jadhav, Palombo, & Datta, 2014; Matéos et al., 2015), with κ-casein being most susceptible to its action, followed by β- and α_{s1}-casein (Recio, García-risco, Ramos, & López-fandiño, 2000; Zhang, Bijl, & Hettinga, 2018). Plasmin hydrolyses in the order β->α_{s1}->α_{s2}-casein, but hardly hydrolyses κ-casein (Rauh, Johansen, et al., 2014a; Zhang et al., 2018). In view of the differences in preferences of AprX and plasmin towards caseins, differences in AprX- and plasmin-induced protein destabilization of UHT milk, such as gelation and sedimentation, have been frequently reported (Rauh, Sundgren, et al., 2014b; Matéos et al., 2015; Zhang et al., 2018). However, the influence of protease on fat destabilisation has been studied much less. It has been

observed that in full-fat UHT milk, bacterial proteases lead to the formation of sediment or a custard-like gel (Visser, 1981; Harwalkar, 1992), whereas native proteases cause a creamy surface layer (Kohlmann, Nielsen, & Ladisch, 1991; Hardham, 1998; Rauh, Sundgren, et al., 2014b). However, the underlying mechanisms of how these two categories of proteases destabilize fat globules in UHT milk have not been unequivocally elucidated.

This study thus aims to compare the differences between AprX and plasmin in hydrolysing FSAP and the subsequent consequences for fat stability in UHT milk. To assess this, full-fat UHT milk samples were hydrolysed with high levels of AprX or plasmin to induce fast destabilisation of milk fat globules.

2.   Materials and methods

2.1   Enzymatic hydrolysis

Commercial full-fat UHT milk was purchased from a local supermarket. The type of processing used on the UHT milk was direct sterilization by steam infusion heat treatment according to additional information obtained from the manufacturer. The protein and fat content of the UHT milk was 3.6% and 3.5% (w/v), respectively, as analysed by MilkoScan 134A/B (Foss Electric, Hillerød, Denmark). To have a low starting degree of hydrolysis, we used milk samples that were not older than 1 month after manufacturing. To prevent spoilage during storage, 0.02% sodium azide was added as preservative. The protease AprX was isolated from *Pseudomonas fluorescens migula* (DSM 50120, Deutsche Sammlung von Mikroorganismen) and purified as described by Zhang et al. (2018). Plasmin from bovine plasma was purchased from Sigma-Aldrich (Roche 10602370001), which was suspended in 3.2 mol/L ammonium sulfate solution.

The enzyme concentrations were determined in a preliminary study. Activity of 1 µl plasmin/mL milk and the equivalent activity of AprX were found to be able to induce obvious changes to fat stability in the model systems within 24 hours of incubation at 37 ℃. In the main experiment, crude AprX extract was weighed into full-fat UHT milk samples at a concentrations of 84 µg/mL, whose activity matched 1 µl plasmin/mL milk. The activity of plasmin and AprX was determined using the azocasein assay as described by Dufour et al. (2008). Samples were collected at 4 hour intervals over 24 hours of incubation at 37 ℃, after which the samples were directly used for physical stability measurement and FSAP analysis.

2.2   Characterization of physical stability during proteolysis

2.2.1   Measuring fat content in different layers

The samples for fat content measurement were placed in a standing position without disturbance. After incubation, as explained in **Section 2.1**, a pipette tip was slowly inserted along the inner wall of the tube until reaching the bottom without stirring the milk.

Then the same volume of the bottom, middle and top layer was collected successively from this vial. Lipid extraction from full-fat UHT milk samples was optimised based on the methods of Lu, Pickova, Vázquez-Gutiérrez, and Langton (2017), using a mixture of hexane and isopropanol (3:2, v/v). The milk sample was mixed with the organic solvent mixture at a ratio of 1:5 (v/v) in a capped Eppendorf tube. The tubes were incubated at 60 ℃ for 15 min with occasional gentle shaking to aid lipid extraction (Baümler, Crapiste, & Carelli, 2010). Then the upper organic phase was collected in a weighed tube, dried under a stream of nitrogen and weighed again, with the increase in weight used to calculate the fat content.

2.2.2   Stability analysis by LUMiFuge

The physical stability of the hydrolysed samples was examined with the LUMiFuge (L.U.M.GmbH, Berlin, Germany). LUMiFuge is an optical stability analyser which accelerates physical destabilization by centrifugal forces and exposes the sample cells to near infrared light while measuring the transmission continuously during the centrifugation. High concentrations of particles yield low transmission profiles and vice versa. Prior to analysis, but after incubation, the samples were diluted 10 times with simulated milk ultrafiltrate (SMUF, pH 6.8) to reduce the turbidity of full-fat UHT milk and facilitate the optical measurement. SMUF was prepared according to Jenness (1962), with the following composition: 11.6 mmol/L KH_2PO_4, 3.70 mmol/L $C_6H_5K_3O_7 \cdot H_2O$, 6.09 mmol/L $C_6H_5Na_3O_7 \cdot 2H_2O$, 1.03 mmol/L K_2SO_4, 8.05 mmol/L KCl, 8.97 mmol/L $CaCl_2 \cdot 2H_2O$, and 3.20 mmol/L $MgCl_2 \cdot 6H_2O$. The sample was then homogenized by gently shaking from side to side, after which 400 µL sample was inserted to LUMiFuge sample cells (LUM 2mm, PC, Rectangular synthetic cell (110-131xx), Berlin, Germany). The LUMiFuge measurements were carried out at 2300 *g* for 43 min at room temperature, and transmission was measured for 255 cycles with a duration of 10 s at a near-infrared wavelength of 865 nm. These parameters were set according to Sunds (2016).

To quantify the emulsion stability of the samples, the integrated transmission percentage against time was used. This will further be referred to as the "instability index" in this study. The

instability index was automatically calculated with the LUMiFuge software SEPView 6.3. Lower instability index values indicate better emulsion stability.

2.2.3   Size distribution of milk particles

Particle size distribution analysis was performed using laser light diffraction (Mastersizer 3000, Malvern Instruments, Ltd, Malvern, UK). Samples diluted 50 times with MilliQ water were injected directly into the dispersion cell under agitation at 1500 rpm. A higher stirring intensity was avoided to prevent disintegration of aggregates. The droplet size was calculated with a refractive index of 1.45 and 1.33 for the droplet and continuous phase (MilliQ water), respectively. The particle absorption index was set as 0.002.

2.2.4   Viscosity

The viscosity of the samples was measured in a stress controlled rheometer (MCR302, Anton Paar), using a double-gap concentric cylinder geometry (MCR 72). A solvent trap was used to prevent evaporation. The samples were stabilized for 2 min. Subsequently, the viscosity was monitored by exponentially increasing the shear rate from 0.01 to 500 s^{-1}. Measurements were performed at 20 ˚C.

2.3   FSAP isolation

The method was optimized based upon previously described methods by McPherson, Dash, and Kitchen (1984); McCrae, Hirst, Law, and Muir (1994) and Lu et al. (2016). The FSAP isolation method was divided into two parts, namely cream separating and washing. Cream was separated from full-fat UHT milk samples by ultracentrifugation at 60,000 g for 1 hour at 20 ˚C using an L60 Beckman ultracentrifuge (Beckman, Fullerton, CA). To aid the separation of fat droplets from milk serum, 0.6 mol/L sucrose was incorporated before centrifugation as density-increasing agent. Pre-experiments showed that after ultracentrifugation with the aid of sucrose, the cream layer of UHT milk samples was as thick as that of raw milk, indicating most milk fat ended up in the cream layer. Immediately after ultracentrifugation, the samples were immersed into an ice bath for 15 min to solidify the cream layer. The cream was then collected into a new tube and washed as described next. To wash away the proteins that are loosely attached or entrapped in the cream layer, the collected cream was washed in three steps: 1) submersion of the cream layer (at 1:10; w/v) in SMUF with added sucrose (35% w/v); 2) centrifugation at 21,000 g for 10 min at 10 ˚C; 3) discarding the washing solution, and collection of the cream layer. After repeating the 3-step washing procedure three times, the washed cream fraction was weighed, and the FSAP were analysed with RP-HPLC and SDS-PAGE.

2.4   Characterization of protein profile in FSAP during proteolysis

2.4.1   RP-HPLC

The protein composition was determined by Reversed Phase High Pressure Liquid Chromatography (RP-HPLC, Thermo Scientific™ UltiMate 3000) equipped with an Aeris Widepore 3.6 μm XB-C18 column, 250×4.6 mm (Phenomenex, Utrecht, the Netherlands), according to the method described by Zhang et al. (2018). Protein standards (β-casein, α_{S1}-casein, α_{S2}-casein, κ-casein, α-lactalbumin and β-lactoglobulin; all with purities of 70–98%, all from Sigma-Aldrich) were used to validate the elution times of milk proteins. The resulting chromatograms were analyzed by Chromeleon 7.1.2 software. The changes in α_{s2}-casein could not be quantitatively described due to the co-elution of the peaks of α_{s2}-casein with breakdown products of protein hydrolysis, as also explained by Rauh, Sundgren, et al. (2014a, b).

2.4.2   SDS-PAGE

SDS-PAGE was performed under reducing conditions to complement RP-HPLC results. The sample buffer, reducing agent, gels, running buffer and antioxidant agent were all purchased from Invitrogen (Carlsbad, USA). The washed cream was diluted 30 times with SMUF, 5 μL diluted cream was mixed with 5 μL 4× concentrated NuPAGE® LDS sample buffer, 2 μL 10× concentrated NuPAGE® sample reducing agent and 10 μL MilliQ water. Then the mixture was centrifuged at 425 g for 1 min and heated at 70˚C in a heating block (Labtherm Graphit, Liebisch, Germany) for 10 min. Samples were loaded onto NuPAGE® Novex® 12% Bis-Tris gels. The BlueRay Prestained Protein Marker 10-180 kDa (Jena, Germany) was applied as a reference. NuPAGE®MES buffer with addition of 0.5 mL NuPage® antioxidant was used for running the gels. Electrophoresis was performed using an XCell SureLock™ unit (Invitrogen, Paisley, UK) at constant voltage (120 V). The gels were Coomassie-stained.

2.5   Characterization of microstructure of fat globules

2.5.1   Confocal laser scanning microscopy (CLSM)

The distribution of lipid and protein and their aggregation were observed with a Zeiss LSM 510-META 18 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The stock solutions of lipid-specific stain Bodipy 505/515 (Invitrogen Molecular Probes, Carlsbad, CA, USA, 5 mg/mL in 70% ethanol) and protein-specific stain rhodamine B (Sigma-Aldrich, 10 mg/mL) were mixed and diluted in SMUF to a final concentration of 1 mg/mL. One μL of

mixed stain was added to 99 µL milk sample, vortexed and dropwise transferred to a cavity slide.

The CLSM was connected to an inverted microscope (Axiovert 200M) with differential interference contrast (DIC). Bodipy 505/515 was excited with an Argon laser (488 nm) and emission wavelengths between 505 nm and 530 nm were detected. A He-Ne laser operated at a excitation wavelength of 543 nm and emission wavelengths of 560 nm and higher was used to detect rhodamine B. All images were acquired using a Plan-Apochromat 63× oil immersion objective (1.4 NA) with a resolution of 512×512 pixels, and the pinhole of the microscope was set to 84 µm. The samples stained with both dyes were analysed by multi-channel tracking to enable the visibility of the dyes.

2.5.2   Cryostation scanning electron microscope (Cryo-SEM)

To analyse the morphological changes of fat globules in UHT milk after enzymatic hydrolysis, Cryo-SEM images were taken at distinct times after incubation with proteases. A milk sample of 10 µL was transferred to a copper hollow rivet and quickly plunged into liquid nitrogen for snap-freezing. The sample holder was transferred to a cryopreparation system (MED 020/VCT 100, Leica, Vienna, Austria), in which the samples were fractured and freeze dried to remove water vapor contamination. Then the samples were sputter coated with a layer of 15 nm Tungsten. Samples were cryo-shielded transferred into the field-emission scanning microscope (FEI Magellan 400, FEI, Eindhoven, The Netherlands) equipped with a Leica cold stage for cryo-SEM. The analysis was performed at a working distance of 4.2 mm, with SE detection at an acceleration voltage of 2 kV. The particle size distribution was analyzed by Image J software (1.51f, National Institutes of Health, USA).

3.   Results and discussion

3.1   Change of physical stability

3.1.1   Change of fat content in different layers
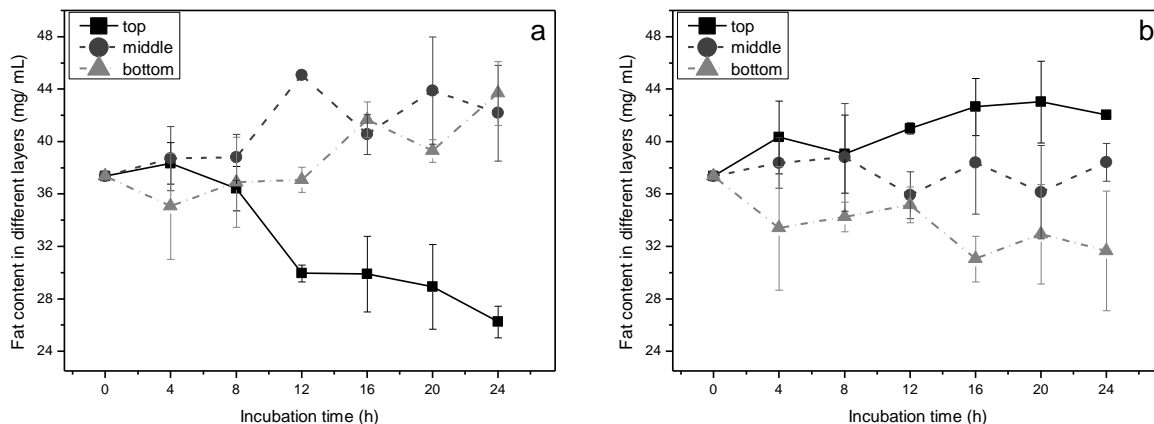
Fig. 1. Fat content in the top, middle and bottom layer of full-fat UHT milk samples incubated with AprX (a) and plasmin (b).

The fat redistribution was monitored by measuring the fat content in the top, middle and bottom layers of the sample tubes. As shown in **Fig. 1a**, the samples incubated with AprX, fat in the top layer started to decrease from 8 h onwards, whereas, the fat content in the middle and bottom layers increased, indicating fat started to sediment. On the contrary, in plasmin-hydrolysed samples the fat content in the bottom layer decreased after 0 h, along with an increase of fat content in the top layer (**Fig. 1b**), indicating the fat globules floated to the surface. Similar plasmin-induced redistribution of fat in different layers of UHT milk during storage has been reported by Malmgren et al. (2017), who observed an increase in fat content of the top layers and, at the same time, a reduction in the lower layers.

3.1.2   Stability test by LUMiFuge

Due to the high turbidity, fat separation was difficult to judge visually in full-fat UHT samples until the sample clarified. Therefore, LUMiFuge was used to aid in analysing the particle migration by calculating the instability index according to the transmission profiles under centrifugal forces. As shown in **Fig. 2**, the instability index increased during incubation in both AprX- and plasmin-containing samples, indicating that sample destabilisation happened in both systems. For the samples incubated with AprX, the instability index rapidly increased between 4 h and 12 h, wherein creaming gradually changed into sedimentation, as shown by the shape of transmission profiles (**Fig. S1**). In plasmin-containing samples, the instability index

increased at a slower pace compared to AprX-containing samples, with more creaming and less sedimentation formed, as inferred from the transmission profiles.
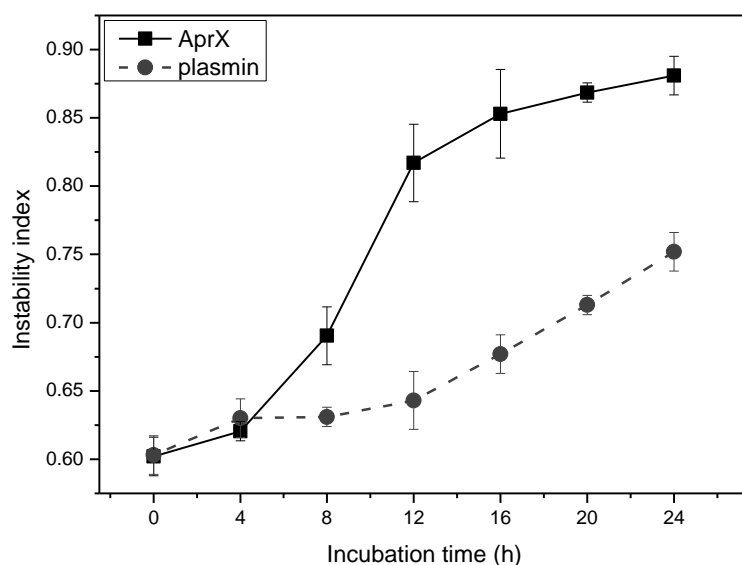
Fig. 2. Instability index obtained by LUMiFuge of full-fat UHT milk samples incubated with AprX (a) and plasmin (b). All the samples were 10-fold diluted to facilitate the optical measurement.

3.1.3   Size distributions
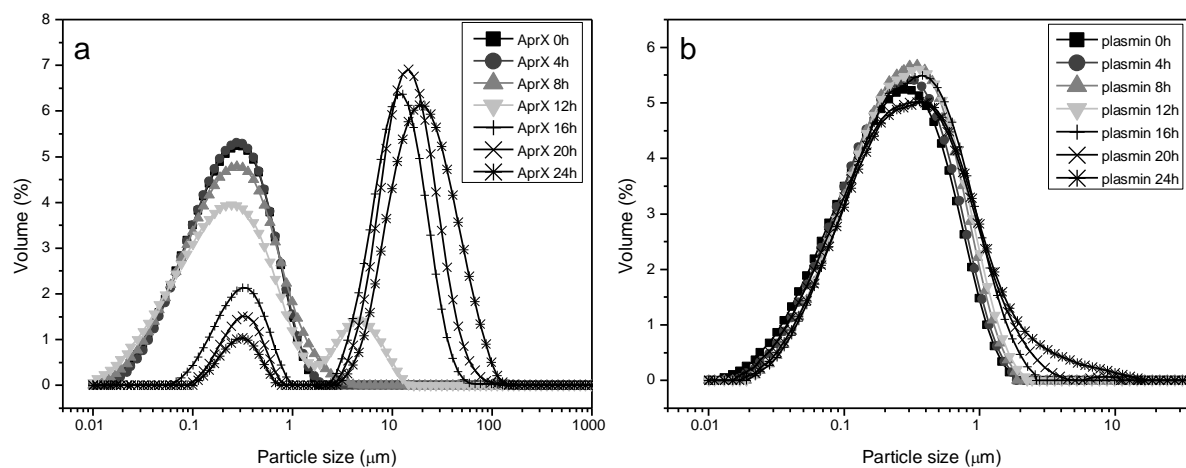
Fig. 3. Volume based particle size distribution in blank full-fat UHT milk samples incubated with AprX (a) and plasmin (b).

The changes in the size distribution of all the colloidal particles in full-fat UHT milk, which include casein micelles, fat globules, and the agglomerates that may form, were monitored during hydrolysis by AprX and plasmin (**Fig. 3**). The blank full-fat UHT milk at 0h incubation had a pseudo-monomodal size distribution centred at approximately 200 nm, which are actually

two overlapping distributions with the casein micelles being smaller and the fat globules being larger than the average. A similar pseudo-monomodal size distribution of full-fat UHT milk has been reported by Matéos et al. (2015).

For the UHT milk containing AprX (**Fig. 3a**), the size distribution started to broaden at 8 h. A new peak appeared in the micron size range at 12 h, and shifted to over 10 μm in the subsequent incubation times. Meanwhile, the peak corresponding to regular particles in the sample before incubation gradually diminished, indicating that the small particles were incorporated in the larger aggregates. This observation is in agreement with earlier findings, where large aggregates ranging from 2-100 μm have been reported in full-fat UHT milk (Matéos et al., 2015). However, in skim UHT milk samples incubated with AprX, these aggregates have also been observed (Baglinière et al., 2013; Zhang et al., 2018), indicating that the underlying mechanism for aggregate formation could be similar. It can also be speculated that fat is entrapped in the formed protein aggregate, similar to cheese production.

Compared to these AprX-containing samples, the size distribution changed to a lesser extent in the plasmin-containing samples. No new peaks appeared, but the average size slightly increased and a population of particles with a size larger than 2 μm arose, indicating that larger particles were formed during the hydrolysis by plasmin. Similar changes in the size distribution have also been found in semi-skim UHT milk (1.5% fat, w/v) with residual plasmin activity during storage (Rauh, Sundgren, et al., 2014b). But the plasmin-induced changes of particle size distribution in the skim UHT milk (0.07% fat, w/v) was slightly different, where the peak corresponding to native casein micelles shifted, instead of broadening to a larger size (Zhang et al., 2018), which may be due to the fact that in skim milk there are only casein micelles, making the peak sharper, whereas in non-skim milk, the peaks of casein micelles and fat globules overlap.

3.1.4   Viscosity
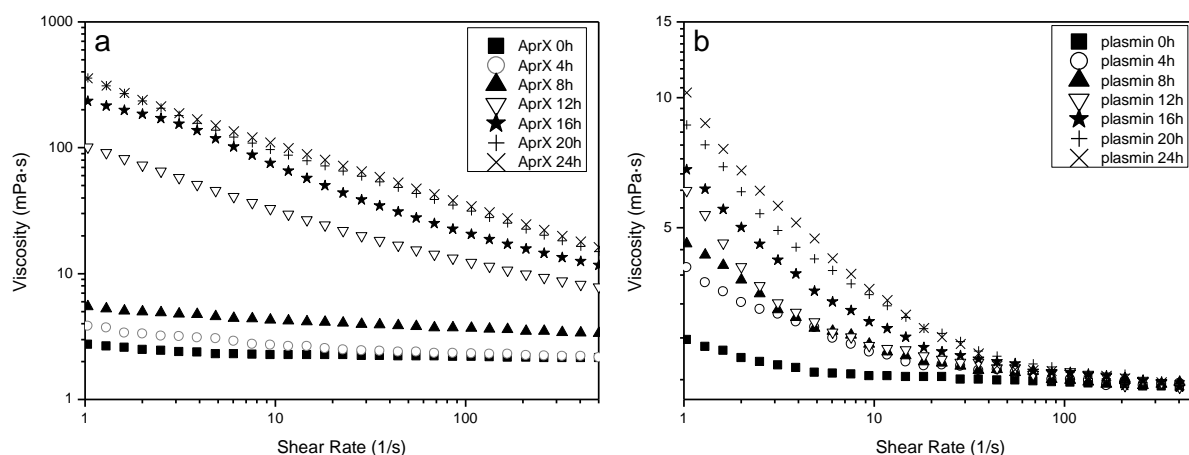
Fig. 4. Viscosity of full-fat UHT milk samples incubated with AprX (a) and plasmin (b) as a function of shear rate.

The flow behavior of milk as determined by a rheometer is closely related to the consumers' perception of viscosity. **Fig. 4** displays the apparent viscosity curves for both AprX- and plasmin-hydrolysed samples. The viscosity of samples with AprX and plasmin both showed shear thinning behavior, and the viscosity increased with increasing incubation time.

In AprX-containing samples, after incubation for 8 hours, the viscosity sharply rose to more than 10 mPa·s (at 20 °C) (**Fig. 4a**), which is generally regarded as a manifestation of age gelation in UHT milk (Datta & Deeth, 2001; Deeth & Lewis, 2016). This coincided with the time that the sedimentation started (**Fig. 1**) and large agglomerates were formed (**Fig. 3**). Therefore we can conclude that a gel network formed after 8 h. In comparison, the viscosity in plasmin-containing samples rose steadily, indicating that the changes in the microstructure were gradual and progressive. In our study, none of the plasmin-containing samples reached a viscosity higher than 10 mPa·s (**Fig. 4b**). But fat floating was detected (**Fig. 1**), showing that creaming happened earlier than gelation in our plasmin-containing UHT milk.

Comparing these two systems, AprX induced a greater increase in viscosity and a less pronounced shear thinning behavior compared to plasmin, which both reflected that AprX induced the formation of stronger network structures than plasmin.

3.2   Change of FSAP content and composition

3.2.1   Change of FSAP content
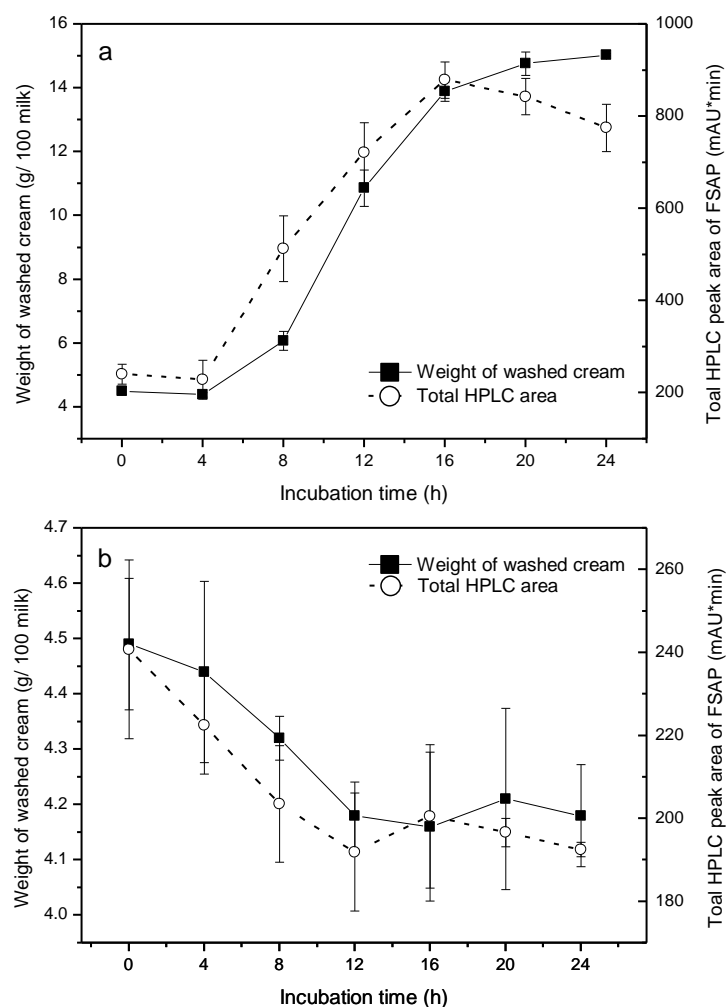
Fig. 5. The weight of washed cream and the total HPLC peak area of FSAP after integration of UV absorbance at 214 nm of full-fat milk samples incubated with AprX (a) and plasmin (b).

To explain how the fat stability may be influenced by the hydrolysis of FSAP, the samples with AprX or plasmin were further studied for their FSAP content and composition. Because the same aount of milk fat ended up in the cream layer after ultracentrifugation, the differences in the weight of cream layer per unit milk was mainly attributed to the different content of protein and the water associated with protein. As shown in **Fig. 5**, in the milk with AprX, the weight of cream started to increase rapidly after 8 h, and remained constant after 20 h. In milk with plasmin, the weight of the cream layer decreased in the first 12 hours, and remained constant afterwards. Similar trends as for weight of cream were seen in both systems when FSAP concentration in the washed cream was quantified using HPLC and expressed as the total HPLC

peak area (**Fig. 5**). Both measurements showed that more protein was trapped in the cream layer along with the hydrolysis by AprX, whereas, part of the FSAP seems to get released to the serum when hydrolysed by plasmin.

3.2.2 Change of FSAP composition
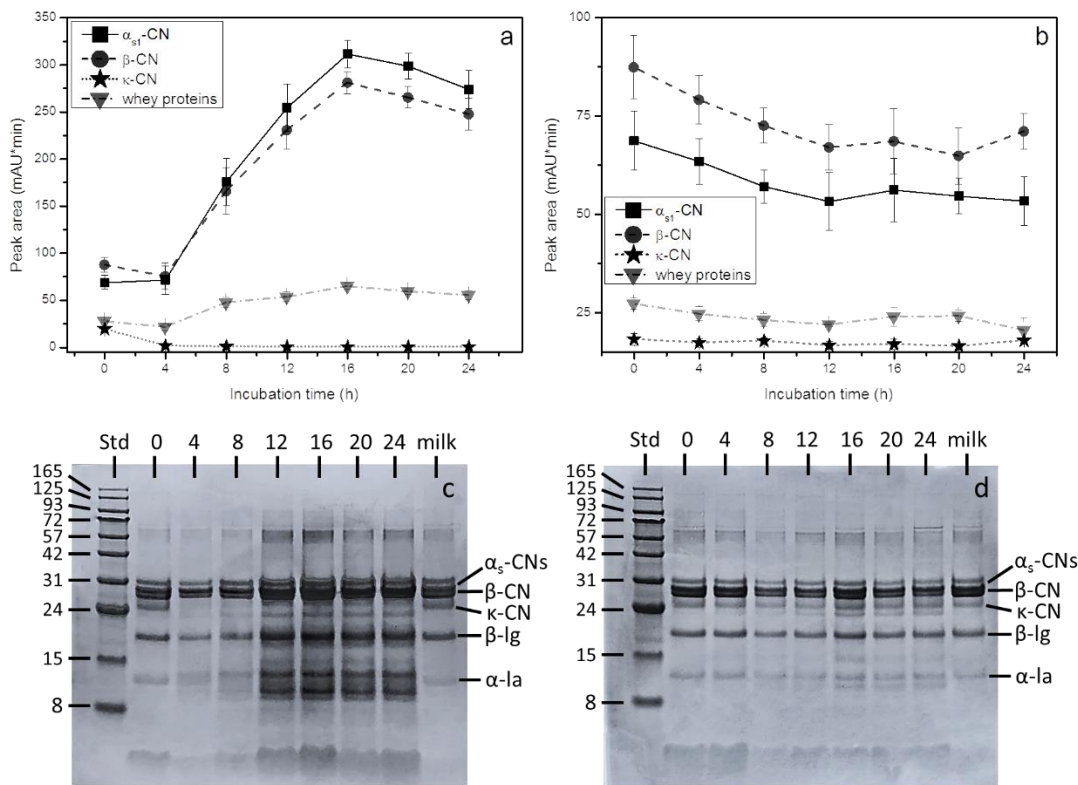
Fig. 6. Changes of α_{s1}-casein, β-casein, κ-casein and total whey proteins in full-fat UHT milk samples incubated with AprX (a) and plasmin (b); SDS-PAGE patterns of full-fat UHT milk samples incubated with AprX (c) and plasmin (d). For (c) and (d): α_s-CNs: α_{s1}+ α_{s2} caseins; Std: molecular mass standards; milk: full-fat UHT milk without addition of protease; the numbers above the gel indicate the incubation time with protease in hours.

The changes of FSAP composition during incubation of the full-fat UHT milk samples with AprX or plasmin were studied using RP-HPLC and SDS-PAGE (**Fig. 6**). In AprX-hydrolysed samples, α_{s1}- and β-caseins remained constant in the first 4 hours, but started to increase sharply between 4 and 16 h, after which they declined slightly (**Fig. 6a**). Meanwhile, almost all the κ-casein was rapidly hydrolysed within the first 4 hours. Besides that, the peak area of whey proteins increased gradually upon hydrolysis. On the contrary, during plasmin incubation, α_{s1}- and β-caseins in FSAP decreased continuously, while κ-casein and whey proteins remained constant. The SDS-PAGE results confirmed the changes with incubation time found with RP-

HPLC (**Fig. 6c, 6d**). New bands of protein breakdown products appeared in the gel for both sets of samples.

3.3   Microstructure of fat globules with hydrolysed FSAP

3.3.1   CLSM
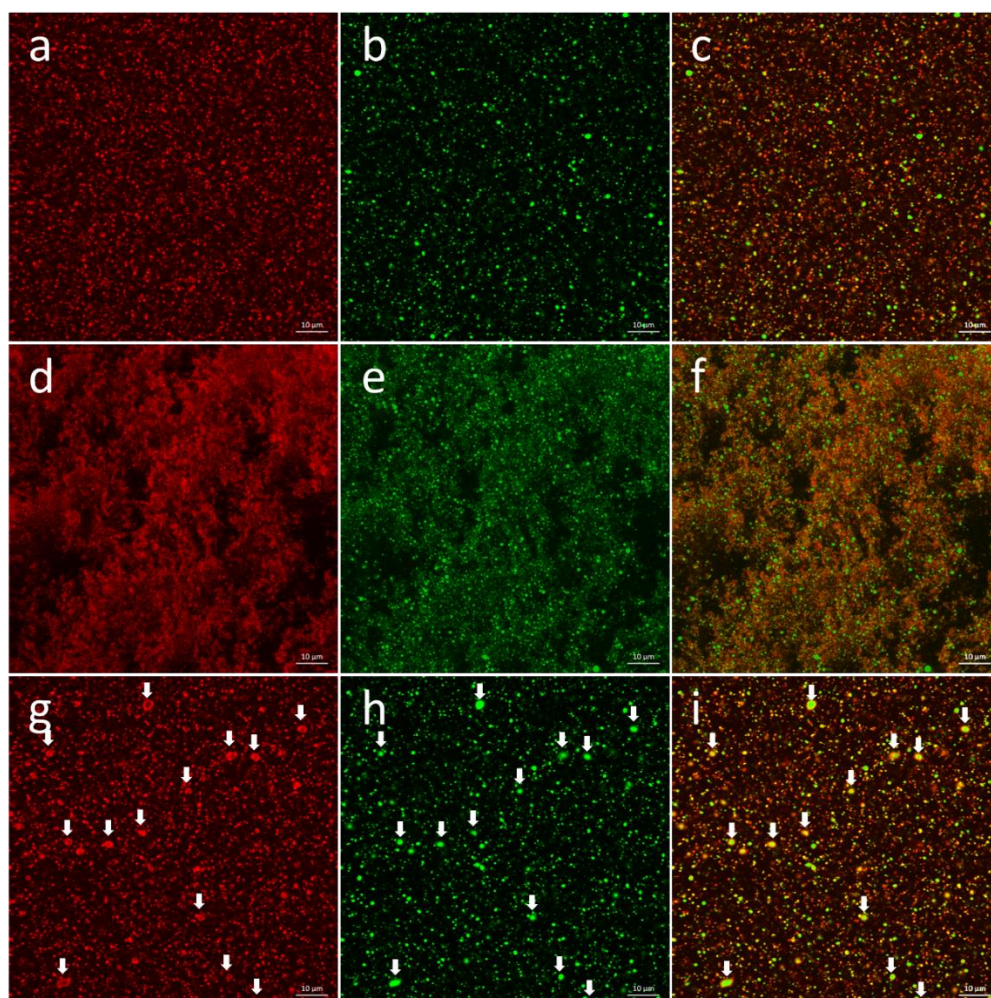
Fig. 7. Confocal micrographs showing the microstructure of blank full-fat UHT milk (a-c), full-fat UHT milk samples incubated with AprX (d-f) or plasmin (g-i) at 37 ℃ for 16 hours. Column 1, lipid channel (Bodipy 505/515 staining); column 2, protein channel (rhodamine B staining); and column 3, merged channels of lipid and protein. White arrows indicate coalesced fat globules. The scale bars are 10 μm in length.

The microstructure of the blank and full-fat UHT milk with AprX or plasmin was visualised by confocal laser scanning microscopy after incubation for 16h (**Fig. 7**). These samples were selected because the FSAP fraction in both milk samples seemed to not change further from this time onwards (**Fig. 5, 6**). In the blank full-fat UHT milk, both the casein micelles (**Fig. 7a**)

and the fat globules (**Fig. 7b**) had uniform sizes and were homogeneously distributed. No aggregation was observed (**Fig. 7a-c**).

In the AprX-hydrolysed samples, on the other hand, a remarkably different state of the fat and protein was observed. The microstructure was composed of a dense protein gel network (**Fig. 7d**) in which the fat globules were embedded (**Fig. 7e, f**). Compared to traditional dairy products, the AprX-induced network structure is more compact than the open gel in full-fat yoghurt where colloidal calcium phosphate is dissolved and the κ-casein brush collapses (Torres, Rubio, & Ipsen, 2012). But the network is similar to chymosin-induced milk gel (Ong, Dagastine, Kentish, & Gras, 2011), which agrees with the results discussed in **Section 3.1.3**.

In plasmin-hydrolysed samples, we observed some coalesced fat globules covered by a "protein shell", as indicated by the white arrows in **Fig. 7g-i**. These fat globules were bigger than those in blank and AprX-hydrolysed samples, which is in line with the increase of the particles in the range over 2 μm, that was observed in the size distribution results (**Fig. 3b**), and indicates fat coalescence in these samples. The ascending or descending of these coalesced fat globules is determined by the protein to fat ratio. In our case, the relative amount of protein associated with the fat globules decreased upon hydrolysis by plasmin (**Fig. 5, 6**). As a result, the average density of fat globules would drop and the density of fluid would increase. Therefore, according to Stokes' law, the coalesced fat globules would float to the surface, which is in agreement with our findings in **Fig. 1b**.

3.3.2   Cryo-SEM
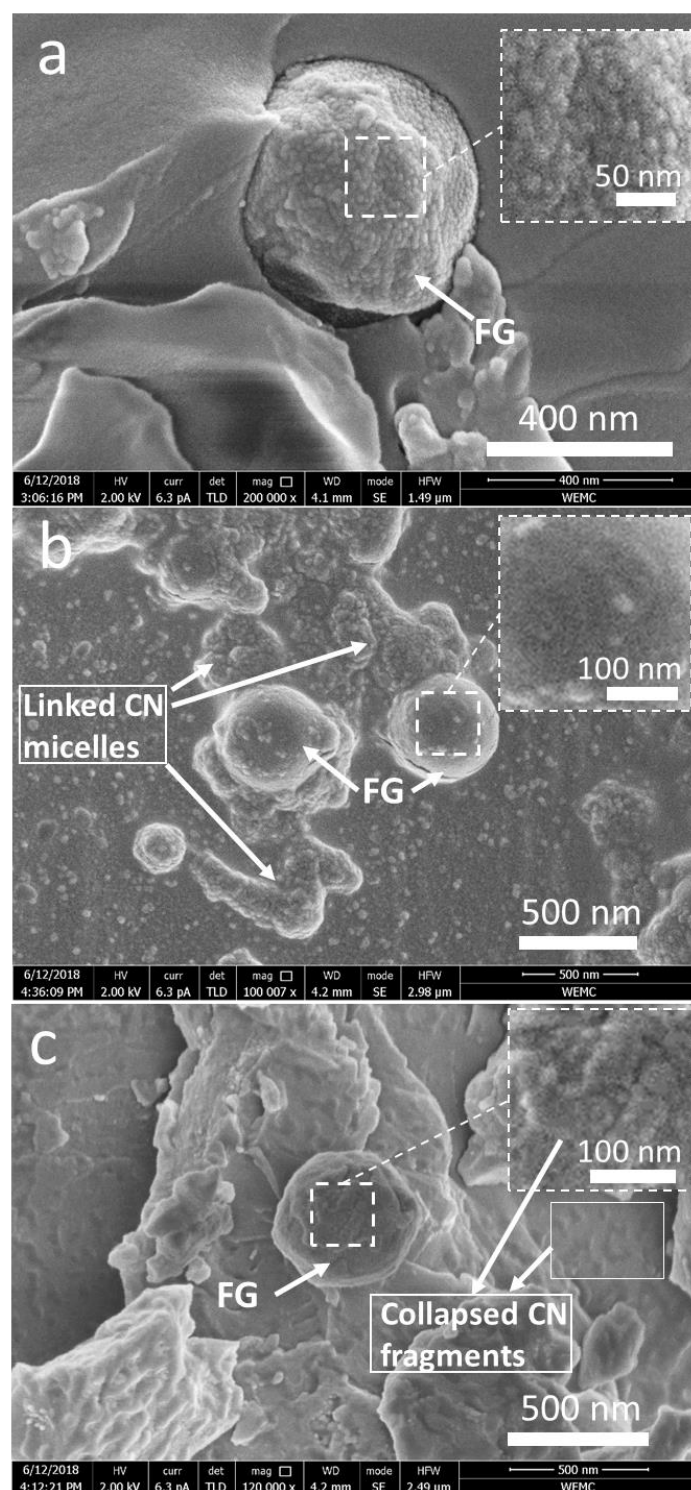
Fig. 8. Cryo-SEM images of fat globule(s) in blank full-fat UHT milk (a), full- fat UHT milk samples incubated with AprX (b) and plasmin (c) at 37 ℃ for 16 hours. The inserts show the enlargement of the fat globule surface. The scale bars are shown in the images. FG: fat globules. The linked and collapsed casein micelles as discussed in the text are pointed out in (b) and (c), respectively.

Cryo-SEM images were taken to investigate the morphologic changes of the fat globules in UHT milk, and meant as examples to further strengthen our theory. Fat globules could be easily distinguished from casein micelles because casein micelles were smaller than fat globules and present as uniform, not perfectly spherical-shaped particles. The sizes of casein micelles in UHT milk ranged from 50-200 nm, with an average diameter of about 100 nm (**Fig. S2**). Kamigaki, Ito, Nishino, and Miyazawa (2018) reported a similar distribution of casein micelle sizes based on the observation of casein micelles in raw milk, with an average diameter of about 140 nm. the smaller average diameter observed in our samples may be explained by the disruption of the micellar structure during homogenization (Dalgleish, Spagnuolo, & Goff, 2004).

The fat globules, in both the blank and hydrolysed samples, were much rounder and bigger than casein micelles, with the diameter of most fat globules ranging from 200 nm-500 nm, which is in agreement with Lopez (2005), who suggested that the volume-weighted average diameter of fat globules in homogenized milks is in the range 0.2-0.5 μm. In the blank full-fat UHT milk, as shown in Fig. 8a, the surface of the fat globules appears to be covered by woolly structures, protruding from the bulk of the fat globule. These structures with a diameter of about 10 and 20 nm are assumed to be casein micelle fragments, which originate from casein micelles that have been pulled apart, and have then spread on the fat globules during homogenization (Dalgleish et al., 2004). These woolly structures probably contain many κ-caseins on their surface, because they were absent after incubation with AprX, as shown in **Fig. 8b**. In addition, in AprX-hydrolysed samples, fat globules appeared to be embedded in an aggregated protein network (**Fig. 8b**). The two findings for the AprX-hydrolysed samples agree with the interpretation of both the amount of cream layer and its protein content (**Fig. 5a**), as well as the CLSM pictures (**Fig. 7d-f**). The surface conformation of fat globules in plasmin-hydrolysed samples (**Fig. 8c**) was more difficult to interpret. Deduced from the FSAP hydrolysis by plasmin (**Fig. 6b**), and the "protein shell" observed in the CLSM pictures (**Fig. 7g-i**), the structure covering the surface of fat globules might consist of collapsed casein micelle fragments.

3.4   Comparison between AprX- and plasmin-induced fat destabilization

FG: fat globules
⬤ : casein micelle fragments with κ-casein
⬤ : casein micelle fragments with cleaved κ-casein
⬤ : collapsed casein micelle fragments
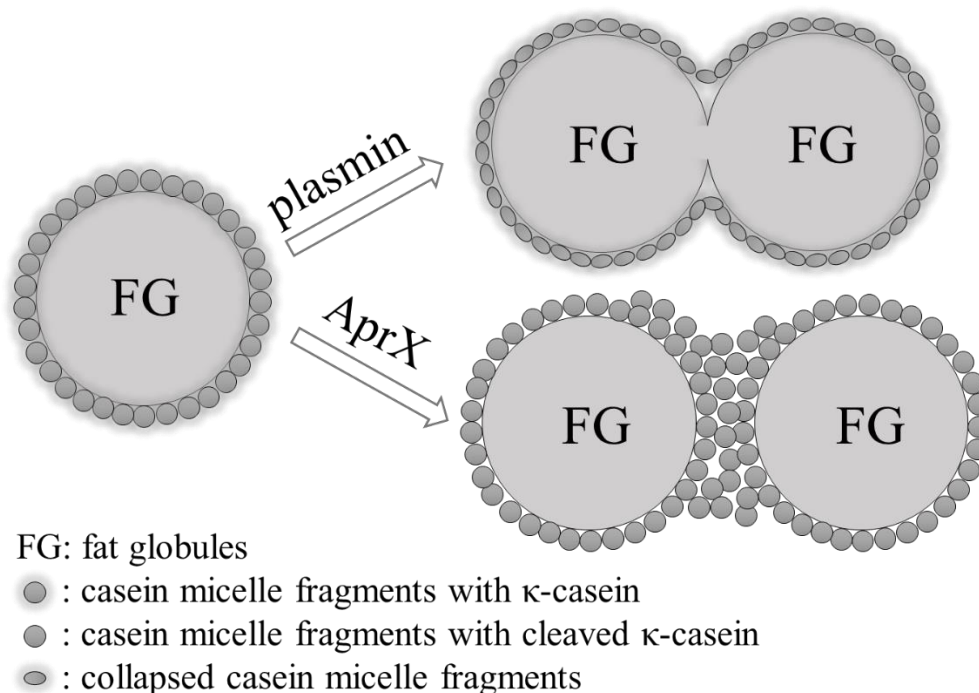
Fig. 9. Simplified scheme describing the different mechanisms of fat destabilization in full-fat UHT milk induced by AprX and plasmin.

When comparing the physical and chemical changes in full-fat UHT milk samples hydrolysed by AprX and plasmin, different mechanisms of fat destabilization can be proposed, as schematically illustrated in **Fig. 9**.

AprX destabilizes fat globules in full-fat UHT milk by flocculation (**Fig. 9**). In AprX-containing samples, since AprX specifically cleaved the protruding κ-casein (**Fig. 5a, c**), casein micelles, together with the casein micelle fragments absorbed on the surface of fat globules, quickly destabilize. Consequently, a network is formed due to the reduced steric and electrostatic repulsion. Being part of the three-dimensional gel network, even though fat globules remain as individual entities, they are closely embedded in the continuous protein network (**Fig. 7d-f, Fig. 8b**). From the perspective of fat globules, more proteins, which are the micellar casein aggregated with the existing FSAP were absorbed (**Fig. 5**). As a result, the hydrodynamic diameter increased (**Fig. 3a**), as well as the viscosity (**Fig. 4a**). Furthermore, due to the adsorption of additional milk proteins with the fat globules, which increases their density, fat globules tended to sink under gravitational force (**Fig. 1a**).

By contrast, the mechanism responsible for the physical instability of fat globules in full-fat UHT milk induced by plasmin is fat globule partial coalescence (**Fig. 9**). In plasmin-containing

samples, plasmin preferentially hydrolysed α- and β-caseins (**Fig. 6b, d**), probably causing collapse of the FSAP which mainly consists of casein micelle fragments (**Fig. 8c**). As a consequence, the fat globules did not longer repel each other, instead, several individual fat globules merged into a larger fat droplet (**Fig. 7g-i**). These coalesced fat droplets lead to an increase in the particle size (**Fig. 3b**) and viscosity (**Fig. 4b**). Because some of FSAPs were cleaved by plasmin, less protein was present on the surface of the fat droplets (**Fig. 5**), and the fat globules thus have a lower density and cream (**Fig. 1b**). It should also be noted that a creamy layer on the top is commonly accompanied by a gel on the bottom when plasmin is present (Kohlmann et al., 1991; Stoeckel et al., 2016b; Visser, 1981).

4. Conclusions

This study elucidated the mechanisms of fat destabilization in UHT milk as a consequence of proteolytic degradation by bacterial protease AprX and native protease plasmin. Our results have shown that AprX induces sedimentation of milk fat, being embedded in a protein gel network, caused by the fast hydrolysis of κ-casein leading to flocculation of casein micelles and fat globules. Plasmin, on the other hand, can induce creaming in full-fat UHT milk, because the hydrolysis of α- and β-caseins disrupts the casein micelle fragments on the milk fat globule surface, causing the fat globules to coalesce and ascend. The knowledge gained from this study may be used for determining the protease responsible for the full-fat UHT milk destabilisation during storage. Overall, both enzymes can bring about destabilization in full-fat UHT milk, thus actions should be taken to decrease the activity of both enzymes in the production chain.

Supplementary materials
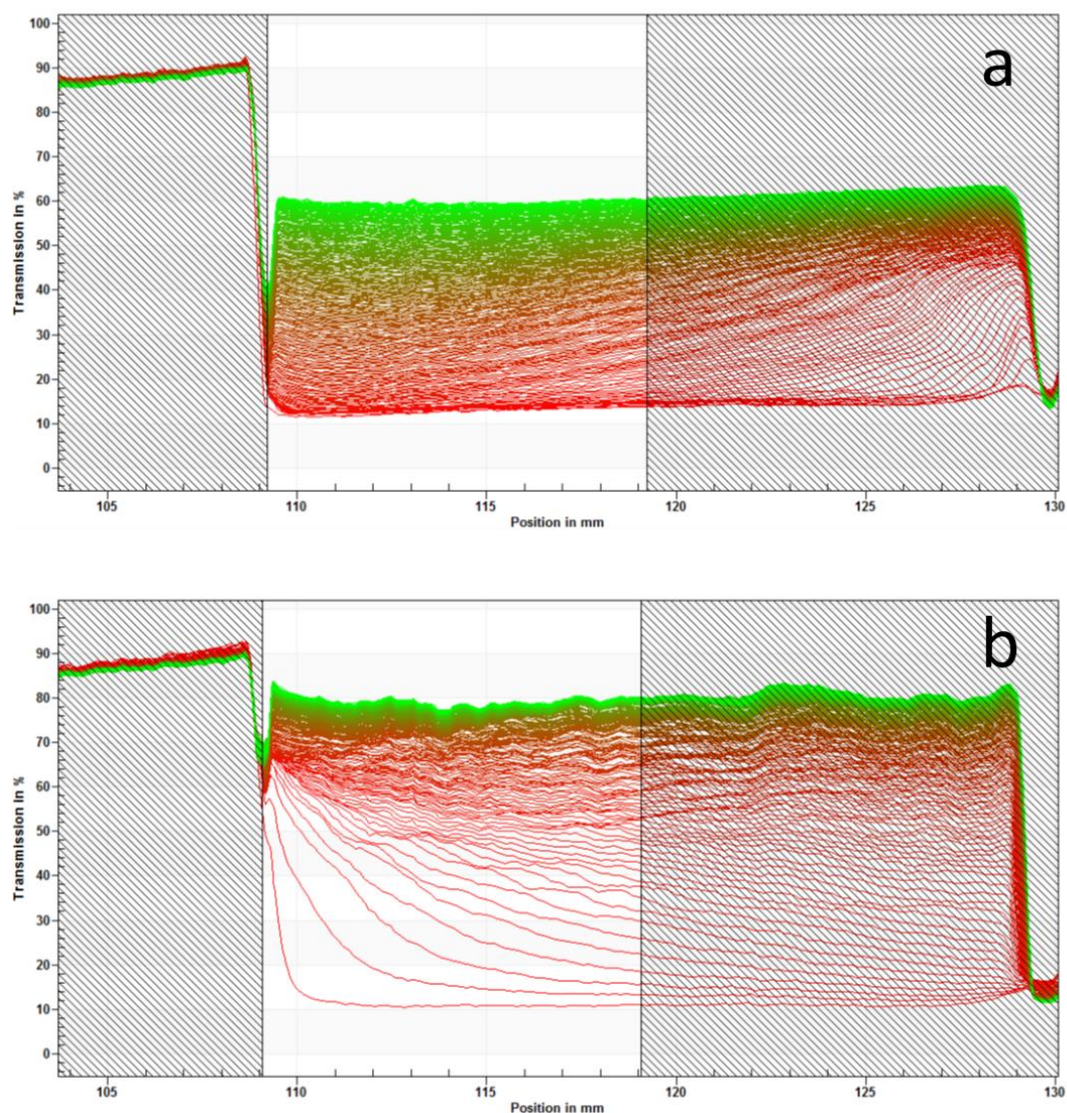
Fig. S1. Representative LUMiFuge transmission profiles of creaming (a) and sedimentation (b) in full-fat UHT milk samples, illustrating measured transmission as function of the local position on the sample cell. All the samples were 10-times diluted to facilitate the optical measurement. Red curves indicate the first measurements, which becomes green over time of analysis. The unshaded part was automatically selected for calculation of instability index.
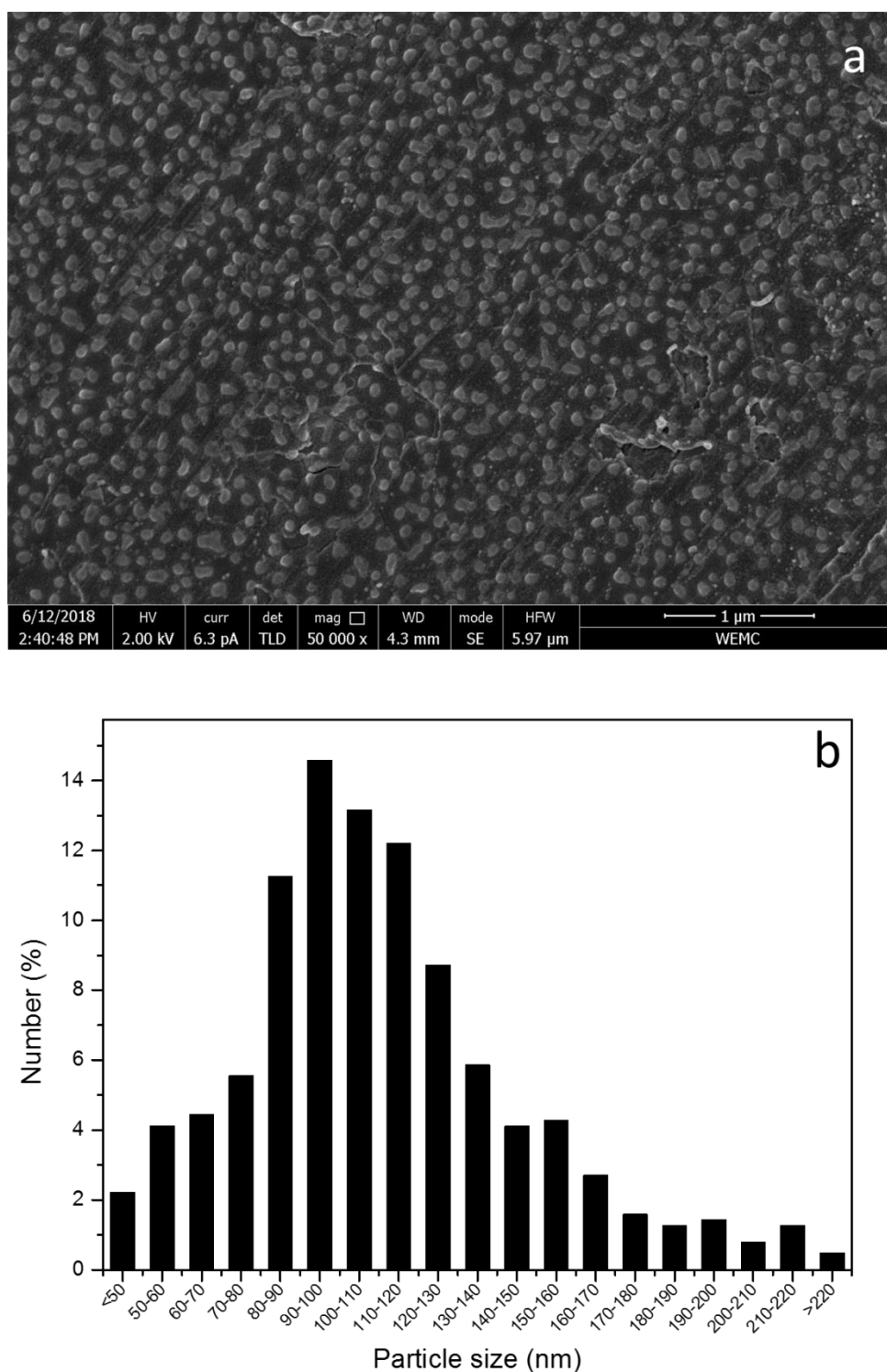
Fig. S2. Cryo-SEM images of casein micelles in blank full-fat UHT milk (a), the scale bar is 1 μm in length; and the particle size distribution analysis was performed by Image J (n=630, mean=110.8 nm) (b).

Chapter 5

Low temperature inactivation of protease AprX from *Pseudomonas* may not be feasible in UHT milk: a peptidomic study

Abstract

Destabilization of UHT milk during its shelf life can be promoted by the residual proteolytic activity attributed to the protease AprX from *Pseudomonas*. To better understand the hydrolysis patterns of AprX, and to evaluate the feasibility of using low temperature inactivation (LTI) for AprX, the release of peptides through AprX activity on milk proteins in both skim and full-fat UHT milk was examined using LC-MS/MS-based peptidomics analysis. Milk samples were either directly incubated to be hydrolysed by AprX, or preheated at LTI condition (60 ℃ for 15 min) and then incubated. Peptides and parent proteins (proteins from matched peptide sequences) were identified and quantified. Then the peptides were mapped, and the cleavage frequency of amino acids in the P1/P1' positions were analysed, after which the influence of LTI and the potential bitterness of the formed peptides were determined. Our results showed a total of 2488 peptides that were identified from 48 parent proteins, with the most abundant peptides originating from κ-casein and β-casein. AprX could also hydrolyse other proteins in milk non-specifically. Except for decreasing the bitterness potential in skim UHT milk, LTI did not significantly reduce the AprX-induced hydrolysis of milk proteins. The effects of LTI on skim and full-fat UHT milk was inconsistent. Therefore, inactivation of AprX by LTI may not be feasible in UHT milk production.

Chapter 5   Low temperature inactivation of protease AprX in UHT milk: a peptidomic study

1. Introduction

The storage of raw milk under refrigeration conditions allows the growth of psychrotrophic bacteria (Sørhaug & Stepaniak, 1997). Among all psychrotrophic bacteria, the *Pseudomonas* genus is predominant in milk (Decimo, Morandi, Silvetti, & Brasca, 2014; von Neubeck et al., 2015; Yuan et al., 2017). The majority of pseudomonads can produce a specific extracellular metalloprotease AprX, which can hydrolyse α_s-, β- and κ-caseins. In addition, AprX is highly heat resistant and can therefore remain active after ultra-high temperature (UHT) processing (typically 135-150 ℃ for 2-10 s) (Stoeckel et al., 2016b). Therefore, different types of destabilization of UHT milk, such as age gelation, fat separation and bitter flavour, have been attributed to AprX activity (Fairbairn & Law, 1986; Baglinière et al., 2013; Matéos et al., 2015; Andreani et al., 2016).

In order to increase the stability of UHT milk during shelf life, the AprX level should be minimized, by implementing good hygiene practices to control the pseudomonads which can produce AprX, and enhancing inactivation of AprX by adjusting UHT processing, especially in milk destined for export or stored at high ambient temperatures. Currently, there are two main mechanisms that can play a role in the inactivation of AprX. The first is thermal inactivation, which for the highly heat resistant AprX refers to the inactivation at UHT temperatures. However, the D-value (the time required to reduce the enzyme activity to 10% of its original value) for the inactivation of AprX is as high as 2.7±1.4 min at 130 ℃ (Stoeckel et al., 2016b), meaning that reducing AprX activity using current UHT regimes is difficult without introducing detrimental effects of increased heating to its sensory properties and to the heat-sensitive whey proteins. The second mechanism is low temperature inactivation (LTI), which is based on the fact that autolysis of AprX can occur at lower temperatures corresponding roughly to the denaturation temperature T_d. At these temperatures, AprX enzyme molecules are present as a mixture of 1) folded, active, proteolytic, compact molecules and 2) (partially) unfolded, inactive molecules. The native state can protect the enzymes from autolysis, while the (partially) unfolded structure renders the enzyme susceptible to intermolecular autolysis, thereby decreasing AprX activity (Schokker & van Boekel, 1998b; Stoeckel et al., 2016b).

LTI treatments at 50-60 ℃ for inactivating bacterial proteases, especially AprX, have been reported in many studies (Barach, Adams, & Speck, 1976, 1978; West, Adams, & Speck, 1978; Kroll & Klostermeyer, 1984; Diermayr, Kroll, & Klostermeyer, 1987; Schokker & van Boekel, 1998b; Glück et al., 2016). But conflicting results have been obtained in these studies with respect to the effects of LTI on extending the shelf life of UHT milk. Driessen (1983) reported

that preheating the milk for 60 min at 55 ℃ before UHT processing could improve the quality of UHT milk, by retarding gelation, proteolysis, bitterness and transparency of the milk. In contrast, Kocak and Zadow (1985b, 1989) found that LTI before UHT processing did not significantly inhibit proteolysis during storage of UHT milk, but LTI after UHT treatment doubled the shelf life by retarding the onset of age gelation. Three-fold extension of shelf life was also reported by West et al. (1978), who LTI treated skim milk after UHT heating.

The inconsistency observed in the inhibition of proteolysis in LTI-treated UHT milk samples was probably due to the physico-chemical changes partly dependent on initial proteolysis of the milk protein system (Kocak & Zadow, 1985b), because during LTI, besides AprX itself, the milk proteins can also be hydrolysed as substrates. An LTI-based autolysis can only be positively effective if the hydrolysis of milk proteins during LTI is less than the hydrolysis that may have been caused by the inactivated AprX. Otherwise, the low temperature treatment risks to increase the hydrolysis of milk proteins, which will in turn lead to an earlier development of destabilization during storage (Stoeckel et al., 2016b). However, hydrolysis of milk proteins during LTI and subsequent incubation remains poorly studied. Peptidomic technologies have been used to examine the quality of dairy products by identifying and quantifying peptides in milk (Dallas & Nielsen, 2018), and to examine the proteolytic activity of microorganisms on bovine milk proteins (Dallas et al., 2016). Hence, a peptidomic approach was employed in the current study to elucidate the milk protein hydrolysis.

In addition, a uniform understanding of the hydrolysis patterns of AprX on caseins is still lacking due to limited studies. Earlier studies often assessed the hydrolysis of milk proteins by AprX qualitatively, by counting the numbers of identified peptides. The order of cleavage sites from each casein was found to be β->α_{s1}->κ->α_{s2}-caseins (Gaucher et al., 2011; Baglinière et al., 2013; Matéos et al., 2015), whereas studies which characterized the decrease in intact caseins showed the order κ->α_s->β-casein using electrophoresis (Adams, Barach, & Speck, 1976) and κ->β->α_s-casein using RP-HPLC (Zhang, Bijl, & Hettinga, 2018). Given the inconsistent conclusions drawn from different approaches, there is a need for a more systematic, quantitative, analysis using up-to-date mass-spectrometric detection methods to better discriminate the preference of AprX towards specific parts of the caseins.

Therefore, this study first aims to supplement the knowledge of AprX hydrolysis patterns on milk proteins from a peptidomic perspective. The second objective of this study was to determine the feasibility of using a LTI treatment as a means of inactivating AprX in UHT milk,

by comparing the peptidome in the AprX-hydrolysed UHT milk samples with and without a LTI treatment.

2.   Materials and methods

2.1   Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2   Determination of the LTI condition

AprX was isolated and purified from the strain *Pseudomonas fluorescens* Migula 1895 (DSM 50120, Deutsche Sammlung von Mikroorganismen, Braunschweig) using the method described by Zhang et al. (2018). Two mg purified AprX was heated for 15 min at different temperatures ranging from 40 ℃ to 80 ℃ in 10 mL skim and full-fat UHT milk, as well as 10 mM phosphate buffered saline (PBS, pH 6.7). The holding time was 15 min, because the extent of inactivation of AprX did not markedly enhance when heated for longer than 15 min (based on the results of pre-experiments, data not shown). Samples were immediately cooled on ice for 10 min to stop the LTI treatment. A control sample prepared without LTI heating was taken as a reference corresponding to 100% of proteolytic activity. The proteolytic activity was determined with at 42 ℃, which is the optimal temperature for AprX, with azocasein assay as described by Zhang et al. (2018). Assays were performed in triplicate.

2.3   Sample preparation

Commercial skim and full-fat UHT milk were purchased from a local supermarket, the milk was subjected to direct sterilization by steam infusion heat treatment. To keep a low starting degree of hydrolysis, we used milk samples that were not older than 1 month after manufacture. To inhibit development of the microorganisms, 0.02% sodium azide and 0.0005% bronopol were added. Milk composition was determined to be 3.76, 3.77 g protein/100 mL milk, and 0.07, 3.67 g fat/100 mL milk in skim and whole milk, respectively, as determined by MilkoScan 134A/B (Foss Electric, Hillerød, Denmark). Skim and full-fat UHT milk samples without addition of AprX were used as untreated controls (coded SM for skim milk, FM for full fat milk). Crude AprX extract was added to the other samples at a concentration of 0.2 mg/mL milk. The samples without LTI treatment were coded SM+AprX and FM+AprX. The samples with AprX that were first treated with LTI and then incubated were coded SM+AprX+LTI and

FM+AprX+LTI. All samples were prepared in triplicates and incubated at 42 ℃ for 5 h in order to have the milk proteins hydrolysed by untreated and LTI-treated AprX.

2.4   Peptides extraction

Peptides were extracted using the methods previously described by Dingess et al. (2017), with modifications. Briefly, right after the incubation, the samples were mixed with the same volume of 200 g/L trichloroacetic acid (TCA) solution. The samples were mixed by vortexed at high speed for 10 seconds, then centrifuged at 3,000 *g* at 4 ˚C for 10 min, both intact proteins and fat were removed by the TCA precipitation. The supernatant was collected and cleaned by a solid phase extraction (SPE) C18 column clean up step prior to analysis by LC-MS/MS. Stage tips containing LiChroprep C18 column material LiChroprep® RP 18 (25-40 µm) were made in-house as described previously (Lu et al., 2011). The C18+ Stage tip column was washed 2 times with 200 µL methanol, then 4 µL of 50% column material LiChroprep C18 in methanol was added. The µColumn was conditioned with 100 µL of 1 mL/L formic acid (HCOOH).Then 50 µL of the peptide samples were loaded on the C18+ Stage tip column followed by washing with 100 µL of 1 mL/L HCOOH. Peptides were eluted with 50 µL of 70% acetonitrile/30% 1 mL/L HCOOH from the C18+ Stage tip column. The samples were then dried in a vacuum concentrator (Eppendorf Vacufuge®) at 45 ℃ for 20 to 30 min until the volume of each sample decreased to 15 µL or less. Samples were reconstituted to 50 µL with 1 mL/L HCOOH for analysis by LC-MS/MS. Validation of the reproducibility of the peptides extraction method has been previously reported by Dingess et al. (2017).

2.5   LC-MS/MS

Peptide analysis was performed on a nano-LC/LTQ-OrbitrapXL (Thermo Fisher Scientific, Bremen, Germany) system. A sample volume of 18 µL was injected onto a pre-concentration column (prepared in-house) and peptides were eluted onto a 0.10×200 mm Magic C18 resin analytical column with an acetonitrile gradient at a flow rate of 0.5 µL/min. The gradient elution increased from 5% to 30% acetonitrile in water with 1 mL/L HCOOH in 50 min. The column was then washed using a fast increase in the percentage acetonitrile to 50% (with 50% water and 1 mL/L HCOOH in both the acetonitrile and the water) in 3 min. A P777 Upchurch microcross was positioned between the pre-concentration and analytical column. An electrospray potential of 3.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line of the micro-cross. Full scan positive mode FTMS spectra were obtained in the LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA) between an m/z of 280 and 1400 at a resolution of 15,000. MS/MS scans of the most abundant singly, doubly, and

triply charged peaks in the FTMS scan were recorded in data-dependent mode in the Orbitrap at a resolution of 7,500 (MS/MS threshold=10,000, 45 s exclusion duration) using a contaminant m/z mass list to prevent selection of contaminants.

2.6   Data analysis

MS/MS spectra from each run were analysed with MaxQuant v.1.6.0.1 with the Andromeda search engine (Cox & Mann, 2008). Parent proteins were quantified based on label free quantification (LFQ) with a minimum ratio count of 2 peptides, whereas peptides were quantified based on raw intensities reported by MaxQuant. Match between runs and unspecified digestion settings were used. A false discovery rate (FDR) of 0.01 was set for both the peptide spectrum match level and the protein level. First search, for mass recalibration, was done using a major bovine milk proteins list downloaded from UniProt on 09-2017. A bovine milk protein database was downloaded from UniProt on 14-09-2017 and used for Andromeda searches with a minimum peptide length of 7 and a maximum length of 50 amino acids. A standard contaminants list containing human keratin and trypsin sequences was included in the search. Variable modifications used included phosphorylation of serine, threonine and tyrosine, oxidation of methionine, acetylation of the protein N-terminus and deamidation of asparagine and glutamine.

Filtering and statistics on the MaxQuant output was done with Perseus v.1.6.0.7, as detailed by Tyanova et al. (2016). The protein and peptide text files from MaxQuant were loaded into Perseus. Once data were loaded, they were filtered for removal of reverse and non-bovine contaminant sequences. Data was log10 transformed, and grouped by the fat content of the UHT milk, addition of AprX and LTI treatment. Data were filtered by rows based on valid values for a minimum of 50% occurrences of each protein or peptide sequence. Missing intensity values were imputed across the entire matrix, using random values from a normal distribution with a variation of 0.3 and a downshift of 1.8, meant to simulate expression below the limit of detection (Tyanova et al., 2016). Once imputed, values were normalized based on z-score which Perseus calculated by subtracting the median intensity from individual intensities followed by division by the standard deviation.

2.7   Statistical analysis

Differences in protein and peptide profiles were assessed in Perseus v.1.6.0.7 and tested across AprX hydrolysed (with or without LTI treatment) groups by ANOVA. For ANOVA, S0 was set to 0, with permutation-based FDR, where FDR was set to 0.05, which are the default settings

in Perseus. For volcano plots, two sided t-tests were used to determine differences caused by LTI treatment. For this analysis, the FDR was set to 0.05 and S0 was set to 0.1. The S0 value here allows for testing of artificial variance within groups and controls for differences between means, which required a larger absolute difference between groups (Tusher, Tibshirani, & Chu, 2001). Throughout, results were reported as means±standard deviation (SD) unless noted otherwise.

2.8   Enzyme Predictions

Enzymes responsible for the cleavage of proteins in blank UHT milk samples was predicted with the web-based software EnzymePredictor (Vijayakumar et al., 2012). Enzymes were evaluated and classified based on the total number of cleavages and their odds ratio (OR). These values were used to determine the degree of participation of certain enzyme in the hydrolysis of the proteins identified. The N-terminal and C-terminal cleavages of individual parent proteins were also assessed.

2.9   Bitterness assessment

Potential bitterness of peptides was evaluated by applying Ney's Q-rule based on peptide hydrophobicity (Ney, 1971). An average hydrophobicity Q of the peptide is calculated as the sum of the amino acids side chains' hydrophobicity divided by the number of amino acid residues of the peptide. Peptides with a Q value>1400 cal/mol are considered to potentially be bitter (Ney, 1971).

2.10   Frequency of amino acids in the P1 and P1' position

Enzyme specificity of AprX was represented using the subsite nomenclature from Schechter and Berger (Schechter & Berger, 1967). Amino acid residues are designated P1, P2, P3, P4, etc. in the N-terminal direction from the cleavage bond. Likewise, the amino acid residues in C-terminal position are designated P1', P2', P3', P4', etc., as shown in the following model:

$$PN\text{- - - - -}P4\text{-}P3\text{-}P2\text{-}P1 \neq P1'\text{-}P2'\text{-}P3'\text{-}P4'\text{- - - - -}PC'$$
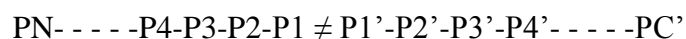
The scissile peptide bonds (the bond susceptible to cleavage) at the amino and carboxyl termini for each peptide were identified from the MS/MS analysis and the milk proteins amino acid sequence. The frequency of amino acids in the P1 and P1' position was studied based on all the peptides identified in five milk proteins (β-, α_{s1}-, α_{s2}, κ-casein and β-lactoglobulin) in all the AprX hydrolysed groups.

The cleavage frequency of every amino acid in the P1 or P1' position was calculated using the number of detected peptide bonds involving a specific amino acid in P1 or P1' position divided by the total number of peptide bonds involving this amino acid residue in P1 or P1' position (Matéos et al., 2015).

3. Results and discussion

3.1 Determination of the LTI condition

As shown in **Fig. 1**, the activity of AprX decreased to a different extent after 15 min of preincubation at different temperatures from 40 to 80 ℃. The most pronounced decrease in activity was found at 60 ℃ in skim and full-fat UHT milk and at 58 ℃ in PBS buffer, with 73%, 77% and 57% residual activity, respectively. Heating at 60 ℃ for 15 min was thus used as the LTI condition in our study.
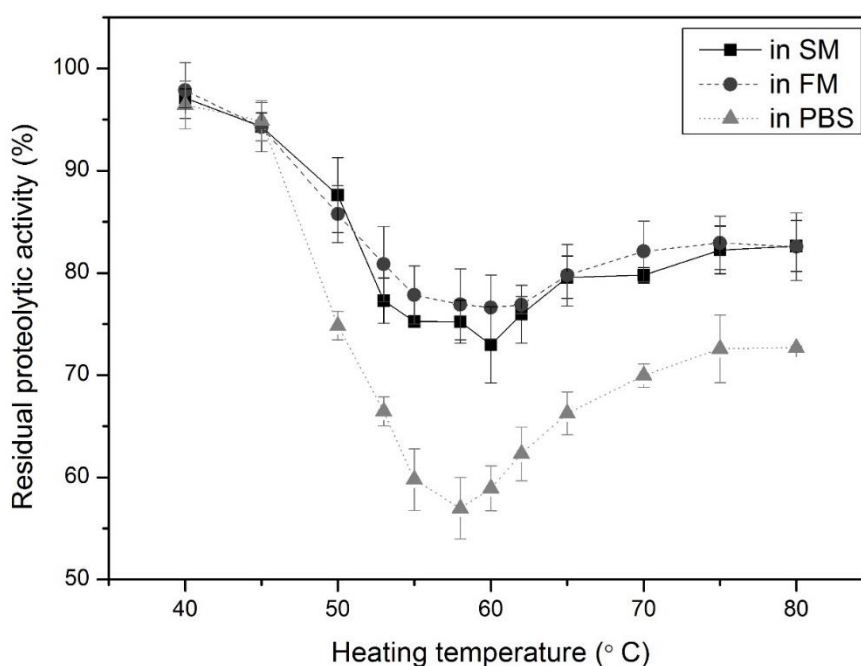
Fig. 1. Residual proteolytic activity of AprX after thermal treatment in skim UHT milk, full-fat UHT milk and 10mM phosphate buffered saline (PBS, pH 6.7). Ten mL of UHT milk or PBS buffer with addition of 2mg AprX was preincubated for 15 min at different temperatures from 40 to 80 ℃. Results are expressed as percentage of the enzyme activity without heated.

The decrease of AprX activity at 60 ℃ was most probably caused by the intermolecular autoproteolysis of the unfolded AprX molecules by native AprX molecules (Schokker & van Boekel, 1998a), as explained in the introduction. Similar temperature of maximum inactivation

has been observed for different *Pseudomonas* species (Glück et al., 2016; Stoeckel et al., 2016b). On the other hand, less AprX activity was lost in milk than in PBS (p<0.01), indicating milk proteins reduced AprX inactivation by autoproteolysis, which may be due to the milk proteins acting as a competitive substrate (Matéos et al., 2015).

3.2   Parent proteins

As reflected by lower inactivation in milk than in PBS, during the LTI milk proteins may also be hydrolysed by native AprX. To study the impact of LTI on UHT milk proteins, proteins and peptides were profiled across the 6 sample groups. Parent protein profiles were qualitatively and quantitatively assessed. A total of 48 detected parent proteins were correlated to a total of 2488 identified peptides, as detailed in **Table S1**. The order of peptide numbers detected in the main milk proteins was β-casein (738)>$α_{s1}$-casein (576)>$α_{s2}$-casein (279)>β-lactoglobulin (216)>κ-casein (190), with these five proteins representing 80.35% of the total peptide number. However, for quantitative comparisons, peptide intensity, rather than peptide presence, was used for further data analysis in this study.

To assess differences in parent protein levels between groups, hierarchical k-means clustering was done. As shown in **Fig. 2a**, large differences could be seen between LFQ intensities of control UHT milks and milks incubated with AprX. Samples also clustered according to the fat content, but not according to the LTI treatment. The increase in abundance of all peptides indicates extensive hydrolysis of the milk proteins.

Comparing the total intensity of the five abundant proteins (**Fig. 2b**), all the hydrolysed samples were significantly higher (P<0.005) than the untreated UHT samples. Besides, the control group FM was found to be significantly lower than SM (P<0.01), which may be due to different processing backgrounds in different batches of milk. Except for these, no significant differences in the total peptide intensity were found among the hydrolysed groups, regardless of fat content and LTI treatment, indicating LTI did not decrease the overall degree of hydrolysis.
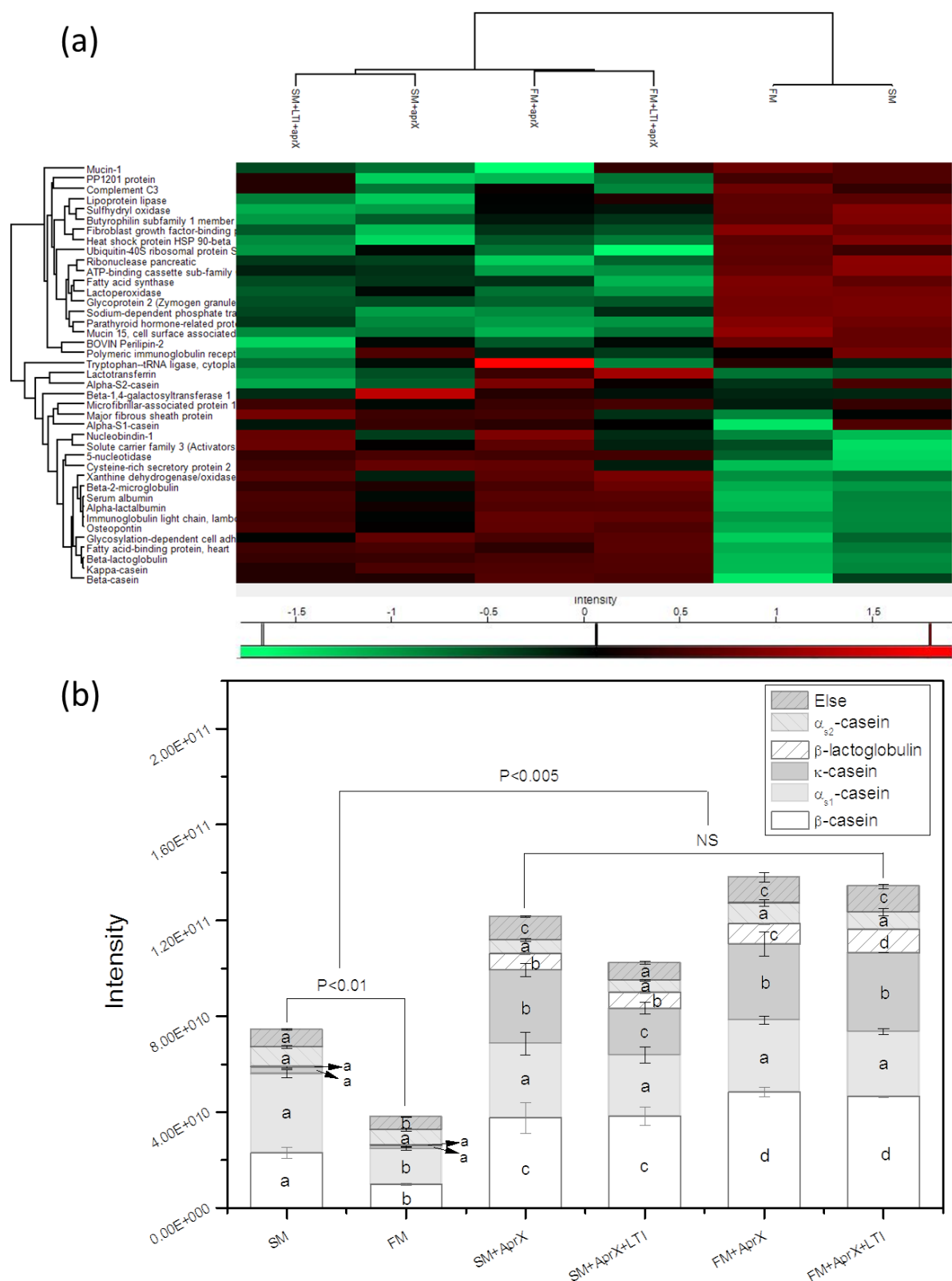
Fig. 2. Quantitative hierarchical k-means clustering shows samples as the column tree, parent proteins as the row tree, with lower intensities plotted as green with an increasing color scale from blank to red as the z-score normalized protein's LFQ intensity increase (a); Peptide LFQ intensity by parent proteins, different letter stands for significant difference p<0.01 (b).

The peptide intensity of the five principal parent proteins (α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, and β-lactoglobulin) were compared separately (**Fig. 2b**). In control samples (SM and FM), the peptides intensity originated from the parent proteins in the order α_{s1}-casein>β-casein>α_{s2}-casein>κ-casein>β-lactoglobulin, probably reflecting proteolysis by the native milk proteases, or by processing/storage (Ebner, Baum, & Pischetsrieder, 2016). After hydrolysis by AprX, the increase in peptide intensity was compared. It turned out that the total intensity of peptides from κ-casein and β-casein increased by the highest amounts, in both skim and full-fat UHT milk, indicating κ-casein and β-casein were most hydrolysed by AprX. This result is in accordance with the consensus that both κ-casein and β-caseins are preferential substrates for extracellular bacterial proteases (Law, Andrews, & Sharpe, 1977; Snoeren & Van Riel, 1979; Matéos et al., 2015).

The intensity of peptides from β-lactoglobulin also rose significantly (P<0.001). There was barely any peptides detected in the control samples (**Fig. 2b**), indicating that β-lactoglobulin mostly stayed intact in normal UHT milk, but they were hydrolysed intensively by AprX. Whey proteins have been considered so far to be relatively insensitive to the action of psychotropic bacterial proteases (Fairbairn & Law, 1986; Mu, Du, & Bai, 2009; Zhang et al., 2015). Nevertheless, based on our results, β-lactoglobulin was also readily hydrolysed by AprX, as also observed on the electrophoretic gel in our previous study (Zhang et al., 2018). Alpha-caseins are also reported to be substrates for AprX, even though to a lesser extent than κ- and β-casein (Zhang et al., 2018). However, no increase was found in the intensity α_{s1}- and α_{s2}-casein derived peptides compared to control samples. This indicates that alpha-caseins are relatively insensitive towards AprX hydrolysis.

On top of these five above-mentioned major milk protein, peptides from in total 25 parent proteins were found to be significantly higher in intensity after the hydrolysis by AprX, as listed in **Table S1**. In these proteins, we found several milk fat globule membrane (MFGM) proteins like mucins, butyrophilin; and many enzymes like lactoperoxidase, lipoprotein lipase, fatty acid synthase, sulfhydryl oxidase. This indicates that AprX can hydrolyse almost all proteins and is not specifically active on limited number of proteins. Due to the low peptide intensities of these proteins compared to the major proteins, the indigenous proteases were not identified by LC-MS/MS. But plasmin was found to be one of the two enzymes with the highest likelihood of activity when predicting the potential enzymes responsible for cleaving the proteins in blank UHT milk samples with EnzymePredictor. Enzymes that are the most likely to be active are

those with a combined high number of total cleavages and a high odds ratio, which means plasmin and trypsin in this study (**Fig. S1**).

3.3   Peptides

3.3.1   Peptide mapping

After comparing the total peptide intensities of the five most abundant parent proteins, we mapped the peptides of β-, κ-, α_{s1}- a_{s2}-casein and β-lactoglobulin in relation to the overall protein sequence to visualize the hydrolysis patterns of AprX on these proteins. Other parent proteins were not further studied because they have no known influence on UHT milk stability. The peptidomic profiles of FM and FM+AprX samples are shown in **Fig. 3** as the examples of control and treatment groups, respectively. The peptidomic profile of other control and treated sample pairs were similar to **Fig. 3**, as shown in **Fig. S2**.

Regarding the control sample FM, overall, high sequence coverage (β-casein 100%, α_{s1}-casein 89%, α_{s2}-casein 89%, κ-casein 67%, β-lactoglobulin 61%) was found. The number of peptides identified in the control UHT milk was relatively high; similar profiles for control UHT milk have also been reported by Baglinière et al. (2017). Most peptides detected came from β- and α_{s1}-caseins (**Fig. 3A1, 3C1**), while fewer peptides came from α_{s2}- and κ-casein (**Fig. 3D1, 3B1**), and β-lactoglobulin (**Fig. 3E1**). These peptides could either be naturally occurring in bovine milk by the actions of native proteases like plasmin (Dallas et al., 2013; Dallas, Weinborn, et al., 2014a), or have resulted from the cleavage of peptide bonds during severe UHT heating (Singh & Fox, 1995; Ebner et al., 2016).
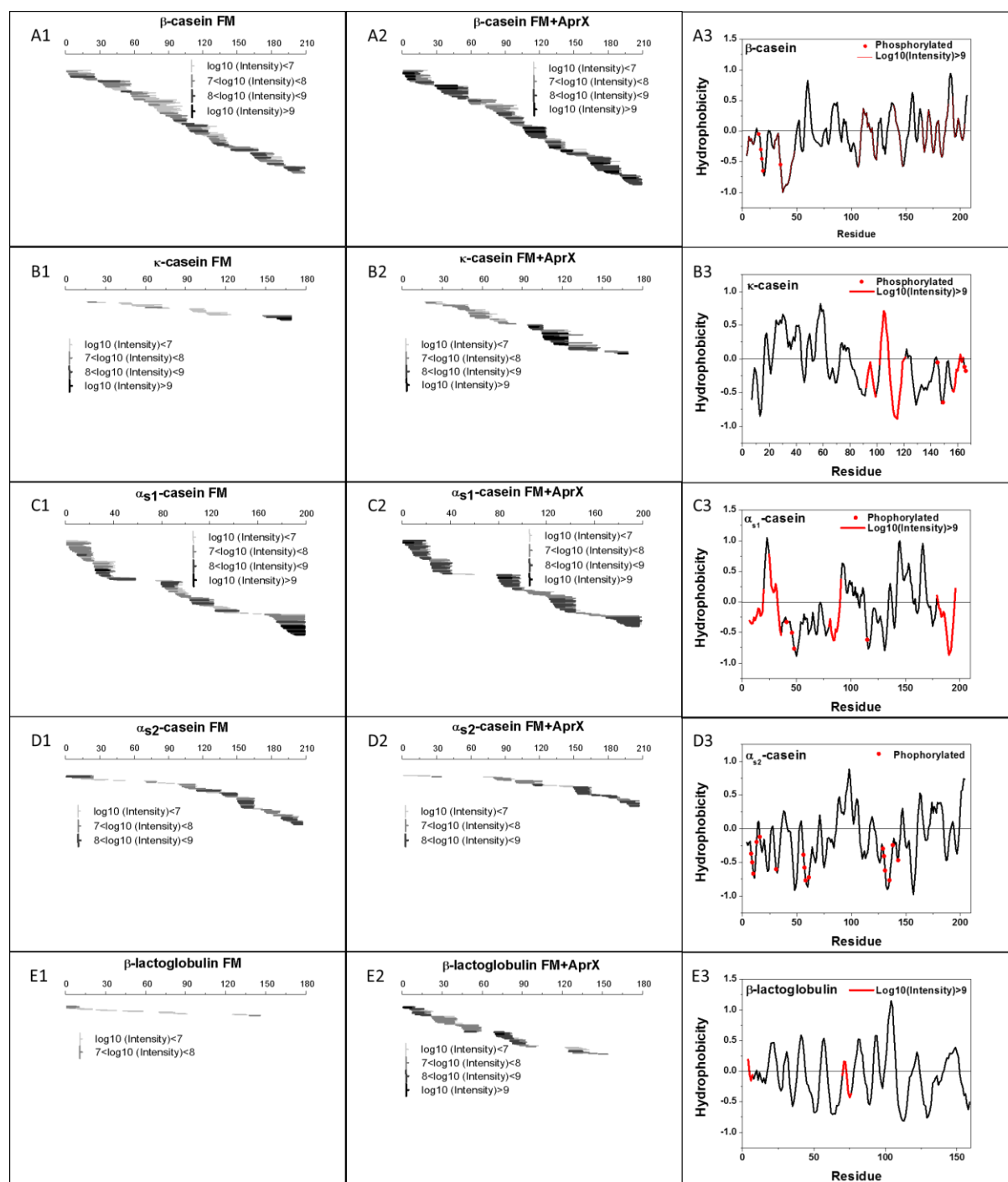
Fig. 3. Peptidomic profile in FM (1), FM+AprX (2) and hydrophobicity distribution (3) in β-casein (A), κ-casein (B), $α_{s1}$-casein (C), $α_{s2}$-casein (D) and β-lactoglobulin (E), respectively. Peptidomic profile was analyzed by a nano-LC/LTQ and Orbitrap. MS/MS, peptides with different intensity were distinguished by color and width, the intensity showed in the figure were the average value of triplicates. hydrophobicity was calculated according to Sweet and Eisenberg (1983), positive values indicate hydrophobic and negative values, hydrophilic regions. Peptides with intensity higher than 10^9 in FM+AprX were highlighted in red, and the detected phosphorylated clusters by LC-MS/MS were marked with (●).

With regard to the hydrolyzed FM samples, β-casein was readily cleaved all over the molecule (**Fig. 3A1, 3A2**). Beta-casein is preferentially hydrolysed by AprX because of its high concentration in the aqueous phase and particular free position in casein micelles (Holt, 1992). Strong proteolysis also characterized κ-casein (**Fig. 3B**). Even though the number of identified peptides coming from κ-casein was much lower than the other caseins (**Table S1**), the total intensity of the peptides from κ-casein ranked second after β-casein. The low number of identified peptides may be because the hydrolysis of κ-casein was focused on the peptide bonds Ger_{104}- Phe_{105} and Phe_{105}-Met_{106}, as also reported by Gaucher et al. (2011); Baglinière et al. (2013); Stuknytė et al. (2016). Compared with chymosin in cheese making, AprX will not only cleave the peptide bond Phe_{105}-Met_{106} of κ-casein, but also non-specifically cleave around the region of soluble hydrophilic glycomacropeptide (**Fig. 3B2**), which, like chymosin hydrolysis, leads to casein micelle destabilisation. **Fig 3C, 3D** show that the sequences of $α_{s1}$- and $α_{s2}$-casein were almost entirely covered by the peptides detected, except for the phosphorylated regions. Because phosphorylated peptides are typically less well ionised, they are therefore relatively difficult to identify by LC/MS (Solari, Dell'Aica, Sickmann, & Zahedi, 2015). Beta-lactoglobulin, the most abundant whey protein in bovine milk, has been regarded to be resistant to digestion because of its compactly folded structure (Dallas, Smink, et al., 2014b). This explains its low number and intensity of peptides in FM (**Fig. 3E1**). It is noteworthy that after incubation with AprX, the peptide number and intensity increased markedly, but the peptides originated from the same area of the protein sequence. This implies that AprX hydrolysed β-lactoglobulin by cleaving the same areas as the native milk proteases.

The peptidomic profile of milk proteins after the action of AprX has also been mapped by Gaucher et al. (2011); Baglinière et al. (2013); Matéos et al. (2015). However, the number of detected peptides in the present study was significantly higher than in these studies, which may be attributed to the different analytical approaches, making direct comparison between these studies difficult.

3.3.2   P1 and P1'

Fig. 4 shows the cleavage frequency of amino acids in the P1 and P1' position for all the peptides obtained after hydrolysis of β-, $α_{s1}$-, $α_{s2}$, κ-casein and β-lactoglobulin by AprX. AprX has a broad specificity, as apparent from the peptide mapping shown in **Fig. 3**. Overall, all the basic amino acid residues (His, Lys, Arg), aliphatic amino acid Leu, sulfur-containing residue Met, and aromatic amino acid Phe in both P1 and P1' position led to a strong cleavage of the peptide bond. Conversely, the presence of acidic amino acid residues (Asp, Glu) or the sulfur-

containing residue Cys in both P1 and P1' position, Ile in P1 position and Pro in the P1' position appeared unfavourable for the cleavage of the peptide bond by AprX. Matéos et al. (2015) have shown that when purified caseins were incubated with the *Pseudomonas* LBSA1 extracellular protease for 24 h, peptide bonds around basic amino acid residues and Phe were preferentially cleaved, while at the same time, peptide bonds around cysteine and proline were poorly cleaved, which is in accordance with our findings. However, the preference or disfavour of AprX for other amino acid residues, as reported by Matéos et al. (2015), was not replicated in our results. The different findings may be explained by differences in enzyme selectivity at different stages of hydrolysis (O'donoghue et al., 2012), or differences in the analytical methods used.
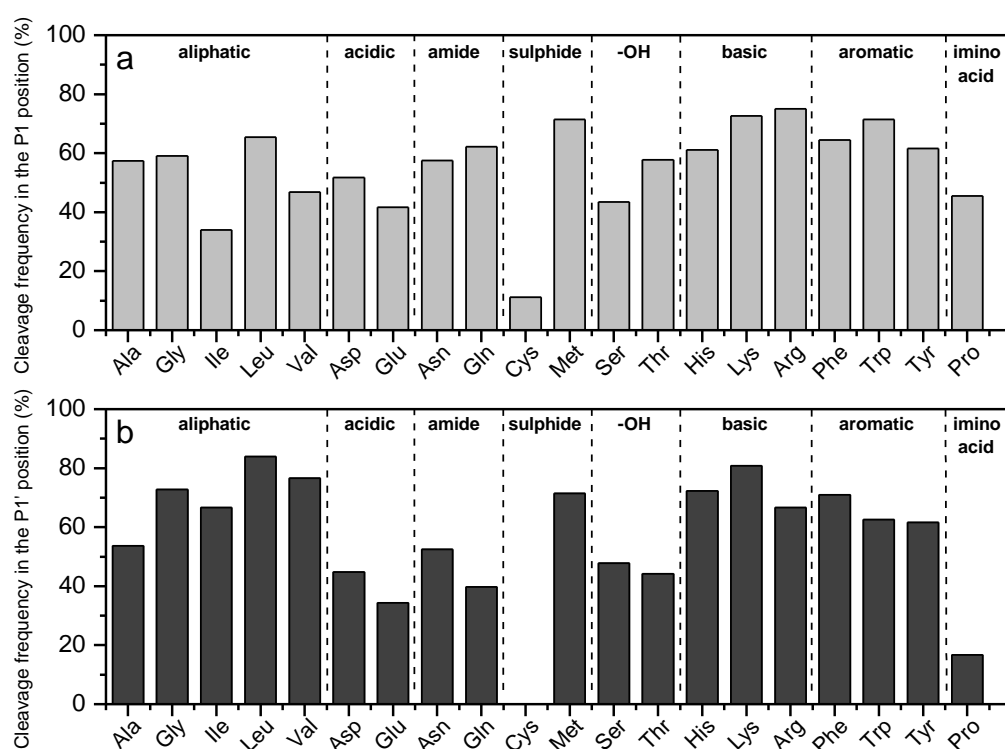
Fig. 4. Cleavage frequency for each amino acid in the P1 (a) and P1' (b) position according to their respective proportion. The analysis was based on the peptides identified in β-, α_{s1}-, α_{s2}, κ-casein and β-lactoglobulin in all the AprX hydrolysed samples. Amino acids were grouped by dashed line based on the side chain structure.

3.4   Effects of LTI

3.4.1   Differences in peptides

Even though the peptide mapping showed similar patterns in AprX-hydrolysed skim and full-fat milk irrespective of LTI treatment, some peptide sequences were found significantly

different by ANOVA. Volcano plots (**Fig. 5**) were made to visualize the differences of peptide intensities between groups with and without LTI treatment; details of the significantly different sequences are presented in **Table S2**.
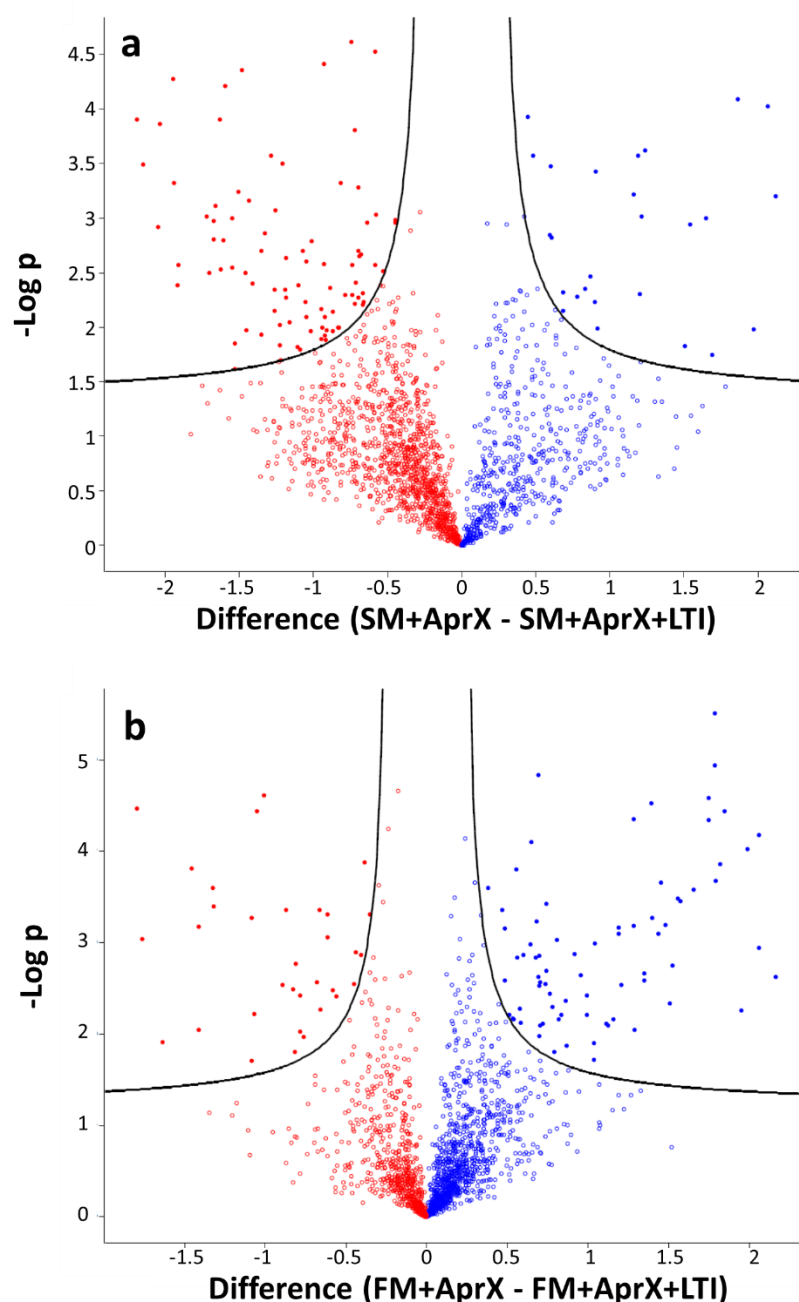
Fig. 5. Volcano plots depicting log 10 fold change in peptide intensity between groups (x axis) and −log p value (y-axis). All significant peptides are represented as filled circles above the line, and non-significant peptides are unfilled below the line. Peptide intensity distribution between SM+AprX and SM+AprX+LTI; peptides higher in SM+AprX are red and peptides higher in SM+AprX+LTI are blue (a). Peptide intensity distribution between FM+AprX and FM+AprX+LTI; peptides higher in FM+AprX are red and peptides higher in FM+AprX+LTI are blue (b).

As listed in **Table S2**, in total 115 and 108 peptides were found to be significant different (p<0.05) by LTI treatment in skim milk and full-fat milk, respectively. In the SM+AprX group, 89 peptides were found to be higher in intensity than in SM+AprX+LTI group, while the other way around, 26 peptides were higher in LTI groups. In full fat milk, however, the number of peptides with significantly higher intensity was found to be higher in LTI group (75 peptides) than in non-LTI group (33 peptides). Therefore, LTI seemed not to result in generally increased or decreased peptide levels.

Among all the different peptides, we paid particular attention to the caseinomacropeptide (CMP) fragments (sequences in the f(106-169) region in κ-casein), because these are very relevant for casein micelle stability. Based on our results (bolded in **Table S2**), 4 (3 higher in SM+AprX, 1 higher in SM+AprX+LTI) and 6 (2 higher in FM+AprX, 4 higher in FM+AprX+LTI) CMP fragments were found to be significantly different by LTI treatment in SM and FM, respectively. Again, no consistent conclusions could be drawn on the effects of LTI in reducing AprX-induced hydrolysis. Overall, the results indicate that LTI does not seem to lead to increased stability of AprX-contaminated milk.

3.4.2   Bitterness assessment

Bitterness due to formation of hydrophobic peptides is frequently encountered in UHT milk (Rauh et al., 2014a; Nielsen et al., 2016). In our study, in total 877 peptides were identified to have a Q value above 1400 cal/mol, which is an evaluation criteria of a bitter peptide (Nielsen et al., 2016). We multiplied the Q value of these potentially better peptides (Q>1400 cal/mol) with their intensity detected in different samples to quantitatively compare the bitterness potential. As shown in **Fig. 6**, the bitterness potential of the groups hydrolysed by AprX was significantly higher than the two blank groups, because hydrolysis gave rise to more hydrophobic peptides, which can contribute to bitterness (Jansson et al., 2014). Comparing among the AprX-hydrolysed groups, SM+AprX group was found to have higher bitterness potential than SM+AprX+LTI, indicating that LTI treatment may reduce the formation of bitter peptides in skim milk, although the absolute differences between groups is small. In full fat milk, however, LTI treatment did not influence the bitterness potential significantly. Therefore, for both peptide release as well as potential bitterness, no consistent conclusion on a possible advantage of LTI could be drawn.
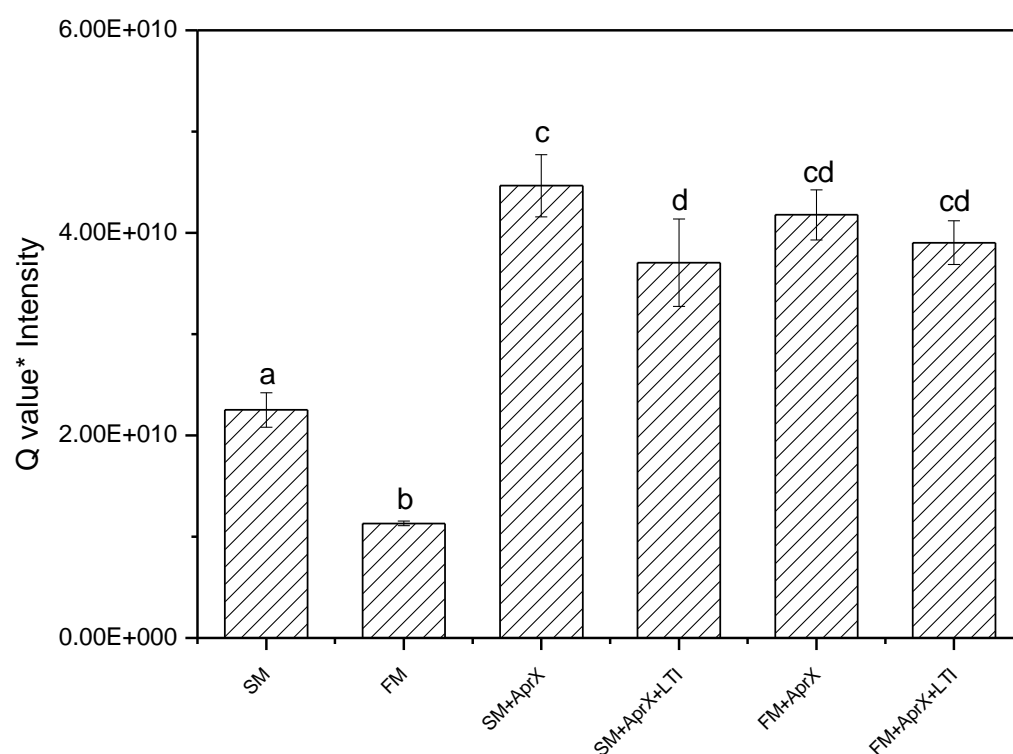
Fig. 6. Quantitative comparison of bitterness potential, expressed as the sum of Q value*Intensity of all the peptides with a Q value>1400 cal/mol in the sample.

4. Conclusions

The present study showed that the protease AprX produced by *Pseudomonas* could hydrolyse proteins in UHT milk, particularly κ-casein and β-casein. Identification of the peptides showed high sequence coverage, and analysis of cleavage sites revealed AprX did not have a strong specificity for specific amino acids. An LTI at 60 ℃ for 15 min could partially inactivate AprX activity in UHT milk. However, this process did not significantly reduce the subsequent proteolysis of milk proteins, especially in full-fat UHT milk. This indicates that during LTI, besides AprX itself, mainly the milk proteins were hydrolysed. Therefore, the feasibility of using a low temperature heat treatment as a means of inactivating AprX may be limited in UHT milk.

Supplementary materials

Table S1: Details of parent proteins from which peptides were derived. The last column indicate peptides from this parent protein were detected to have significant higher intensity in the AprX-hydrolysed samples than the blank UHT milk samples.

Protein IDs	Fasta headers	Peptide counts (all)	Mol. weight [kDa]	Sequence lengths	Higher in AprX
P02666	Beta-casein	738	25.107	224	✓
P02662	Alpha-S1-casein	576	24.529	214	✓
P02668	Kappa-casein	190	21.269	190	✓
P02754	Beta-lactoglobulin	216	19.883	178	✓
P02663	Alpha-S2-casein	279	26.018	222	✓
P80195	Glycosylation-dependent cell adhesion molecule 1	124	17.151	153	✓
Q0IIA4	Glycoprotein 2 (Zymogen granule membrane)	27	59.232	534	✓
P81265	Polymeric immunoglobulin receptor	65	82.434	757	✓
P31096	Osteopontin	51	30.904	278	✓
P80025	Lactoperoxidase	25	80.641	712	✓
P18892	Butyrophilin subfamily 1 member A1	35	59.276	526	✓
Q27960	Sodium-dependent phosphate transport protein 2B	21	75.825	693	✓
P80457	Xanthine dehydrogenase/oxidase	5	146.79	1332	
P17248	Tryptophan--tRNA ligase, cytoplasmic	2	53.811	476	
Q9TUM6	BOVIN Perilipin-2	24	49.368	450	✓
P00711	Alpha-lactalbumin	17	16.246	142	
P11151	Lipoprotein lipase	7	53.377	478	✓
Q5EA98	Microfibrillar-associated protein 1	1	51.977	439	
P01888	Beta-2-microglobulin	2	13.677	118	
Q05927	5-nucleotidase	2	62.965	574	✓
Q71SP7	Fatty acid synthase	5	274.55	2513	✓
P02769; P49065	Serum albumin	10	69.161	606;607; 607	
Q9MZ06	Fibroblast growth factor-binding protein 1	8	26.188	234	✓
Q1RMN8;A4IFI0	Immunoglobulin light chain, lambda gene cluster	4	24.536	234;235	
A7E340	Mucin 15, cell surface associated	7	35.685	330	✓
P08037	Beta-1,4-galactosyltransferase 1	4	44.842	402	
Q9XS94	Major fibrous sheath protein	2	93.987	848	✓
Q5EA54	Solute carrier family 3 (Activators of dibasic and neutral amino acid transport), member 2	1	63.21	572	
P58073	Parathyroid hormone-related protein	5	20.408	177	✓
Q3ZCL0	Cysteine-rich secretory protein 2	3	27.453	244	
Q2UVX4	Complement C3	4	187.25	1661	✓
A6QQA8	Sulfhydryl oxidase	2	62.974	567	✓
Q76LV1;Q76LV2	Heat shock protein HSP 90-beta	3	83.252	724;733	✓
Q0P569	Nucleobindin-1	4	54.982	474	

Protein IDs	Fasta headers	Peptide counts (all)	Mol. weight [kDa]	Sequence lengths	Higher in AprX
Q4GZT4	ATP-binding cassette sub-family G member 2	1	72.724	655	✓
P61823	Ribonuclease pancreatic	1	16.461	150	
Q8WML4	Mucin-1	3	58.091	580	✓
P24627	Lactotransferrin	2	78.056	708	
P10790	Fatty acid-binding protein	1	14.779	133	
P62992;P63048;P0CG53;P0CH28	Ubiquitin-40S ribosomal protein S27a	2	17.965	156;128;305;690	
Q6QRN7	PP1201 protein	2	33.948	308	
P35541	Serum amyloid A protein	2	14.516	130	
P01035	Cystatin-C	1	16.265	148	
Q95122	Monocyte differentiation antigen CD14	1	39.666	373	
P38408	Guanine nucleotide-binding protein subunit alpha-14	1	41.498	355	
Q0VCX2	78 kDa glucose-regulated protein	1	72.399	655	
Q3T178	Vacuolar protein sorting-associated protein 28 homolog	1	25.484	221	

Table S2: Differing peptide sequences by LTI.

	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
Peptides higher in SM+AprX	EELNVPGE	Beta-casein	-0.5843	<0.0001	L	E	E	I	19	26	8
	ESLSSSEESITR	Beta-casein	-0.7138	0.0039	V	E	R	I	29	40	12
	SITRINKK	Beta-casein	-1.0485	0.0025	E	S	K	I	37	44	8
	ITRINKK	Beta-casein	-1.5296	0.0241	S	I	K	I	38	44	7
	INKKIEK	Beta-casein	-1.1594	0.0090	R	I	K	F	41	47	7
	IEKFQSEEQQQTEDELQDKIHPFAQTQS	Beta-casein	-2.1872	0.0001	K	I	S	L	45	72	28
	FQSEEQQQTEDELQDKIHPF	Beta-casein	-0.6608	0.0059	K	F	F	A	48	67	20
	FQSEEQQQTEDELQDKIHPFAQTQS	Beta-casein	-0.5741	0.0009	K	F	S	L	48	72	25
	SEEQQQTEDELQD	Beta-casein	-0.6987	0.0054	Q	S	D	K	50	62	13
	TEDELQDKIHP	Beta-casein	-0.9307	<0.0001	Q	T	P	F	56	66	11
	KIHPFAQTQS	Beta-casein	-0.9446	0.0068	D	K	S	L	63	72	10
	HPFAQTQS	Beta-casein	-1.6230	0.0030	I	H	S	L	65	72	8
	HPFAQTQ	Beta-casein	-1.2578	0.0009	I	H	Q	S	65	71	7
	TQTPVVVPPFLQPEVMGVSKVKEA	Beta-casein	-0.8663	0.0072	L	T	A	M	93	116	24
	QPEVMGVSKVKEA	Beta-casein	-0.8839	0.0044	L	Q	A	M	104	116	13
	VMGVSKVKE	Beta-casein	-1.2599	0.0046	E	V	E	A	107	115	9
	MGVSKVKEA	Beta-casein	-0.6874	0.0023	V	M	A	M	108	116	9
	GVSKVKE	Beta-casein	-1.4552	0.0032	M	G	E	A	109	115	7
	KEAMAPK	Beta-casein	-1.3473	0.0020	V	K	K	H	114	120	7
	EAMAPKH	Beta-casein	-1.4540	0.0106	K	E	H	K	115	121	7
	FTESQSLTL	Beta-casein	-0.9198	0.0120	P	F	L	T	134	142	9

Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
WMHQPHQPLPPTVMFPPQS	Beta-casein	-1.0136	0.0016	S	W	S	V	158	176	19
VMFPPQS	Beta-casein	-1.1825	0.0054	T	V	S	V	170	176	7
SQSKVLPVP	Beta-casein	-0.7365	0.0027	L	S	P	Q	181	189	9
KAVPYPQRD	Beta-casein	-1.3501	0.0116	Q	K	D	M	191	199	9
KAVPYPQ	Beta-casein	-1.0703	0.0020	Q	K	Q	R	191	197	7
KHQGLPQEVL	Alpha-S1-casein	-1.6726	0.0011	I	K	L	N	22	31	10
HQGLPQE	Alpha-S1-casein	-1.9143	0.0041	K	H	E	V	23	29	7
FGKEKVNE	Alpha-S1-casein	-1.4346	0.0007	V	F	E	L	47	54	8
VPSERYLGYLEQLL	Alpha-S1-casein	-0.5292	0.0031	D	V	L	R	101	114	14
KKYKVPQ	Alpha-S1-casein	-1.9093	0.0027	L	K	Q	L	117	123	7
LHSMKEG	Alpha-S1-casein	-1.0177	0.0109	R	L	G	I	135	141	7
HSMKEGIHAQQKEPM	Alpha-S1-casein	-1.3291	0.0014	L	H	M	I	136	150	15
IHAQQKEP	Alpha-S1-casein	-1.2844	0.0003	G	I	P	M	142	149	8
HAQQKEPMIG	Alpha-S1-casein	-1.5918	<0.0001	I	H	G	V	143	152	10
HAQQKEP	Alpha-S1-casein	-1.2237	0.0096	I	H	P	M	143	149	7
AQQKEPM	Alpha-S1-casein	-1.5439	0.0010	H	A	M	I	144	150	7
IGVNQEL	Alpha-S1-casein	-1.6067	0.0016	M	I	L	A	151	157	7
PSFSDIPNPIGSENSEKTTMP	Alpha-S1-casein	-1.4067	0.0040	A	P	P	L	192	212	21
PNPIGSENSEKTTMP	Alpha-S1-casein	-1.0467	0.0081	I	P	P	L	198	212	15
ALNEINQ	Alpha-S2-casein	-0.6641	0.0062	K	A	Q	F	96	102	7
KLTEEEKNR	Alpha-S2-casein	-1.1815	0.0023	T	K	R	L	167	175	9
FLKKISQ	Alpha-S2-casein	-1.2629	0.0071	N	F	Q	R	178	184	7
KISQRYQKF	Alpha-S2-casein	-1.7191	0.0010	K	K	F	A	181	189	9
PQYLKTVYQHQ	Alpha-S2-casein	-0.7207	0.0061	L	P	Q	K	192	202	11

Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
KTVYQHQK	Alpha-S2-casein	-1.6593	0.0008	L	K	K	A	196	203	8
TVYQHQKAM	Alpha-S2-casein	-1.1891	0.0046	K	T	M	K	197	205	9
KAMKPWIQPK	Alpha-S2-casein	-1.2092	0.0003	Q	K	K	T	203	212	10
KAMKPWIQPKT	Alpha-S2-casein	-0.8662	0.0109	Q	K	T	K	203	213	11
PWIQPKTKVIP	Alpha-S2-casein	-0.5818	0.0027	K	P	P	Y	207	217	11
KVIPYVR	Alpha-S2-casein	-1.0524	0.0059	T	K	R	Y	214	220	7
VLSRYPS	Kappa-casein	-0.9401	0.0101	Y	V	S	Y	52	58	7
VLSRYPSYG	Kappa-casein	-0.8316	0.0101	Y	V	G	L	52	60	9
YGLNYYQQKP	Kappa-casein	-1.1033	0.0152	S	Y	P	V	59	68	10
NYYQQKP	Kappa-casein	-0.6384	0.0011	L	N	P	V	62	68	7
YYQQKPV	Kappa-casein	-1.6701	0.0016	N	Y	V	A	63	69	7
YQQKPVAL	Kappa-casein	-0.9452	0.0068	Y	Y	L	I	64	71	8
AVRSPAQ	Kappa-casein	-0.9103	0.0106	A	A	Q	I	87	93	7
TMARHPHPH	**Kappa-casein**	**-1.9399**	**0.0005**	**T**	**T**	**H**	**L**	**115**	**123**	**9**
TMARHPHPHLSF	**Kappa-casein**	**-0.7175**	**0.0002**	**T**	**T**	**F**	**M**	**115**	**126**	**12**
VQVTSTA	**Kappa-casein**	**-0.6943**	**0.0005**	**T**	**V**	**A**	**V**	**183**	**189**	**7**
AMAASDISLLDAQSAP	Beta-lactoglobulin	-1.5055	0.0006	L	A	P	L	39	54	16
LDAQSAPLR	Beta-lactoglobulin	-0.6976	0.0020	L	L	R	V	48	56	9
VEELKPTPEGDLEILL	Beta-lactoglobulin	-0.6786	0.0022	Y	V	L	Q	59	74	16
LKPTPEGDLE	Beta-lactoglobulin	-0.7845	0.0051	E	L	E	I	62	71	10
KKIIAEKT	Beta-lactoglobulin	-2.0448	0.0012	Q	K	T	K	85	92	8
KIPAVFK	Beta-lactoglobulin	-1.9462	<0.0001	T	K	K	I	93	99	7
VDDEALEK	Beta-lactoglobulin	-2.0315	0.0001	E	V	K	F	144	151	8

Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
IRLSFNPT	Beta-lactoglobulin	-0.7458	<0.0001	H	I	T	Q	163	170	8
NKPEDETH	Glycosylation-dependent cell adhesion molecule 1	-0.9185	0.0132	L	N	H	L	21	28	8
LISKEQIVI	Glycosylation-dependent cell adhesion molecule 1	-0.4470	0.0010	D	L	I	R	62	70	9
ISKEQIVIR	Glycosylation-dependent cell adhesion molecule 1	-0.7384	0.0051	L	I	R	S	63	71	9
IVIRSSRQPQ	Glycosylation-dependent cell adhesion molecule 1	-2.1481	0.0003	Q	I	Q	S	68	77	10
SSRQPQSQNPKLP	Glycosylation-dependent cell adhesion molecule 1	-1.2167	0.0199	R	S	P	L	72	84	13
SSRQPQSQNPKLPLS	Glycosylation-dependent cell adhesion molecule 1	-1.6997	0.0032	R	S	S	I	72	86	15
SSRQPQSQNP	Glycosylation-dependent cell adhesion molecule 1	-1.5439	0.0028	R	S	P	K	72	81	10
RQPQSQNPKLPL	Glycosylation-dependent cell adhesion molecule 1	-1.2258	0.0147	S	R	L	S	74	85	12
KQSNSKY	Immunoglobulin light chain, lambda gene cluster	-1.1014	0.0041	S	K	Y	A	188	194	7
QRPPKIQVY	Beta-2-microglobulin	-1.4791	<0.0001	I	Q	Y	S	22	30	9
DGVAKLS	Complement C3	-0.9203	0.0081	D	D	S	I	402	408	7
DRITGGKDFRDIES	Lipoprotein lipase	-0.8172	0.0005	A	D	S	K	29	42	14
DELKRQEVS	Nucleobindin-1	-1.0866	0.0161	L	D	S	R	98	106	9
KPDPSQKQT	Osteopontin	-0.9264	0.0027	L	K	T	F	45	53	9

	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
	SVAYGLKSRSKKF	Osteopontin	-0.8283	0.0102	D	S	F	R	155	167	13
	AAPAGAAIQS	Polymeric immunoglobulin receptor	-0.6642	0.0049	K	A	S	R	576	585	10
	AGEIQNKAL	Polymeric immunoglobulin receptor	-0.9442	0.0127	R	A	L	L	587	595	9
	GSSKALVSTLVPLA	Polymeric immunoglobulin receptor	-0.4460	0.0011	S	G	A	L	627	640	14
	DTHKSEIAHRF	Serum albumin	-1.6288	0.0001	R	D	F	K	24	34	11
	VKSVASLG	Xanthine dehydrogenase/oxidase	-1.5281	0.0141	Q	V	G	G	342	349	8
Peptides higher in SM+AprX+LTI	LEELNVPGEIVE	Beta-casein	0.5915	0.0014	E	L	E	S	18	29	12
	VMFPPQSVLS	Beta-casein	0.6033	0.0015	T	V	S	L	170	179	10
	VLSLSQSKVLPVPQ	Beta-casein	1.6872	0.0179	S	V	Q	K	177	190	14
	LSQSKVLPVPQKAVPYPQRDMPIQA	Beta-casein	0.6002	0.0003	S	L	A	F	180	204	25
	VPYPQRDMPIQA	Beta-casein	1.1906	0.0003	A	V	A	F	193	204	12
	RDMPIQAFLL	Beta-casein	0.6851	0.0048	Q	R	L	Y	198	207	10
	RPKHPIKHQGLPQEVLNENL	Alpha-S1-casein	1.9692	0.0104	A	R	L	L	16	35	20
	HQGLPQEVLNENLL	Alpha-S1-casein	0.4842	0.0003	K	H	L	R	23	36	14
	VAPFPEVFGKEKVNEL	Alpha-S1-casein	0.8345	0.0045	F	V	L	S	40	55	16
	KVPQLEIVPNSAEERLHSMKEG	Alpha-S1-casein	2.0654	<0.0001	Y	K	G	I	120	141	22
	LEIVPNSAEERLHSMKEG	Alpha-S1-casein	1.1587	0.0006	Q	L	G	I	124	141	18
	EGIHAQQKEPMIGVNQ	Alpha-S1-casein	0.9144	0.0102	K	E	Q	E	140	155	16
	IHAQQKEPMIGVNQELA	Alpha-S1-casein	1.8628	<0.0001	G	I	A	Y	142	158	17
	QGPIVLNPWDQVK	Alpha-S2-casein	1.2117	0.0010	Y	Q	K	R	116	128	13
	LINNQFLPYPYYA	Kappa-casein	1.2366	0.0002	A	L	A	K	71	83	13
	INNQFLPYPYYA	Kappa-casein	0.9053	0.0004	L	I	A	K	72	83	12

	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
	FLPYPYYA	Kappa-casein	1.2008	0.0050	Q	F	A	K	76	83	8
	QILQWQVL	Kappa-casein	0.7815	0.0053	A	Q	L	S	93	100	8
	ILQWQVL	Kappa-casein	0.6821	0.0071	Q	I	L	S	94	100	7
	RHPHPHLSF	**Kappa-casein**	**0.8695**	**0.0034**	**A**	**R**	**F**	**M**	**118**	**126**	**9**
	VEELKPTPEGDLEIL	Beta-lactoglobulin	0.4452	0.0001	Y	V	L	L	59	73	15
	ILNKPEDETHLEAQPTDASAQFI	Glycosylation-dependent cell adhesion molecule 1	2.1153	0.0006	A	I	I	R	19	41	23
	LNKPEDETHLEAQPTDASAQFIRNLQ	Glycosylation-dependent cell adhesion molecule 1	1.5057	0.0150	I	L	Q	I	20	45	26
	KPEDETHLEAQPTDASAQF	Glycosylation-dependent cell adhesion molecule 1	0.8986	0.0059	N	K	F	I	22	40	19
	VGVHPPLQGSSHGAAAIGQPSGELRL	Beta-1,4-galactosyltransferase 1	1.6472	0.0010	L	V	L	R	54	79	26
	KAFLDSRTRL	Lactoperoxidase	1.5394	0.0011	N	K	L	K	47	56	10
Peptides higher in FM+AprX	EKFQSEEQQQTEDELQDKIHP	Beta-casein	-1.3218	0.0003	I	E	P	F	46	66	21
	LQPEVMGVS	Beta-casein	-0.4501	0.0028	F	L	S	K	103	111	9
	VSKVKEAMAPK	Beta-casein	-0.8267	0.0032	G	V	K	H	110	120	11
	AMAPKHKEMPFP	Beta-casein	-0.6816	0.0027	E	A	P	K	116	127	12
	APKHKEMPFP	Beta-casein	-1.0089	<0.0001	M	A	P	K	118	127	10
	TESQSLT	Beta-casein	-1.0848	0.0005	F	T	T	L	135	141	7
	HQPHQPLPPT	Beta-casein	-1.0521	<0.0001	M	H	T	V	160	169	10
	VLPVPQKAVPYPQRDMPIQA	Beta-casein	-0.6612	0.0004	K	V	A	F	185	204	20
	AVPYPQRDMPIQA	Beta-casein	-0.7613	0.0107	K	A	A	F	192	204	13
	KHIQKEDVPSER	Alpha-S1-casein	-1.4111	0.0007	Q	K	R	Y	94	105	12

| Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
HIQKEDVPSER	Alpha-S1-casein	-0.5579	0.0039	K	H	R	Y	95	105	11
IQKEDVPSERY	Alpha-S1-casein	-0.6132	0.0005	H	I	Y	L	96	106	11
KEDVPSE	Alpha-S1-casein	-1.4112	0.0090	Q	K	E	R	98	104	7
LGYLEQLLR	Alpha-S1-casein	-0.3543	0.0005	Y	L	R	L	107	115	9
NSEKTTMP	Alpha-S1-casein	-0.8696	0.0004	E	N	P	L	205	212	8
ITVDDKH	Alpha-S2-casein	-0.7826	0.0094	K	I	H	Y	86	92	7
TVDDKHY	Alpha-S2-casein	-0.7824	0.0037	I	T	Y	Q	87	93	7
LTEEEKNRL	Alpha-S2-casein	-0.5805	0.0033	K	L	L	N	168	176	9
VYQHQKAM	Alpha-S2-casein	-1.0697	0.0060	T	V	M	K	198	205	8
KAMKPWIQP	Alpha-S2-casein	-0.4065	0.0014	Q	K	P	K	203	211	9
AMKPWIQPKTK	Alpha-S2-casein	-1.4551	0.0002	K	A	K	V	204	214	11
TKVIPYVR	Alpha-S2-casein	-0.6573	0.0054	K	T	R	Y	213	220	8
VLSRYPSYGLNYYQQKPVA	Kappa-casein	-1.0824	0.0196	Y	V	A	L	52	70	19
YYQQKPVAL	Kappa-casein	-0.3850	0.0001	N	Y	L	I	63	71	9
TMARHPHPH	**Kappa-casein**	**-1.6366**	**0.0121**	**T**	**T**	**H**	**L**	**115**	**123**	**9**
TMARHPHPHLS	**Kappa-casein**	**-0.8927**	**0.0029**	**T**	**T**	**S**	**F**	**115**	**125**	**11**
TPEVDDEALEKFDKAL	Beta-lactoglobulin	-0.6142	0.0009	R	T	L	K	141	156	16
IVIRSSRQPQ	Glycosylation-dependent cell adhesion molecule 1	-1.7614	0.0009	Q	I	Q	S	68	77	10
LPLSILKE	Glycosylation-dependent cell adhesion molecule 1	-0.4394	0.0013	K	L	E	K	83	90	8
AHVKQVL	Lactotransferrin	-0.8082	0.0017	A	A	L	L	624	630	7
AGEIQNKA	Polymeric immunoglobulin receptor	-1.3216	0.0004	R	A	A	L	587	594	8
DTHKSEIAHRF	Serum albumin	-0.8169	0.0158	R	D	F	K	24	34	11

	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
	KLPTQKT	Xanthine dehydrogenase/oxidase	-1.7934	<0.0001	A	K	T	E	318	324	7
Peptides higher in FM+AprX+LTI	RELEELNVPGEIVESLS	Beta-casein	0.5408	0.0069	A	R	S	S	16	32	17
	IEKFQSEEQQQTEDELQDKIHPF	Beta-casein	0.6972	0.0014	K	I	F	A	45	67	23
	ELQDKIHP	Beta-casein	1.5036	0.0046	D	E	P	F	59	66	8
	KIHPFAQTQS	Beta-casein	0.6829	0.0006	D	K	S	L	63	72	10
	HPFAQTQ	Beta-casein	1.2820	0.0006	I	H	Q	S	65	71	7
	SLPQNIPPLTQTPVVVPPFLQPEVMG	Beta-casein	2.1611	0.0024	N	S	G	V	84	109	26
	QPEVMGVSKVKEA	Beta-casein	0.7390	0.0020	L	Q	A	M	104	116	13
	VMGVSKVKE	Beta-casein	1.3490	0.0026	E	V	E	A	107	115	9
	MGVSKVKEA	Beta-casein	1.0357	0.0125	V	M	A	M	108	116	9
	SKVKEAM	Beta-casein	0.8323	0.0061	V	S	M	A	111	117	7
	KYPVEPFTES	Beta-casein	0.7219	0.0076	P	K	S	Q	128	137	10
	QSWMHQPH	Beta-casein	1.7925	0.0002	L	Q	H	Q	156	163	8
	RDMPIQA	Beta-casein	0.6770	0.0014	Q	R	A	F	198	204	7
	RPKHPIK	Alpha-S1-casein	2.0576	0.0012	A	R	K	H	16	22	7
	RPKHPIKHQG	Alpha-S1-casein	2.0585	<0.0001	A	R	G	L	16	25	10
	HPIKHQGLPQE	Alpha-S1-casein	1.2809	<0.0001	K	H	E	V	19	29	11
	HQGLPQEVLNENLLRF	Alpha-S1-casein	0.5776	0.0052	K	H	F	F	23	38	16
	HQGLPQEVLNENLL	Alpha-S1-casein	0.6000	0.0014	K	H	L	R	23	36	14
	FVAPFPEVFGKEKVNEL	Alpha-S1-casein	0.6913	0.0024	F	F	L	S	39	55	17
	PEVFGKEKVNEL	Alpha-S1-casein	0.7808	0.0050	F	P	L	S	44	55	12
	FGKEKVNE	Alpha-S1-casein	0.8642	0.0043	V	F	E	L	47	54	8
	FGKEKVNEL	Alpha-S1-casein	1.1872	0.0007	V	F	L	S	47	55	9

|	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
	HIQKEDVPS	Alpha-S1-casein	1.2070	0.0029	K	H	S	E	95	103	9
	HIQKEDVPSE	Alpha-S1-casein	1.8426	<0.0001	K	H	E	R	95	104	10
	IQKEDVPSER	Alpha-S1-casein	1.5730	0.0004	H	I	R	Y	96	105	10
	ERYLGYLEQ	Alpha-S1-casein	0.3803	0.0003	S	E	Q	L	104	112	9
	SAEERLHSM	Alpha-S1-casein	1.3892	<0.0001	N	S	M	K	130	138	9
	LHSMKEGIHAQQKEPM	Alpha-S1-casein	0.9538	0.0023	R	L	M	I	135	150	16
	LHSMKEGIHAQQ	Alpha-S1-casein	1.5561	0.0003	R	L	Q	K	135	146	12
	HSMKEGIHAQQKEPM	Alpha-S1-casein	1.3995	0.0005	L	H	M	I	136	150	15
	SMKEGIHAQQKEPM	Alpha-S1-casein	1.5232	0.0018	H	S	M	I	137	150	14
	MKEGIHAQQKEPMIGVNQELAY	Alpha-S1-casein	1.1235	0.0080	S	M	Y	F	138	159	22
	KEGIHAQQKEP	Alpha-S1-casein	0.6405	0.0010	M	K	P	M	139	149	11
	IHAQQKEP	Alpha-S1-casein	1.7443	<0.0001	G	I	P	M	142	149	8
	HAQQKEP	Alpha-S1-casein	0.8158	0.0069	I	H	P	M	143	149	7
	PIGSENSEKTTMPLW	Alpha-S1-casein	0.6979	0.0105	N	P	W	-	200	214	15
	SENSEKTTMPLW	Alpha-S1-casein	0.4700	0.0004	G	S	W	-	203	214	12
	IISQETYKQEK	Alpha-S2-casein	0.7924	0.0156	S	I	K	N	29	39	11
	NREQLSTSEENS	Alpha-S2-casein	0.5119	0.0061	L	N	S	K	139	150	12
	TKLTEEEKNRL	Alpha-S2-casein	1.7827	<0.0001	K	T	L	N	166	176	11
	KISQRYQKF	Alpha-S2-casein	0.8682	0.0132	K	K	F	A	181	189	9
	YLKTVYQHQ	Alpha-S2-casein	1.7842	<0.0001	Q	Y	Q	K	194	202	9
	LKTVYQHQ	Alpha-S2-casein	1.4345	0.0008	Y	L	Q	K	195	202	8
	TVYQHQKAM	Alpha-S2-casein	1.0371	0.0190	K	T	M	K	197	205	9
	KAMKPWIQPKT	Alpha-S2-casein	1.2896	0.0089	Q	K	T	K	203	213	11

Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
YYQQKPV	Kappa-casein	0.9148	0.0013	N	Y	V	A	63	69	7
LINNQFLPYPYYA	Kappa-casein	0.7645	0.0036	A	L	A	K	71	83	13
RHPHPHLS	**Kappa-casein**	**1.9511**	**0.0056**	**A**	**R**	**S**	**F**	**118**	**125**	**8**
KKNQDKTEIPTINT	**Kappa-casein**	**0.4838**	**0.0007**	**P**	**K**	**T**	**I**	**132**	**145**	**14**
KKNQDKTEIPT	**Kappa-casein**	**1.7450**	**<0.0001**	**P**	**K**	**T**	**I**	**132**	**142**	**11**
IASGEPTSTPTTE	**Kappa-casein**	**0.9918**	**0.0038**	**T**	**I**	**E**	**A**	**146**	**158**	**13**
IIAEKTKIP	Beta-lactoglobulin	1.4816	0.0006	K	I	P	A	87	95	9
NENKVLV	Beta-lactoglobulin	1.6553	0.0003	L	N	V	L	104	110	7
LKALPMH	Beta-lactoglobulin	1.1575	0.0069	A	L	H	I	156	162	7
KPEDETHLEAQPTDASAQFIRN	Glycosylation-dependent cell adhesion molecule 1	0.6926	<0.0001	N	K	N	L	22	43	22
KPEDETHLEAQPTDASAQF	Glycosylation-dependent cell adhesion molecule 1	0.6462	<0.0001	N	K	F	I	22	40	19
KPEDETHLEAQPTDASAQ	Glycosylation-dependent cell adhesion molecule 1	1.9871	<0.0001	N	K	Q	F	22	39	18
LISKEQIVI	Glycosylation-dependent cell adhesion molecule 1	0.5336	0.0068	D	L	I	R	62	70	9
ISKEQIVIRSS	Glycosylation-dependent cell adhesion molecule 1	1.0395	0.0010	L	I	S	R	63	73	11
SSRQPQSQNPK	Glycosylation-dependent cell adhesion molecule 1	0.7048	0.0027	R	S	K	L	72	82	11
RQPQSQNP	Glycosylation-dependent cell adhesion molecule 1	0.7420	0.0004	S	R	P	K	74	81	8
RQPQSQNPKLPL	Glycosylation-dependent cell adhesion molecule 1	0.5531	0.0002	S	R	L	S	74	85	12
RQPQSQNPK	Glycosylation-dependent cell adhesion molecule 1	0.8064	0.0009	S	R	K	L	74	82	9
RQPQSQNPKLP	Glycosylation-dependent cell adhesion molecule 1	1.8172	0.0001	S	R	P	L	74	84	11
IVQNNDSTEYG	Alpha-lactalbumin	1.1108	0.0076	A	I	G	L	60	70	11

	Sequence	Protein group	Log 10 fold change	p-value	Amino acid before	First amino acid	Last amino acid	Amino acid after	Start position	End position	Length
	VSREGQEQEGEEMAEYRG	Butyrophilin subfamily 1 member A1	0.6974	0.0030	F	V	G	R	76	93	18
	KNVQTEIVNKHND	Cysteine-rich secretory protein 2	1.1886	0.0008	L	K	D	L	35	47	13
	KLDELKRQEVS	Nucleobindin-1	0.9952	0.0061	T	K	S	R	96	106	11
	DELKRQEVS	Nucleobindin-1	0.7373	0.0028	L	D	S	R	98	106	9
	HELDSASSEVN	Osteopontin	1.4522	0.0002	S	H	N	-	268	278	11
	KPDPSQKQT	Osteopontin	0.7022	0.0081	L	K	T	F	45	53	9
	GAAIQSRAGEIQN	Polymeric immunoglobulin receptor	0.4862	0.0026	A	G	N	K	580	592	13
	AAGGPGAPADPGRPTGYSGSSKA	Polymeric immunoglobulin receptor	0.5806	0.0074	D	A	A	L	609	631	23
	KDLGEEHF	Serum albumin	0.5609	0.0014	F	K	F	K	35	42	8
	KVPQVSTPT	Serum albumin	1.3455	0.0022	R	K	T	L	436	444	9
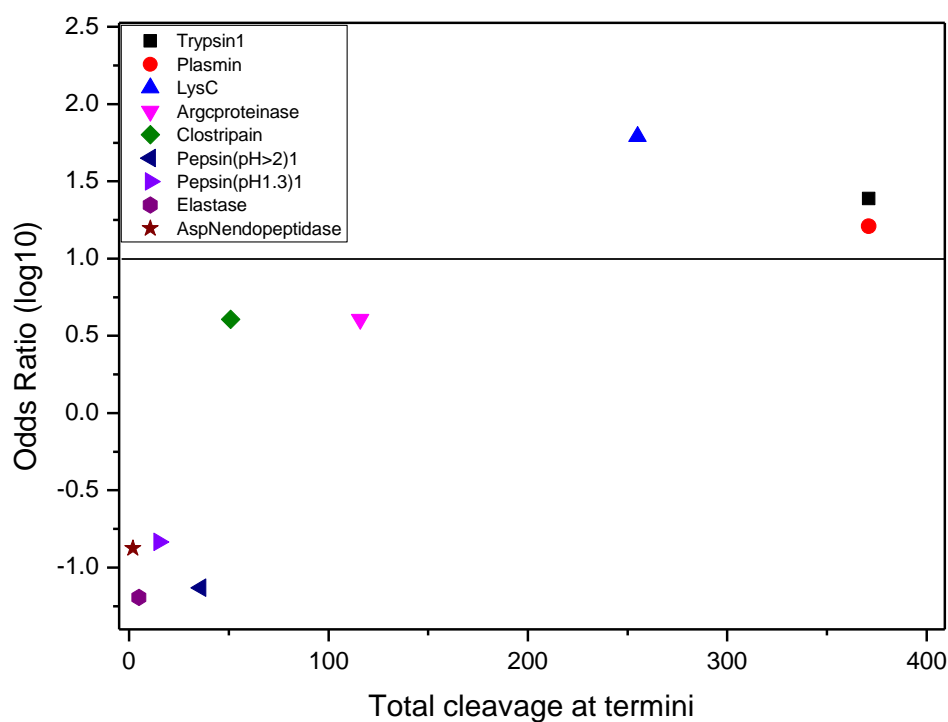
Fig. S1. Scatter plot of the total sites cleaved by an enzyme, at termini (x-axis) and log odds ratio (y-axis). Enzymes that are the most likely to be active in milk are those with a combined high number of total cleavages and a high odds ratio, represented by the top most right corner. For the enzymes that do not have expression in milk, data indicate the presence of other enzymes with similar activity.
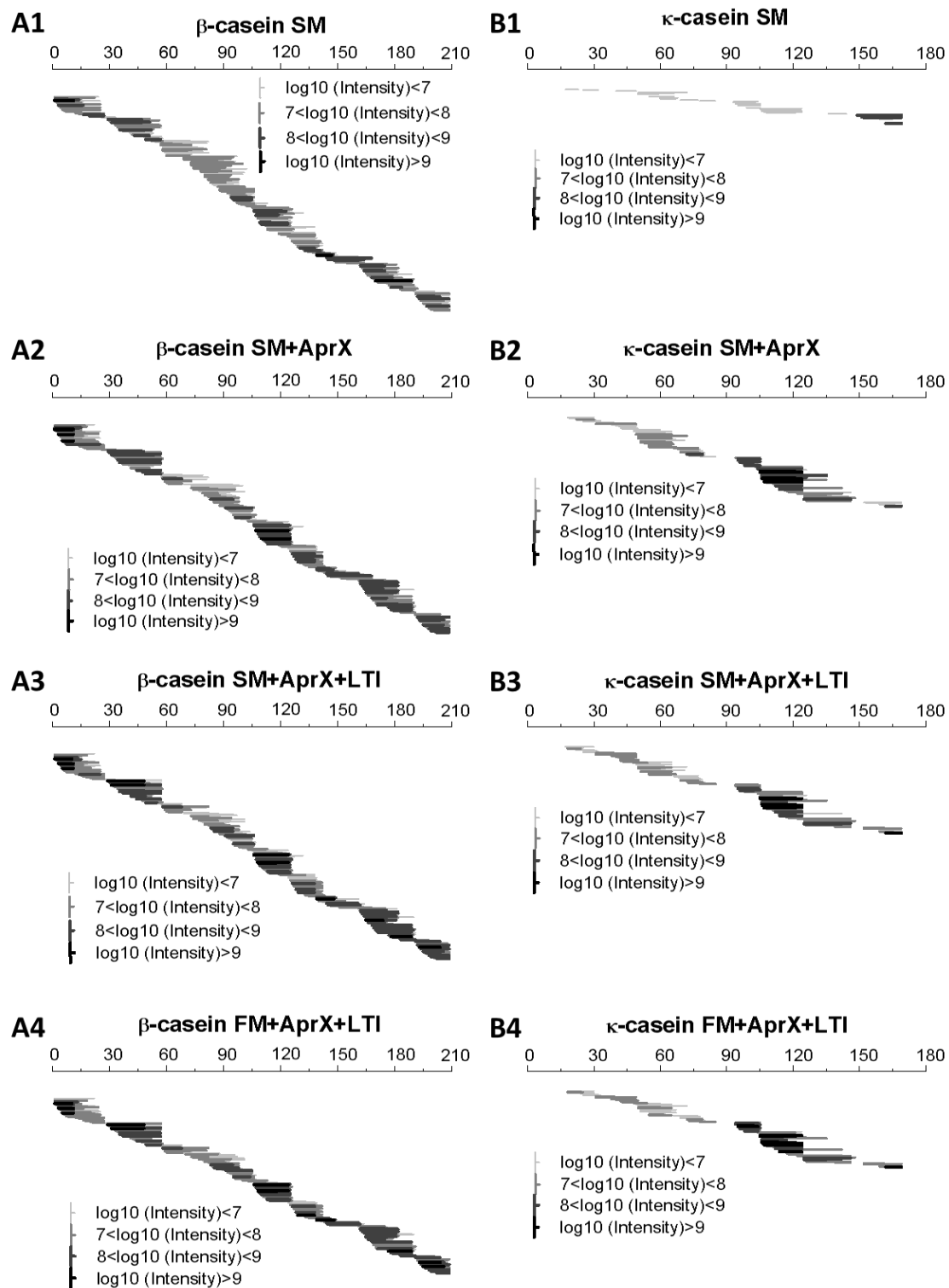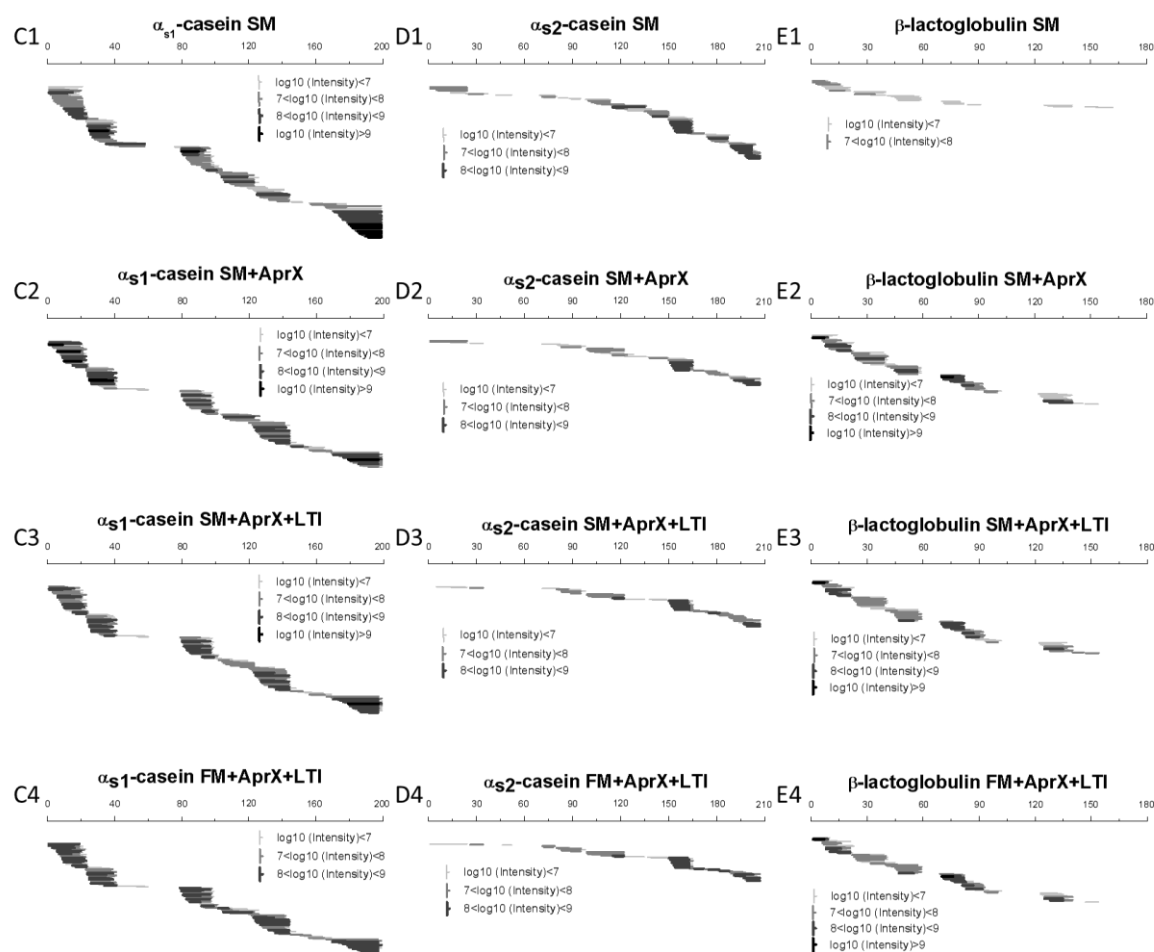
Fig. S2. Peptidomic profile from β-casein (A), κ-casein (B), αs1-casein (C), αs2-casein (D) and β-lactoglobulin (E) in SM, SM+AprX, SM+AprX+LTI and FM+AprX+LTI, respectively, analyzed by a nano-LC/LTQ and Orbitrap. MS/MS. Peptides with different intensity were distinguished by color and width. The intensity showed in the figure were the average value of triplicates.

Chapter 6

General discussion

UHT milk is an indispensable dairy product in many countries. However, its stability during storage can be seriously impaired by proteolysis, caused by high levels of thermoresistant proteases originating from low quality raw milk. Two main categories of problematic proteases in UHT milk are the native protease plasmin and proteases produced by psychrotrophic bacteria. While both categories of proteases have been widely studied previously in UHT milk, there is little direct comparison between their influence, and the enzyme-induced changes were mostly occurring simultaneously with the non-enzymatic reactions in previous studies. Hence, the work in this thesis is focussed on obtaining deeper understanding of the role of bacterial and native proteases on the destabilization of UHT milk. In this way, UHT milk instability is linked with raw milk quality, and suggestions on quality control measures in the whole dairy chain can be given.

The research questions studied in each chapters are: which proteases are involved (**Chapter 2**)? How is the stability of UHT milk influenced by the proteases (**Chapters 3, 4**)? Can we adjust the processing to achieve enhanced inactivation of enzymes (**Chapter 5**)? In this chapter, theoretically, an extension of current theories on the instability of UHT milk during shelf life is obtained, by combining the results of all previous chapters and additional experiments; practically, the sequence of product defects during storage of UHT milk and the detection of the cause of the defects are discussed; at last, recommendations for further study from the viewpoint of both scientific research and industrial dairy processing are given.

1.   New insights in the instability of UHT milk

1.1   New insights in the destabilization of proteins in UHT milk

Age gelation is the most prominent defect caused by protein destabilization in UHT milk. Age gelation is proposed to occur either enzymatically through proteases or non-enzymatically through physicochemical processes.

1.1.1   AprX- and plasmin-induced gelation

In our study, we found that both AprX and plasmin can lead to gelation in skim UHT milk as a result of enzymatic hydrolysis. Our results in **Chapter 3** showed that for gelled samples containing specific proteases, the same pattern and level of hydrolysis was found, irrespective of protease concentration, storage time or storage temperature. This indicates that the onset of enzyme-induced gelation is determined by a specific critical degree of hydrolysis of specific caseins, which differs between enzymes.

It is widely recognised that AprX-induced gelation of UHT milk occurs as a result of hydrolysis of κ-casein that is similar to renneting by chymosin (Nieuwenhuijse & van Boekel, 2003). However, AprX and chymosin also differ in some ways. Chymosin is an aspartic enzyme that can specifically cleave the Phe_{105}-Met_{106} bond of κ-casein, in a rate that is much higher than it cleaves at other positions. Although AprX also preferentially cleaves κ-casein at Phe_{105}-Met_{106}, it is a more generic protease which can also hydrolyse other caseins at almost any position, resulting in the production of a wide range of small and medium-sized, potentially-bitter, peptides (**Chapter 5**). Upon gelation in UHT milk, not only 95% of κ-casein, but also more than 45% of β-casein and 35% of $α_{s1}$-casein have been hydrolysed, indicating that AprX stimulates gelation by both cleaving the hydrophilic tails of κ-casein on the surface, and simultaneously destabilizing the internal part of casein micelles (**Chapter 3**).

While the involvement of plasmin activity in UHT milk instability has been studied intensively, the underlying mechanisms are only known to a limited extent (Chavan, Chavan et al., 2011, Rauh, 2014a). We attempted to provide more insights in the gelation pathways by correlating the physical changes with casein hydrolysis. Combining our results with previous studies, the process of plasmin-induced gelation can be inferred as four steps: penetrating & loosening, disassembling, rearranging, and aggregation & clarification (**Chapter 3**). To be specific, plasmin first penetrates into the casein micelles and hydrolyzes around the regions that are essential for the internal integrity of the casein micelles. When more than 60% of both β- and $α_{s1}$-caseins are hydrolysed, the generated amphiphilic and charged polypeptides tend to rearrange and aggregate into a gel network. Our conclusion is in line with the fact that polypeptides are the main building blocks of plasmin-induced gel in UHT milk, as reported by (De Koning et al., 1985, Manji & Kakuda, 1988).

Besides the pathways of destabilizing milk proteins, the types of bonds and intermolecular forces between proteins in the gel network are also important, but very little is known about this for UHT milk protein gels. We measured the turbidity of the enzyme-induced gels after mixing it with different concentrations of dissociating reagents at pH 6.6, to recreate conditions similar to the final gelation pH (Rauh, Sundgren et al., 2014b). As shown in **Fig. 1a**, treatment with 1 mol/L SDS was not enough to fully solubilize AprX-induced gels, whereas SDS was able to fully solubilize the plasmin-induced gel already at 0.1 mol/L (**Fig. 1a**). It is known that SDS is able to reduce hydrophobic protein-protein interactions (Nelson 1971); the results therefore suggest that hydrophobic forces are a main factor contributing to network assembly in plasmin-induced gelation, but not in AprX-induced gelation. Next to hydrophobic forces,

hydrogen bonding is also known to be involved in maintaining many protein networks. It is found that urea, which can disrupt the hydrogen bonding network (Huppertz, Fox et al., 2018), was able to solubilize AprX-induced gelation at a concentration of 0.5 mol/L, whereas plasmin-induced gelation was less influenced by urea (**Fig. 1b**), indicating that hydrogen bonds are important in AprX-induced gel formation, but less so in plasmin-induced gel formation. In addition, the roles of disulfide bonds and electrostatic interactions were explored by measuring the change in turbidity after mixing with different concentrations of the reducing agent β-mercaptoethanol and NaCl, respectively. Only small differences were observed (**Fig. 1c, d**), suggesting both disulfide bonds and electrostatic interactions make limited contributions to enzyme-induced gelation. Furthermore, it is well recognized that calcium bridges are essential for inducing aggregation and gelation of casein micelles (Corredig and Salvatore 2016), but not for the aggregation of the polypeptides (Nieuwenhuijse and van Boekel 2003); likewise, calcium bridges are expected to play a key role in AprX-induced gelation, but not plasmin-induced gel network formation, although we did not test this. We thus tentatively conclude that hydrogen bonding together with calcium bridges, and hydrophobic interactions are the main forces involved in the AprX- and plasmin-induced gelation, respectively.
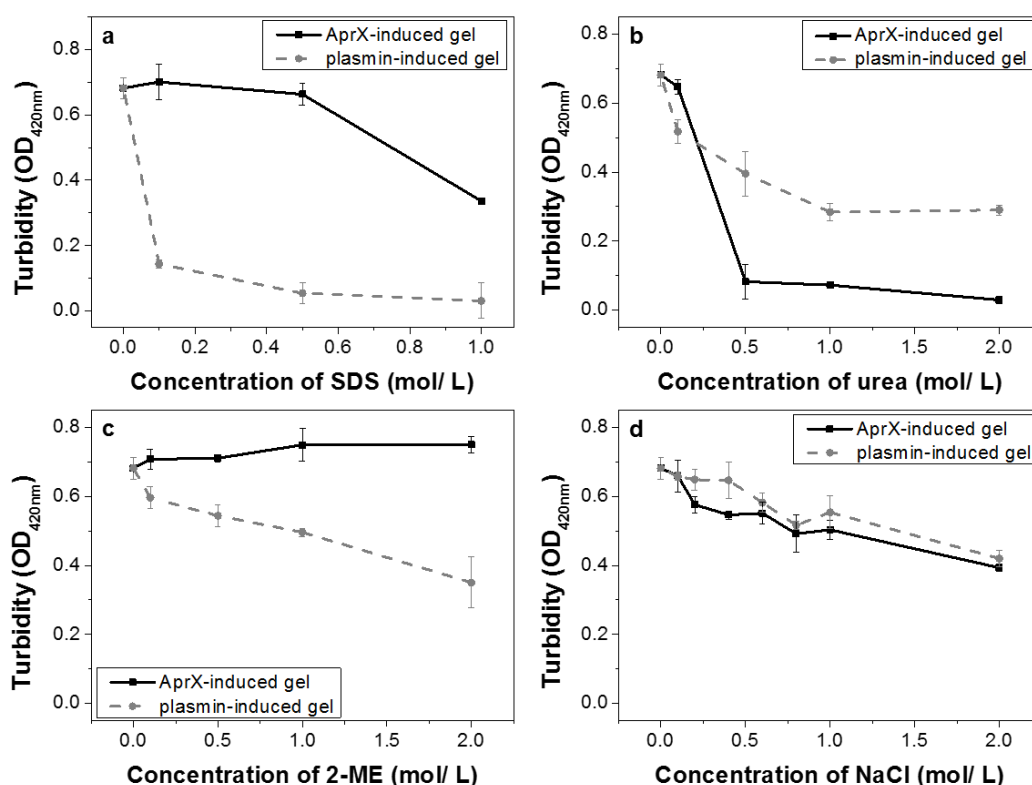
Fig. 1. Turbidity (OD_{420nm}) of the AprX- and plasmin-induced gels treated with different concentrations of dissociating reagents: sodium dodecyl sulfate (SDS) (a); urea(b); β-mercaptoethanol (2-ME) (c); and NaCl (d).

Besides the above discussed enzyme-induced gelation, as described in **Chapter 1**, two other non-enzymatic pathways, which are bridging flocculation involving β-lactoglobulin/κ-casein complexes (βκ-complexes) and gelled sediment caused by high temperature storage, can also lead to destabilization of proteins in UHT milk. The next section will further discuss the relationships between the enzymatic and non-enzymatic induced gelation.

1.1.2   The relationship between enzyme-induced gelation and bridging flocculation

The cases discussed in **Section 1.1.1** all refer to rapid gelation induced by enzymatic proteolysis in model systems. However, the enzyme activity in UHT milk will be much lower in real life, and proteolysis is not the sole trigger of gelation in UHT milk. Therefore, the other pathways of gelation must also be taken into consideration. The gelation of UHT milk after storage for many months at room temperature or below has been proposed to be led by a kind of "bridging flocculation" (Nieuwenhuijse and van Boekel 2003). This model was first proposed by Wilson, Vetter et al. (1963) for concentrated milk, and then extensively studied by McMahon (1996) for UHT-sterilized, UF-concentrated milk. McMahon's theory is that during the long-term storage of UHT milk, the βκ-complexes that are formed during heat treatment, dissociate from casein micelles, and subsequently aggregate into three-dimensional networks, eventually causing the milk protein gel to form. This theory is acknowledged in explaining the mechanism of the slow age gelation in UHT milk stored at a temperature lower than 30 ℃. Other than the explanation from the colloid and food science perspective, the aggregation mechanism has recently been proposed by Holt, Carver et al. (2013) to fit with the intrinsic chaperone action of κ-casein from a biological perspective. A molecular chaperone means a protein can interact with a target protein to either help it acquire its native conformation or stabilize the target protein against aggregation (Holt, Carver et al., 2013). In UHT milk, κ-casein may complex with β-lactoglobulin, resulting in the inhibition of the aggregation and precipitation of β-lactoglobulin. The formed protein aggregates called amyloid fibrils dissociate from casein micelles, and contribute to the gel formation when the fibril-forming proteins become concentrated in solutions over time (Guijarro, Sunde et al., 1998).

Even though various theories have been proposed from different perspectives, this "βκ-complexes-related" theory does not explain the mechanisms underlying cases with enzymatic proteolysis. McMahon (1996) suggested that both plasmin and bacterial proteases can accelerate or cause age gelation, by hydrolyzing the proteins that anchor βκ-complexes to the casein micelles and in this way allowing the release of the complexes from the micelles followed by the formation of a gel. The promotion of the release of βκ-complexes into serum

by the presence of plasmin was also discovered by Crudden, Fox et al. (2005). But the released βκ-complexes may not be the basis for forming a gel. For example, Malmgren (2007) and Rauh, Sundgren et al. (2014b) found that in the UHT milk samples with low activity of plasmin, the gel was composed of the protein fragments from proteolysis by plasmin, and only low concentrations of β-lactoglobulin and κ-casein were found in the formed gel, indicating that the formation and release of βκ-complexes was not the main cause of gelation in their plasmin-containing samples.
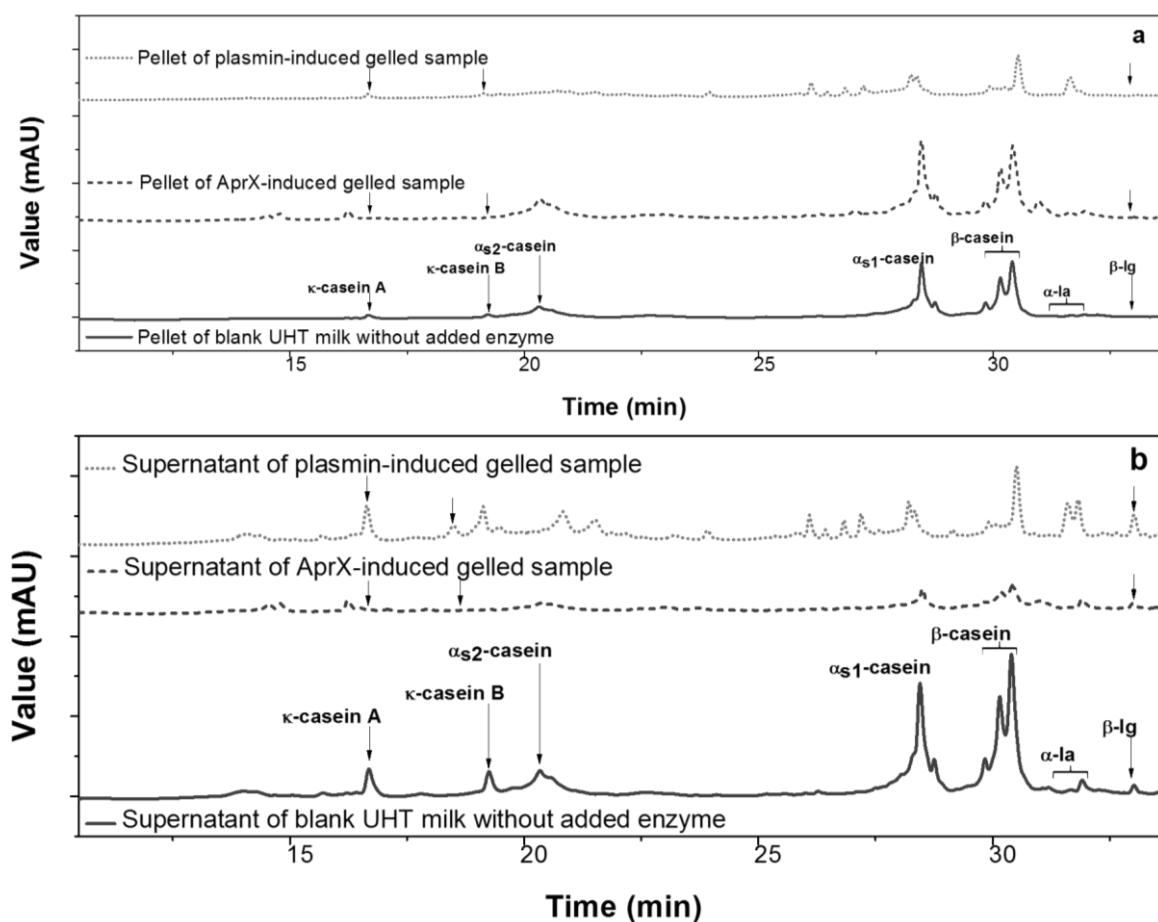
Fig. 2. Protein profiles of gel particles (a) and liquid phase (b) in skim UHT milk hydrolysed by 70 µg/mL AprX and 10 µL/mL plasmin after storage at 37 ℃ for 6 hours, separated by centrifuging at 9,000 g for 5 min at 20 ℃. Washed pellets were dissolved in a solution comprised of 0.1 mol/L Bis Tris buffer, 8 mol/L Urea, 5.37 mmol/L sodium citrate and 19.5 mmol/L DTT (pH 7).

To find out whether βκ-complexes participate in the enzyme-induced gelation or not, we analyzed the protein composition of the gel and the liquid phase of AprX- and plasmin-induced gelled samples. We found that in AprX-induced gelled samples, intact κ-casein was absent in both the gel and the liquid phase (**Fig. 2a**), which is due to the almost complete hydrolysis of

κ-casein by AprX upon gelation (**Chapter 3**). The AprX-induced gel was mainly composed of α- and β-caseins, but without the presence of β-lactoglobulin (**Fig. 2a**). In plasmin-induced gelled sample, as shown in **Fig. 2b**, almost all κ-casein and β-lactoglobulin ended up in the supernatant, meanwhile the gel was mainly made up of intact α- and β-caseins and their breakdown products. Summarizing, bridging flocculation involving βκ-complexes seems to be only relevant for the gelation of UHT milk in the absence of significant enzymatic proteolysis.

1.1.3   The relationship between enzyme-induced gelation and glycation

Since UHT milk can be preserved without refrigeration, the storage temperature may vary greatly in different regions. Among all the reactions happening during the storage of UHT milk, it is the glycation (or Maillard) reaction that is influenced most by temperature. Andrews (1975) found that during several months of storage at temperatures of 30 ˚C, the extent of glycation was several times greater than the heat-induced changes resulting from the UHT process itself. Therefore, the significance of storage temperature and glycation on the enzymatic hydrolysis should be taken into account during long-term storage, especially when storage is in a warm environment.

The impact of storage temperature has been studied extensively and literature results are contradictory in this regard. (Ramsey and Swartzel 1984, Newstead, Paterson et al., 2006, Malmgren, Ardö et al., 2017) discovered that the extent of sedimentation increased with increasing temperature. However, conflicting results were reported by Samel, Weaver et al. (1971), Kocak and Zadow (1985a), who found that gelation occurs more readily at room temperatures (20 to 25 ˚C) than at higher (35 to 40 ˚C) temperatures. The slower gelation at higher temperature was interpreted by Samel, Weaver et al. (1971) to be due to fact that gelation may be inhibited by the glycation, because the casein–lactose interactions involve lysine residues, which are also a prime target of plasmin. This interpretation is reasonable in their study because the enzyme activity in their samples was so low that gelation only happened after the milk had been stored for at least 13 months, which is long enough for considerable glycation to occur. But in short-term studies like our study in **Chapter 3**, the enzymatic hydrolysis occurs much faster than the glycation, implying that the proteolytic gelation is not counteracted by glycation. In short, we can thus infer that impact of temperature on the stability of UHT milk is negatively correlated with protease level because at a higher level of proteolysis destabilisation will happen much fast than glycation. In addition, the hydrolysis by AprX should be less influenced by glycation than plasmin, because the attachment of lactose at the ε-amino group

of lysine during glycation makes specifically the plasmin cleavage sites unrecognisable (Lapolla, Fedele et al., 2004, Bhatt, Cucheval et al., 2014).

Having reflected on, and extended, the existing theories, it can be concluded that the relative impact of βκ-complexes as well as glycation depends on the level of enzyme present, with higher levels of enzymes making these other processes less relevant.

1.2   New insights in the destabilization of fat in UHT milk

Fat globules in UHT milk are an oil-in-water emulsion, which is stabilized by adsorbed proteins, mainly being caseins. Any loss, damage, or change in the adsorbed protein layer may cause instability of the emulsion. Different appearance of fat destabilization in UHT milk induced by bacterial proteases and plasmin has been reported (Kohlmann, Nielsen et al., 1991, Hardham 1998, Rauh, Sundgren et al., 2014b), but the mechanisms underlying the enzyme-induced fat destabilisation by both enzymes are not yet clear. Therefore, we tried to fill this knowledge gap by elucidating the mechanisms of AprX- and plasmin-induced destabilization of fat globules in full-fat UHT milk (**Chapter 4**). We found that in AprX-containing samples, the fast hydrolysis of κ-casein led to flocculation of casein micelles together with fat globules, resulting in protein-rich fat fractions that are dense enough to sediment. In plasmin-containing samples, on the other hand, because the hydrolysis of α- and β-caseins disrupts the casein micelle fragments on the milk fat globule surface, there is insufficient protein material to stabilize the fat globule surfaces, causing the fat globules to coalesce and ascend.

2.   Instability of UHT milk and its diagnosis

This thesis described several kinds of product defects during storage of UHT milk: curd-like gel, weak gel and clarification in **Chapter 3**, sediment and cream layer in **Chapter 4** and bitterness in **Chapter 5**. This shows that proteases decrease the stability of UHT milk based on several concurrent mechanisms, or different phases of a single mechanism, resulting in multiple physical appearances. In this section, the sequence of occurrence and the diagnostic tests for different quality defects will be discussed, in order to help the UHT milk manufacturer to determine the cause of instability depending on the changes observed.
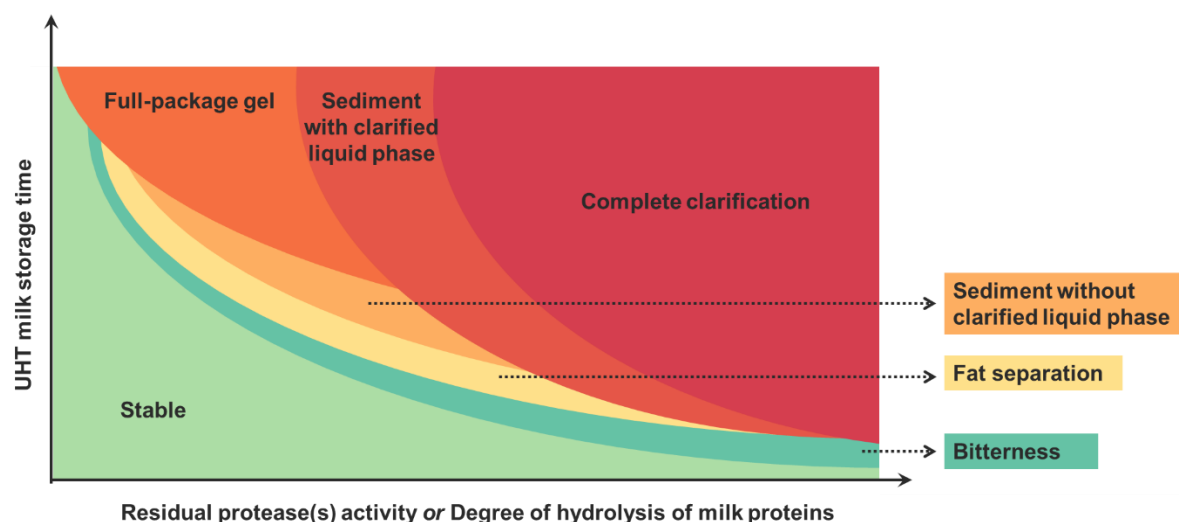
2.1   Sequence of product defects during storage of UHT milk

The casein micelles and fat globules of sterilised milks undergo considerable changes during storage, depending on the temperature and time of storage, as well as the enzymatic activity and enzymatic specificities of the enzyme(s) involved.

Stoeckel, Lidolt et al. (2016a) have reported that during the storage of full-fat UHT milk with residual AprX activity at ambient temperature, the onset of different quality defects started with bitterness, followed by an increase in particle size/viscosity, then cream layer formation, sedimentation, and finally gelation. Adams, Barach et al. (1975) and Gebre-Egziabher, Humbert et al. (1980) also reported that destabilization of UHT milk by *Pseudomonas* proteases was initiated by the development of bitterness, followed by visual changes like particle forming, fat separation and gelation. The level of protein breakdown at which bitterness became perceivable was found to differ considerably for various proteases, and bitterness frequently developed at a much lower degree of hydrolysis for proteolysis by bacterial proteases than endogenous proteases (Mitchell and Ewings 1985). This can be explained by the unspecific cleavage and the formation of many hydrophobic peptides by AprX (**Chapter 5**).

For plasmin-containing samples, bitterness is also perceptible before physical instability (Nieuwenhuijse and van Boekel 2003). In non-skim UHT milk with residual plasmin activity, creaming may happen earlier than gelation, as shown by our results in **Chapter 4**. With respect to the protein, the physical instability starts from sedimentation at the bottom of containers, after which sedimentation develops into a full-package gel at ambient temperature (Kohlmann, Nielsen et al., 1988, Newstead, Paterson et al., 2006, Malmgren, Ardö et al., 2017), or heavy sedimentation of non-gelled precipitate with a clarified liquid phase (Kocak and Zadow 1985a, Enright, Bland et al., 1999, Malmgren, Ardö et al., 2017). The lack of gelation in the samples stored at high temperatures is suggested to be due to extensive protein degradation, which does not allow the physical association of polypeptides needed for gelation (McKellar and Loewy 1981, Mitchell and Ewings 1985, Manji, Kakuda et al., 1986). This agrees with Kohlmann, Nielsen et al. (1988), who showed that slow protein breakdown is conducive to the gel formation.

To sum up, in UHT milk containing residual protease activity, product defects occur in an order: bitterness- fat separation- sedimentation- gelation- clarification, as shown in **Fig. 3**. Visual changes are preceded by sensorial change (bitterness), and fat separation occurs earlier than gelation. The occurrence of these defects depend on the residual protease activity and storage time. The higher the protease activity, the earlier the observation of the defects. A product may sequentially experience some of the defects, depending on the protease activity and storage conditions.

Fig. 3. Sequence of product defects during storage of UHT milk, fat separation occurs only in non-skim UHT milk.

2.2   Visual inspection of the cause of instability

Once the instability is found in one batch of UHT milk, it is necessary for the manufacturer to analyse the cause, in order to avoid the reoccurrence of the problem. Based on the above discussion, we developed a diagnostic graph that can be used for diagnosing the origin of the observed problem.
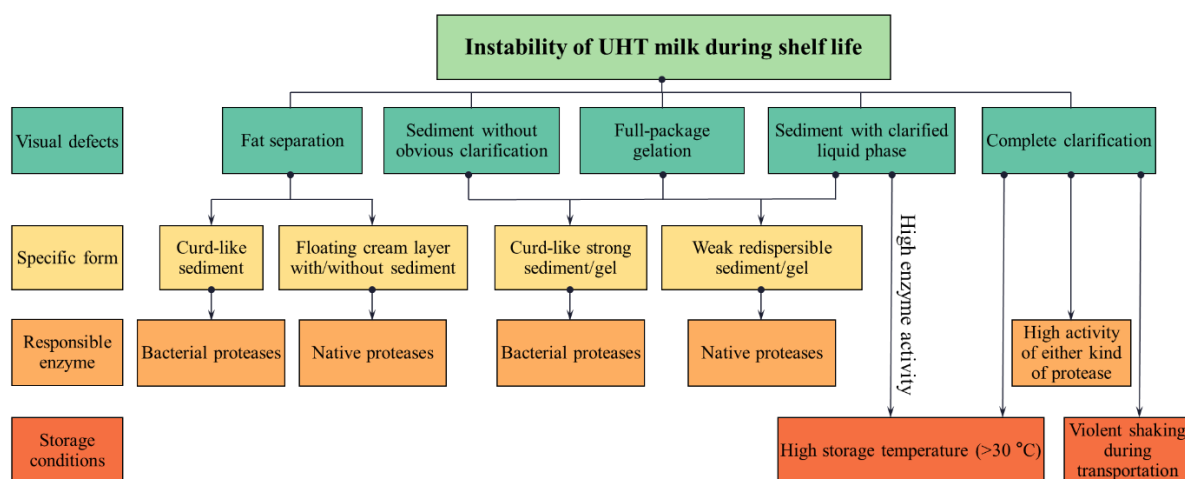
Fig. 4. Diagnostic graph for diagnosing the responsible enzymes based on the visual difference of instability during the shelf life of UHT milk, fat separation occurs only in non-skim UHT milk.

The above decision tree suggests an easy approach to distinguishing the cause(s) of instability in UHT milk by observing the visual defects. The causes may be the responsible residual enzyme(s) alone, or compounded by improper storage conditions, like high temperature or

violent shaking. It's also worth noting that bitterness usually happens earlier than physical defects, and can be caused by either protease.

2.3   Technical detection of the cause of instability

On top of visual inspection, analytical identification is always suggested to confirm the origin of instability. Many analytical detection approaches have been described in **Chapter 2** and **Chapter 3**. Of all the approaches, as mentioned in **Section 1.3**, peptide profiling by mass spectrometric approaches is the most promising approach for the detection of UHT milk quality. For diagnostic use, MALDI-ToF-MS should be considered, because it has as a major advantage that no time-consuming sample preparation is required, as opposed to LC/MS, thus having great potential for the practical application in routine milk analysis. Since the composition of the milk peptide fraction is dependent on the specificity of the enzyme, peptide patterns profiled by MALDI-ToF-MS can potentially be used for the identification of the protease(s). In this context, we performed preliminary trials on UHT milk samples hydrolysed by AprX and plasmin to the same degree of hydrolysis using MALDI-ToF-MS. As shown in **Fig. 5**, the peptide profiles significantly differed between the samples. More peptides were generated in AprX-hydrolysed samples, which was also expected due to its unspecific hydrolysis, while less peaks with higher intensities were found in plasmin-hydrolyzed samples, because of the lysine/arginine specificity of plasmin. This preliminary result reveals great potential of using marker peptides or designing prediction models for the differentiation of bacterial and native proteases in UHT milk, which can be further studied in future.
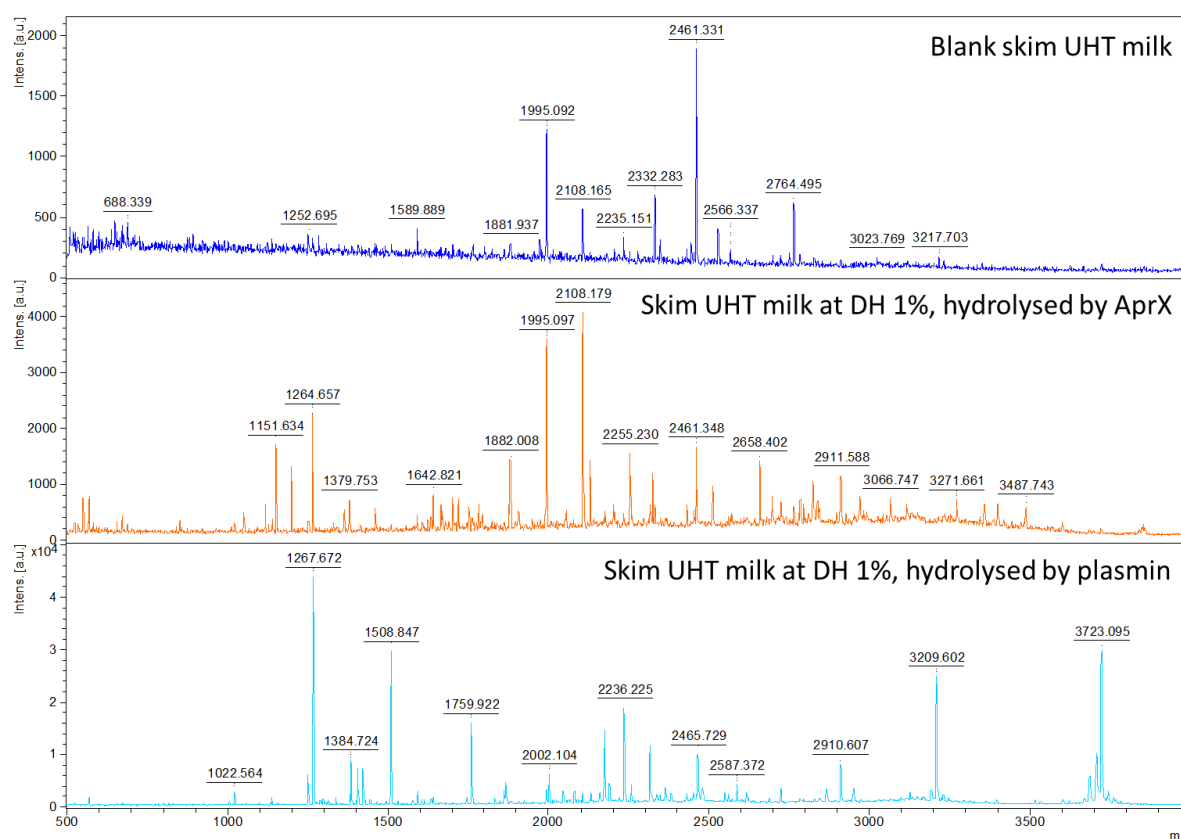
Fig. 5. Comparison of peptide profiles of blank skim UHT milk, and milk hydrolysed by AprX and plasmin to a degree of hydrolysis of 1%.

3. Recommendations for further study

In this thesis, the destabilization of UHT milk caused by enzymatic proteolysis has been intensively studied. Some points have been discussed above, whereas some potential areas for future research are mentioned below. From the viewpoint of scientific research:

- The research described in this thesis focused on the enzymatic factors that can lead to UHT milk instability, however, the enzymatic factors are coexisting with the non-enzymatic factors during storage in real life. From that perspective, research on the enzymatic and non-enzymatic factors should be combined, and their interactions should be determined in studying the mechanism of destabilization of UHT milk;

- The effects of AprX and plasmin were studied separately in this thesis, but there is a chance that residues of both enzymes are present simultaneously, in which case it would be difficult to identify which enzyme is responsible for the destabilization based on current knowledge. It would thus be desirable to understand their combined influence and possible synergetic interactions on destabilizing UHT milk;

- Recently, Raynes, Vincent et al. (2018) investigated the effect of protein variant on the age gelation in UHT skim milk, but no clear trend could be found. Further work is required to investigate the influence of the genetic variance on UHT milk stability.

From the viewpoint of industrial dairy processing, predicting the stability of UHT milk during shelf life based on the raw milk quality is of manufacturers' utmost interest. While, as shown in **Fig. 6**, numerous changes and factors can play roles in this process and their interplay is difficult to ascertain. Therefore, the shelf life prediction may be achieved by adding a detection point right after processing (storage time t=0 in **Fig. 6**), and filling the critical knowledge gaps below:
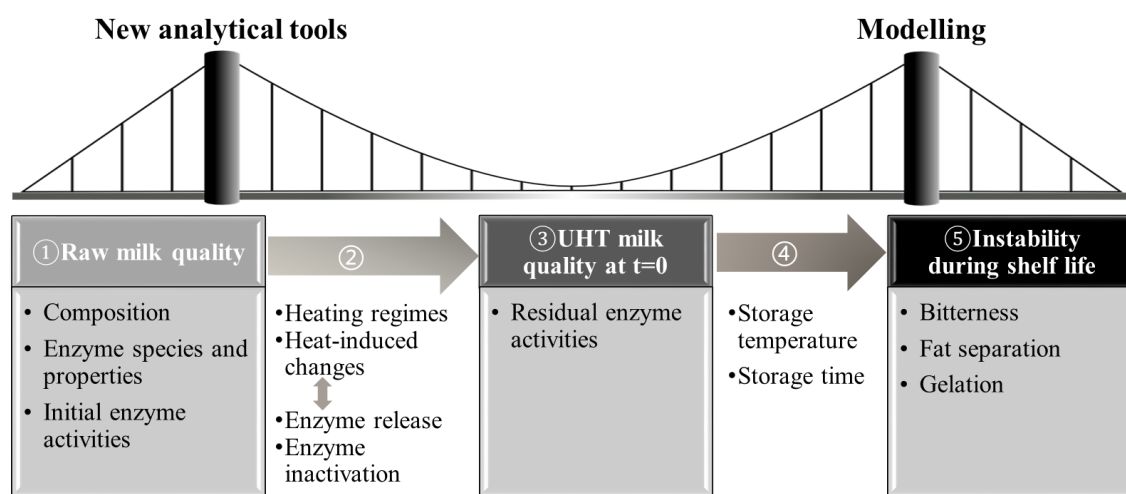
Fig. 6. Critical factors and chances for predicting the stability of UHT milk during shelf life based on the raw milk quality.

- ① Currently, reliable, simple and cheap methods for detecting trace levels of proteases are not available. Therefore, new analytical tools need to be developed for enzyme profiling of milk, including their origin, activity and thermal stability;

- ② Understanding of the influence of milk processing on the release of bacterial proteases is limited. Studying the manner in which the bacterial proteases are released due to processing factors like homogenisation and heating may help decrease their release;

- ③ Due to the difficulty in detecting the trace level of protease(s), especially after UHT processing, detecting the protein breakdown and/or peptide formation during certain incubation times may be a better approach. Peptide profiling using MALDI-ToF-MS, for example, could be an option, as will also be discussed in **Section 3.3**;

- ④→⑤ To predict the instability during long storage, appropriate storage conditions should be set for accelerated testing. Based on the results of such experiments, a prediction model for shelf life can be built.

4. Conclusions

The main conclusions of the research described in this thesis are:

- The residual activity of the protease AprX from psychrotrophic bacteria and the native protease plasmin in raw milk can lead to destabilization of UHT milk during storage.

- AprX can induce destabilization of casein micelles at lower degree of hydrolysis than plasmin.

- Plasmin destabilizes casein micelles and fat globules in UHT milk, mainly by hydrolysing β- and α-caseins, leading to a soft gel and creaming.

- AprX destabilizes casein micelles and fat globules in UHT milk mainly by hydrolysing κ-casein, leading to a curd-like strong gel or sediment.

- The mechanism of protein gelation is the same in both skim and full-fat UHT milk.

- Differences in the appearances, and underlying pathways, between bacterial and native protease-induced destabilization of UHT can be used to diagnose the responsible enzyme for the instability.

- Psychrotrophic bacterial proteases cannot be easily inactivated by adding a process of low heat inactivation, therefore psychrotrophic bacterial proteases can only be controlled from the raw milk level.

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Summary

The demand for UHT milk is steadily increasing worldwide. Meanwhile the problem of destabilization during its shelf life, with age gelation and fat destabilization being the most prominent defects, is still bothering many UHT milk manufacturers. Previous studies have revealed that the main factor of UHT milk destabilization during its shelf life is protein enzymatic hydrolysis. The two main categories of residual proteases in UHT milk are the native protease plasmin and heat resistant bacterial proteases, both of which are closely related with the raw milk quality (**Chapter 1**). However, a complete overview of how the proteases destabilize casein micelles and fat globules in UHT milk is still lacking. Besides, the approaches of preventing and diagnosing the proteases are still poorly understood. Hence, this thesis explored the enzymatic factors associated with destabilization of UHT milk, aiming to provide new insights into the mechanisms of UHT milk instability and to determine the significance of raw milk quality control in UHT milk production.

Compared with the native protease plasmin, the heat resistant proteases produced by psychrotrophic bacteria have been much less studied in UHT milk system. To fill this gap, the progress of current research on AprX was reviewed in **Chapter 2**, as a representative of all extracellular metalloproteases produced by psychrotrophic bacteria in milk. First of all, AprX is a generic protease produced by *Pseudomonas*, which can grow and produce enzyme in raw milk under refrigeration temperatures. Secondly, the flexible structure of AprX determines its high resistance to various conditions, including UHT processing. Thirdly, all AprX from proteolytic strains can readily hydrolyze κ-casein, which will result in solid and compact gels in UHT milk. Due to the problems it can cause, AprX spoilage should be minimized by implementing good hygiene management, cooling and thermization at various stages of the UHT milk production chain. Furthermore, new methods, like multiplex PCR assays and ELISA should be developed to detect "AprX-like" proteases. In addition, innovative strategies for inactivating bacterial proteases are recommended to be studied.

The roles of protease AprX and plasmin in destabilizing casein micelles and fat globules were investigated in the next chapters of the thesis. In **Chapters 3**, skim UHT milk-based model systems with addition of either AprX or plasmin, were used to allow a direct comparison of these enzymes on the destabilization of skim UHT milk. It was shown that AprX induced more solid and compact gels than plasmin, due to its specific hydrolysis of κ-casein. AprX can thereby induce gelation at a lower degree of hydrolysis than plasmin. Upon gelation, the same

Summary

pattern and level of hydrolysis were found in the gelled samples by specific proteases, indicating that there is a critical degree of hydrolysis of caseins that determines the onset of enzyme-induced gelation in UHT milk, irrespective of protease concentration or storage temperature. Based on the knowledge gained in this chapter on the differences in enzyme characteristics, several diagnostic tests to identify the responsible enzyme for age gelation have also been proposed.

In **Chapter 4**, the enzyme-induced fat destabilization is, similar as the protein destabilization in **Chapter 3**, studied by comparing the effects of AprX and plasmin in UHT milk-based model systems. It was shown that fat destabilization in UHT milk could be a consequence of proteolytic degradation of the fat surface-adsorbed proteins, but the physical appearances were different in AprX- and plasmin-containing samples. In AprX-containing samples, κ-casein was rapidly hydrolysed, leading to flocculation of casein micelles and fat globules. Consequently, AprX induced sedimentation of fat globules, which were embedded in a protein gel network. In plasmin-containing samples, on the other hand, the hydrolysis of α- and β-caseins disrupted the casein micelle fragments on the milk fat globule surface, causing the fat globules to coalesce and float to the surface. The results in this chapter provided new insights in the underlying mechanisms of fat destabilization in UHT milk during storage.

After confirming the adverse impacts of AprX and plasmin on the stability of UHT milk, the need of inactivating the enzymes arose accordingly. Where for plasmin, specific temperature regimes are known to inactivate plasmin in UHT milk processing, less is known about such inactivation of AprX. Low temperature inactivation (LTI) has been considered to be a possible way to inactivate AprX. Therefore, in **Chapter 5**, an LC-MS/MS-based peptidomics approach was employed to examine the effects of LTI by comparing the peptidome in the AprX-hydrolysed UHT milk samples with and without LTI treatment. It was shown that although LTI at 60 ℃ for 15 min could partially inactivate AprX activity in UHT milk, during LTI, besides AprX itself, mainly the milk proteins were hydrolysed. On top of that, the effects of LTI on skim and full-fat UHT milk were inconsistent. Therefore, LTI may not be feasible to inactivate AprX during UHT milk production. In addition, based on the assessment of the 2488 identified peptides from 48 parent proteins, the knowledge of AprX hydrolysis patterns on milk proteins was supplemented from a peptidomic perspective. It was shown that except for hydrolysing the major milk proteins in the order of β-casein>κ-casein≈$α_{s1}$-casein>β-lactoglobulin>$α_{s2}$-casein, AprX could also hydrolyse many other milk proteins like milk fat globule membrane proteins

and enzymes. The unspecific hydrolysis revealed that AprX can influence the properties of milk in many prospects.

The properties, the influences on casein micelles and fat globules by AprX and plasmin, and the inactivation of AprX in UHT milk were studied in **Chapters 2 to 6**, which provided a comprehensive understanding of the interaction between raw milk proteases and UHT milk stability. Based on these findings, scientific and practical implications were discussed in **Chapter 6**. To extend the existing theories on the destabilization of proteins in UHT milk, the gelation processes induced by AprX and plasmin were refined. Furthermore, the relationships between enzymatic and non-enzymatic physicochemical processes were discussed. It can be concluded that the relative impact of non-enzymatic physicochemical processes on UHT milk gelation depends on the level of enzyme present, with higher levels of enzymes making these other processes less relevant, due to the shorter time needed for the enzymes to cause destabilisation. To better predict the stability of UHT milk during long storage, based on raw milk quality, future research needs were addressed. In addition, in the light of the various types of instability of UHT milk described in this thesis, potential diagnostic tests to determine the cause of instability depending on the different quality defects were proposed. Finally, the implications for raw milk quality control along the UHT milk production chain were reviewed. In conclusion, this thesis highlights that to attain high stability of UHT milk during shelf life, the best way is to control quality from the raw milk level to reduce the risk from both plasmin and AprX.

Appendix: Implications for increase UHT milk stability in practice

This project is part of PhD program of the Sino-Dutch Dairy Development Centre (SDDDC). The objective of SDDDC is to improve dairy production, safety and quality level throughout the entire dairy chain in China by sharing Dutch dairy expertise. SDDDC was founded in 2013 and sponsored by China Agricultural University, Wageningen University and Research, and FrieslandCampina. Since its start, a total of 35 projects were set-up, covering many links in the dairy chain. This project on UHT milk instability is part of the multidisciplinary project "Quality control throughout Chinese dairy chain".

Ultra-high-temperature (UHT) processed milk was chosen as an important product for improvement because it is the most consumed liquid milk in China. According to the White Paper on China Dairy 2016 (Li et al., 2018), from 2000 to 2016, the percentage of liquid milk in total dairy production rose from 64% to 87%, whereby 40.6% liquid milk consumption was UHT milk (in 2016). UHT technology ensures that the milk quality can be maintained during long-distance transportation at ambient temperature from production sites in northern China to consumers in southern China, where cold chain is more difficult to maintain. Therefore, long shelf life stability of UHT milk is an actual need for the Chinese dairy industry. In this part, suggestions on raw milk quality control are proposed specific for UHT milk production.

The results in this thesis clearly show that both bacterial proteases and native proteases can indeed lead to destabilization of UHT milk during storage. Considering that achieving sufficient inactivation of the protease activity with thermal processes has been proven to be difficult, especially for proteases produced by pseudomonads, a better approach is to prevent the formation of the proteases in the raw milk in the first place. Therefore, actions should be taken to minimize the level of both enzymes, by strict quality management of the entire dairy chain. In this section, the potential vulnerabilities in the dairy chain and the key points for quality monitoring will be discussed.
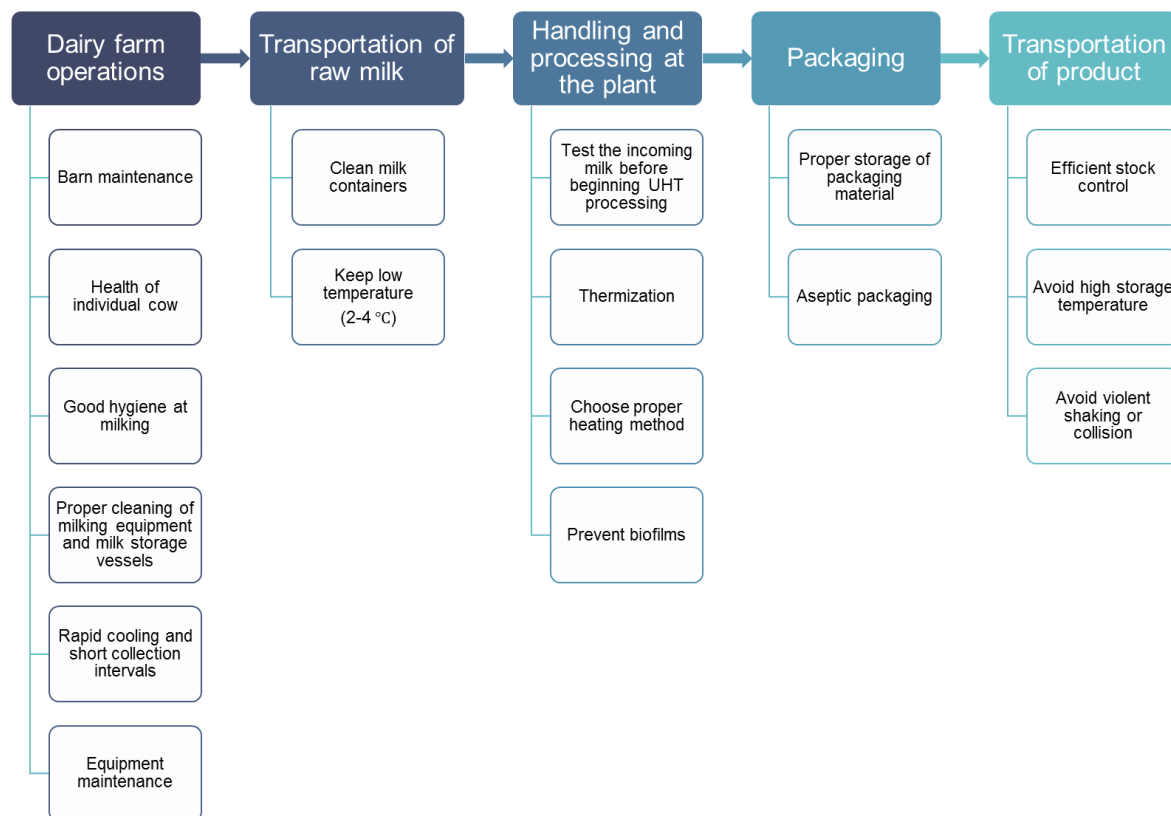
Appendix: Implications in practice

Fig.1. The key points of quality assurance and quality control of UHT milk production.

The key points of quality assurance and quality control of UHT milk production are shown in **Fig. 1**. High quality UHT milk starts from good dairy farm operations. The contamination of milk on the farm will have the greatest influence on not only the raw milk quality, but also the quality of the finished products. Cow health, hygiene practices, and cooling processes are three of the most vulnerable links that may go wrong and lead to an increased level of proteases in the raw milk.

As introduced in **Chapter 1**, increased plasmin level is correlated with elevated somatic cell count (SCC), which is a typical symptom of mastitis. Therefore, to keep a low plasmin level in milk, mastitis should be prevented and controlled using an integrated non-antibiotic strategy (De Vliegher, Fox, Piepers, McDougall, & Barkema, 2012), and cows should have their body health evaluated regularly. Good hygiene practices and fast cooling (sometimes a rapid precooling of milk before entering the bulk tank is necessary) combined with short collection intervals are needed to control the bacterial growth and protease synthesis resulting therefrom. In addition, the aging of the rubber parts of the milking system may form a harbouring place for psychrotrophic bacteria and is hard to be noticed; therefore, equipment must be maintained and rubber parts replaced in time.

During the transportation of raw milk, keeping the tank clean and keeping milk refrigerated at low temperatures (2-4°C) is important. The temperature of incoming milk, together with the parameters discussed below, are recommended parameters for quality control, in order to determine how long the raw milk can be stored, if this raw material is suitable for UHT processing, and whether the heating temperature or time needs to be adjusted. The major test parameters that are suggested are:

- The temperature history of the incoming milk in the production chain, because it can indicate if the milk has been properly chilled; if not properly chilled, it may need to be subjected to shorter storage;
- pH value: even though the protease production *per se* does not lead to a decrease in pH, pH value of milk is an overall indicator of microbiological spoilage. A pH value between 6.65 and 6.8 at 20 ℃ is in the normal range. A pH lower than 6.65 indicates that there is a large risk of high numbers of bacteria, including psychrotrophs, that can produce heat-resistant enzymes, making the milk unsuitable for UHT production;
- Somatic cell count (SCC): high SCC is associated with high activity of native proteases. The upper limits of somatic cell count according to legislation are: 400,000 cells/mL in EU and 750,000 cells/mL in US. If the SCC in the raw milk is close to the upper limit, the indirect heating method is recommended, in view of the higher degree of plasmin inactivation during indirect heating. If only direct heating equipment is available, preheating at 80-95 ℃ for 30 to 180 s is suggested.

Even though some current tests like acidity and alcohol stability can also be used to test the stability of raw milk, they are mostly targeting growth of lactic acid bacteria instead of psychrotrophic bacteria. Therefore, new detection methods specifically aimed at thermostable bacterial and native proteases need to be developed to add direct information to decide the suitability for UHT processing.

Thermization is a heating process (generally 63-65 °C for 15 s) which eliminates most of the microorganisms that can grow at low temperature and thus enable a cold storage of milk for another 1-2 days prior to further processing (Humbert, Campbell, Blankenagel, & Gebre-Egziabher, 1985). Thermization is an effective way to inhibit the proliferation of psychrotrophs as well as the production of heat-stable enzymes. Therefore, all raw milk destined for UHT production should be treated with thermization on arrival at the factory if not further processed immediately.

Appendix: Implications in practice

For the UHT milk destined for export, or if longer shelf life is needed, a longer heating time for both preheating (eg. 90-95 ℃ for 180-90 s) and UHT heating (eg. 125-130 ℃ for >150 s) may be adopted (Stoeckel et al., 2016), although this only helps against plasmin and not bacterial proteases.

At the end of the dairy chain, the final products should be transported under mild conditions if possible, where high temperatures or violent shaking should be avoided, because high temperature can speed up the destabilization, and coalescence of fat droplets may be induced by mechanical collision (Huppertz & Kelly, 2006). Efficient stock control should be managed to shorten the storage time before distribution.

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Curriculum Vitae

Chunyue Zhang was born on 18th March, 1989 in Benxi, Liaoning province, China. After finishing high school in Benxi, she moved to Beijing, to start her bachelor study of Food Science and Engineering at China Agricultural University in 2007. Her BSc degree was completed with a thesis project at the Key Laboratory of Functional Dairy on the "*Heat-induced transient rheological properties of pectin family*", under the supervision of Prof. Xiaojing Leng. Her BSc thesis was awarded as "The Excellent Undergraduate Thesis". In 2011, she got the postgraduate recommendation and started her MSc study in the College of Food Science and Nutritional Engineering, China Agricultural University. She finished her MSc thesis on "*Study of the Controlled Release Property and Antioxidant Activity of Chitosan- Selenium Self-Assembly Nanosystems*", under the supervision of Prof. Xiaojing Leng. After obtaining her MSc degree in 2014, she was selected as the one of the first batch of PhD candidates in the programme of Sino-Dutch Dairy Development Center. She started her PhD research at Food Quality and Design Group, Wageningen University and Research, under the supervision of Prof. Dr Vincenzo. Fogliano, Dr. Kasper Hettinga and Dr. Etske Bijl. The results of her PhD research are presented in this thesis. Currently, Chunyue is working as a junior teacher at Food Quality and Design Group.

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Zhai, X., **Zhang, C**., Zhao, G., Stoll, S., Ren, F., & Leng, X. (2017). Antioxidant capacities of the selenium nanoparticles stabilized by chitosan. ***Journal of Nanobiotechnology***, *15*(1), 4.

Du, B., Tang, X., Liu, F., **Zhang, C**., Zhao, G., Ren, F., & Leng, X. (2014). Antidepressant-like effects of the hydroalcoholic extracts of *Hemerocallis Citrina* and its potential active components. ***BMC Complementary and Alternative medicine***, 14(1), 326.

Liu, F., Jiang, Y., Du, B., Chai, Z., Jiao, T., **Zhang, C.**, Ren F, Leng X. (2013). Design and characterization of controlled-release edible packaging films prepared with synergistic whey-protein polysaccharide complexes, ***Journal of Agricultural and Food Chemistry***, 61, 5824-5833.

Chai, Z., Li, Y., Liu, F., Du, B., Jiao, T., **Zhang C**, Leng X. (2013). Outer Eggshell Membrane as Delivery Vehicle for Polysaccharide/ Protein Microcapsules Incorporated with Vitamin E, ***Journal of Agricultural and Food Chemistry***, 61, 589-595.

Overview of completed training activities

Discipline specific activities

Courses

WGS PhD Workshop Carousel, WGS, Wageningen, NL,2015, 2016

Food Stability, VLAG and Copenhagen University, Copenhagen, Denmark, 2015

Food & Biorefinery Enzymology[1], VLAG, Wageningen, NL, 2015

Reaction Kinetics in Food Science, VLAG, Wageningen, NL, 2016

Advanced Food Analysis[1], VLAG, Wageningen, NL, 2017

Management of Microbiological Hazards in Foods, VLAG, Wageningen, NL, 2017

Advanced Proteomics, VLAG, Wageningen, NL, 2017

Healthy and Sustainable Diets: Synergies and Trade-offs, VLAG, Wageningen, NL, 2017

Dairy Protein Biochemistry, VLAG, Wageningen, NL, 2018

Conferences and meetings

2nd Food Packaging, Shelf Life and Food Safety Conference[2], ELSEVIER and Fraunhofer IVV, Munich, Germany, 2017

3rd Food Structure and Functionality Forum Symposium and 3rd IDF Symposium on Microstructure of Dairy Products[1,2], Food Structure and Functionality Forum and the International Dairy Federation, Montréal, Canada, 2018

General courses

VLAG PhD week, VLAG, Baarlo, NL, 2015

Systematic Approaches to Reviewing Literature, WGS, Wageningen, NL, 2015

Project and Time Management, WGS, Wageningen, NL, 2015

Data Management, WGS, Wageningen, NL, 2016

Mobilising Your Scientific Network, WGS, Wageningen, NL, 2016

Introduction to R, VLAG, Wageningen, NL, 2016

Scientific Writing, WGS, Wageningen, NL, 2017

Philosophy and Ethics of Food Science and Technology, VLAG, Wageningen, NL, 2017

Career Perspectives, WGS, Wageningen, NL, 2019

Additional activities

Preparation of research proposal, FQD, Wageningen, NL, 2015

Dutch Dairy Experience, SDDDC, NL, 2015

PhD study tour to Italy, FQD[2], Italy, 2016

PhD study tour to Australia[1,2], FQD, Australia, 2018

DST meetings and Seminars in FQD[1,2], FQD, Wageningen, NL, 2015-2019

[1] poster presentation; [2] oral presentation;

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences;

WGS: Wageningen Graduate School;

SDDDC: Sino-Dutch Dairy Development Center;

FQD: Food Quality and Design group.