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Comprehensive Study of Proteolysis During Cheese Ripening

ANASTASSIA TAIVOSALO

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Engineering
Department of Materials and Environmental Technology
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Supervisor: Signe Adamberg, PhD
Senior Research Scientist
Department of Chemistry and Biotechnology
Tallinn University of Technology
Tallinn, Estonia

Co-supervisor: Tiina Kriščiunaite, PhD
Head of Chemical and Instrumental Analyses
Center of Food and Fermentation Technologies
Tallinn, Estonia

Opponents: Prof Paul L.H. McSweeney
School of Food and Nutritional Sciences
University College Cork
Cork, Ireland

Assoc Prof Ivi Jõudu
Institute of Veterinary Medicine and Animal
Sciences Chair of Food Science and Technology
Estonian University of Life Sciences
Tartu, Estonia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation, and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Anastassia Taivosalo

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Proteolüüsi detailne iseloomustamine juustu valmimise käigus

ANASTASSIA TAIVOSALO

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List of Publications

These publications form the basis of the thesis and are reproduced in the appendix with permission from the publishers:

I Taivosalo, A., Kriščiunaite, T., Seiman, A., Part, N., Stulova, I., Vilu, R. (2018), **Comprehensive analysis of proteolysis during 8 months of ripening of high-cooked Old Saare cheese**. J. Dairy Sci, 101: 944–967. doi:10.3168/jds.2017-12944

II Kriščiunaite, T., Stulova, I., Taivosalo, A., Laht, T.-M., Vilu, R. (2012), **Composition and renneting properties of raw bulk milk in Estonia**. Int. Dairy J, 23: 45-52. doi:10.1016/j.idairyj.2011.09.013

III Taivosalo, A., Kriščiunaite, T., Stulova, I., Part, N., Rosend, J., Sõrmus, A., Vilu, R. (2019), **Ripening of hard cheese produced from milk concentrated by reverse osmosis**. Foods, *8, 165.* doi:10.3390/foods8050165

Author's Contribution to the Publications

Contribution to the papers in this thesis is:

I The author performed analysis of caseins by CE and prepared the water-soluble extracts of cheeses for LC-MS/MS and UPLC analyses. She interpreted all results (including data treatment by MaxQuant and Python), made all figures and tables, and wrote the manuscript together with T. Kriščiunaite.

II The author was responsible for measurements of caseins by CE, analyzed and interpreted the data obtained from CE, and participated in the writing of the manuscript.

III The author analyzed all data, prepared all the figures and tables, and wrote the manuscript.

Introduction

Simultaneous production and degradation of peptides in ripening cheese is a complex process controlled by various proteolytic enzymes of milk and bacterial origin. In the result of the proteolytic events, the texture of cheese undergoes changes from rubbery grains to creamy and smooth cheese body, while the catabolism of FAA to various flavor compounds defines the cheese characteristic organoleptic quality (Sousa, Ardö, & McSweeney, 2001).

A deep understanding of the mechanisms of casein hydrolysis expands the opportunities for rational optimization of cheese manufacturing process by fine-tuning the technological parameters as well as selecting the starters with desired properties. During the recent decades, the use of mass spectrometry-based techniques have emerged as important tool for monitoring proteolysis in cheese during ripening (Fontenele, Bastos, dos Santos, Bemquerer, & do Egito, 2017; Sforza et al., 2012). There is an expanding interest in cheese peptidomics regarding also the identification and quantification of the peptides, including characterization of bioactive (Nielsen, Beverly, Qu, & Dallas, 2017; Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014) and bitter peptides (Karametsi, Kokkinidou, Ronningen, & Peterson, 2014), as well as phosphopeptides (Ardö, Lilbæk, Kristiansen, Zakora, & Otte, 2007).

The objective of this dissertation was to develop a novel approach, including the application of up-to-date analytical and computational tools, to study casein hydrolysis and to obtain the comprehensive picture of proteolysis in cheese. This thesis focused on a thorough analysis of more than 3,000 water-soluble peptides produced during ripening of the traditional hard cheese Old Saare, using the Python-assisted analysis and visualization of the data obtained by liquid chromatography coupled with tandem mass-spectrometry method (LC-MS/MS). To describe the changes in the proteolytic cascades during cheese ripening and to follow the differences among the hydrolysis of different caseins, the proteolytic coverage maps and enzyme cleavages profiles of caseins were developed. To follow the trends in peptide profiles during cheese ripening, the principal component and clustering analyses were performed on the selection of the most abundant peptides. Eventually, we composed an overall description of proteolysis in cheese during ripening by combining the data on primary proteolysis (capillary electrophoresis; CE), water-insoluble peptides (LC-MS/MS), amino acids (ultra-performance liquid chromatography; UPLC), and N content of different proteinaceous fractions (Kjeldahl method). This novel approach to the analysis of cheese proteolysis introduced in this dissertation forms a basis for future studies allowing a detailed description of casein hydrolysis taking place during cheese ripening.

Quality and sensory properties of cheese greatly depend on protein and fat content, degree of microbial proteolytic activities, coagulation properties of milk, the effects of the cooling and cold storage time on farm, that have been studied thoroughly (Franciosi, Settanni, Cologna, Cavazza, & Poznanski, 2011; Malacarne et al., 2013; Paludetti, Kelly, O'Brien, Jordan, & Gleeson, 2018). However, there is a lack of knowledge on the changes in protein composition taking place during the short-term transportation of raw milk in refrigerated tank trucks. Hence, a part of this dissertation focuses on the elucidation of the extent of possible proteolysis taking place during refrigerated transportation of raw milk from dairy farms to the dairy plant.

Cheese is a product with high nutritional value, which is produced in a wide range of varieties depending on the peculiarities of the manufacturing technology. There has

always been a profound interest towards the use of the novel milk pretreatment technologies to improve the yield and efficiency of cheese production, as well as to enrich cheese milk with valuable components. Along with a large number of studies on the application of ultra- and microfiltration technologies for the concentration of milk (Delgado, Salazar, & García, 2013; Guinee, O'Kennedy, & Kelly, 2006; Karami, Reza Ehsani, Ebrahimzadeh Mousavi, Rezaei, & Safari, 2008), only few studies have focused on the application of reverse osmosis (RO) in cheese manufacturing (Agbevavi, Rouleau, & Mayer, 1983; Barbano & Bynum, 1984). In the present study, cheese was produced from milk concentrated by RO and the primary proteolysis during ripening was investigated.

Abbreviations

CN	casein
CCP	colloidal calcium phosphate
CD	citrate dispersion
CE	capillary electrophoresis
CEP	cell envelope proteinase
DVS	direct vat set
EDTA	ethylenediaminetetraacetic acid
EOF	electroosmotic flow
FA	fatty acid
FAA	free amino acids
GMP	Glycomacropeptide
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc. lactis ssp. *lactis*	*Lactococcus lactis* subspecies *lactis*
LC-MS/MS	liquid chromatography coupled with tandem mass-spectrometry
MHEC	methyl hydroxyethyl cellulose
PCA	principal component analysis
P-site	phosphorylation site
RFT	rennet flocculation time
RO-cheese	cheese made from milk concentrated by reverse osmosis
RO-milk	milk concentrated by reverse osmosis
St.	*Streptococcus*
TN	total nitrogen
TP	total protein
Tris	Trisaminomethane
UPLC	ultra-performance liquid chromatography
WP	whey proteins
WSE	water-soluble extract
α-LA	α-lactalbumin
α_{s1}-CN	alpha$_{s1}$-casein
α_{s2}-CN	alpha$_{s2}$-casein
β-CN	beta-casein
β-LG	β-lactoglobulin
κ-CN	kappa-casein

1 Literature Review

1.1 Milk composition and structure

Composition of milk defines the technological properties, nutritional value, texture and yield of cheese, one of the highly regarded milk products. Processing of milk to cheese leads to the principal changes of milk at its microstructural levels, i.e. proteinaceous and fat matrix.

Milk provides the nutrients, energy and immunological defense for a newborn mammal and is synthesized in the secretory cells in mammary gland. The composition of bovine milk is extremely complex and varies with breed, genetic variation, feed, stage of lactation, health status, and season. The principal constituents of bovine milk are presented in Table 1 (Walstra, Wouters, & Geurts, 2006).

Table 1. Average composition of bovine milk (adapted from Walstra et al., 2006)

Component	Average content (% w/w)
Moisture (water)	87.1
Lactose	4.6
Fat	4.0
Protein	3.3
Caseins	**2.6**
whey proteins	0.56
Mineral substances	0.6
Organic acids	0.17
Miscellaneous	0.15

1.1.1 Lactose

The main carbohydrate dissolved in milk, lactose, is a disaccharide consisting of galactose and glucose residues, linked by β-1,4 glycosidic bond. The conversion of residual lactose to lactate by various microorganisms is one of the biochemical pathways in cheese. Decrease of pH effects the growth of bacteria in cheese and the activity of the enzymes involved in cheese maturation (McSweeney & Sousa, 2000). Acidities in hard and semi-hard cheeses vary between pH 5.0 – 5.3 in the beginning and 5.5-5.7 in the end of ripening.

1.1.2 Lipids

Fat is dispersed in milk serum in the form of fat globules (particle diameter 0.2−20 μm), which are surrounded by a membrane containing polar lipids and proteins (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015; Walstra et al., 2006). Milk lipids consist of tri-, di-, and mono glycerides, phospholipids, sterols, cholesterol, carotenoids, and vitamins A, D, E, K. Triglycerides form about 97−98% of the total lipids in the core of fat globule. The amount and variety of triglycerides strongly impact the physical properties of milk fat, e.g. melting characteristics, solid fat content and rheology. The fatty acids (FA) in the molecules of triglycerides differ widely in chain lengths (2−20 carbon atoms) and degrees of saturation (0−4 double bonds), over 65% of all FAs in milk fat are saturated, 30% mono- and 5% polyunsaturated.

Short- and medium-chain free FA, liberated by enzymatic hydrolysis of triglycerides during cheese ripening also contribute to flavor in mostly soft and mold type cheeses (McSweeney & Sousa, 2000).

1.1.3 Proteins

Bovine milk proteins can be divided into two major fractions: the caseins (CNs) and the globular whey proteins, which comprise about 80% and 20% of total protein, respectively (Fox et al., 2015; Walstra et al., 2006).

CNs are defined as proteins, that precipitate by acidification of milk to pH 4.6, the isoelectric point of casein. According to the primary amino acid sequence, caseins can be divided into four protein species, α_{s1}-, α_{s2}-, β-, and κ-CN, in the approximate proportions of 4: 1: 3.5: 1.5, respectively. Molecular weights of the individual caseins vary from 19000 to 25000 Da, and the average isoelectric point is in the range of pH 4.6−4.8. Casein is heat stable and resist heating at 140 °C for 20 min without coagulation.

During synthesis in the mammary gland, casein faces two post-translational modifications (Farrell et al., 2004; Fox, Guinee, Cogan, & McSweeney, 2000; Walstra et al., 2006). The first reaction is phosphorylation. The phosphate is linked with the casein through the hydroxyl group of serine (rarely threonine) or forms the colloidal calcium phosphate. Thus, an important feature of casein to bind a large amount of calcium makes it nutritionally a very valuable protein (Fox et al., 2015). The second reaction is glycosylation. Only κ-CN is known to be glycosylated via several threonine residues of the hydrophilic C-terminal end of κ-CN sequence (Fox et al., 2000).

Whey proteins are globular proteins with quaternary structures and a particle diameter 3−6 nm (Fox et al., 2015; Walstra et al., 2006). The family of whey or serum proteins consists of α-lactalbumin (α-LA, M_r 14000 Da), β-lactoglobulin (β-LG, M_r 36000 Da), bovine serum albumin, immunoglobulins, lactoferrin, and proteose-peptones. The first two are the dominant bovine whey proteins, and synthesized in the mammary gland, while others originate from the blood. In contrast to the caseins, the whey proteins remain soluble at pH 4.6 at 20−30 °C. During heating the whey proteins partially denature at 70 °C and completely denature at 90 °C for 10 min. Whey proteins do not contain phosphate groups and are relatively rich in sulphur due to significant amounts of amino acids methionine, cysteine, and cystine (Farrell et al., 2004; Fox et al., 2015). Denatured α-LA and β-LG can interact and form the complexes with κ-CN (rarely α_{s2}-CN) in the serum or on the casein micelle through thiol-disulphide exchanges. This interaction has practical interest in cheesemaking, as it affects the renneting properties and firmness of coagulum (Anema, 2007). In (semi-)hard cheeses, only residues of whey proteins remain in the cheese matrix as most of them are removed by whey separation.

The caseins are found in milk in the form of a stable colloidal suspension of spherical aggregates known as casein micelles (Fox et al., 2000). The diameter of the micelle ranges between 20−400 nm with an average of 120 nm, and a mass varies between 10^3–10^9 Da. One micelle consists of about 20,000 casein molecules. The micelles are highly hydrated binding 2−4 grams H_2O per gram of casein, mainly because of the κ-CN layer on the micelle surface. Almost all caseins are incorporated into casein micelles, containing high amount of calcium and inorganic phosphate (Fox et al., 2000). However, a small part of the casein (mostly β-CN and at a lesser extent α_{s1}- and α_{s2}-CN) can dissociate from the casein micelle upon cooling (Pouliot, Pouliot, Britten, Maubois, & Fauquant, 1994). Once the milk is taken from the 37 °C of the udder to the storage

for a day or more at 4 °C, the solubilization of casein occurs. Nevertheless, this process is reversible. Heating the milk back to 30–37 °C returns the casein into the micelle (Pouliot et al., 1994). Colloidal casein scatters light and gives a white appearance to the milk (Walstra et al., 2006).

Caseins are amphiphilic polymers with high levels of hydrophilic and hydrophobic AA residues, forming hydrophobic and hydrophilic sequence fragments. Amphiphilicity of the caseins in aqueous solution motivate caseins to self-assemble into the casein micelles (Fox et al., 2000). Various models have been proposed to describe casein micelle structure (Dalgleish, 2011; Holt, 1992; Horne, 1998), but no complete detailed structure is established up to now. The model of "nanoclusters is considered to be the most relevant model to describe the casein micelle structure (Figure 1).

According to this model, the caseins are associated into micelles by a combination of hydrophobic interactions between groups of different molecules, and calcium phosphate nanoclusters (referred as colloidal calcium phosphate, CCP), linked to the serine residues of the caseins (Figure 1). Only α_{s1}-, α_{s2}-, and β-CN have phosphoseryl clusters (two and more consecutive amino acids are phosphorylated), which link together those caseins through the calcium phosphate nanoclusters. Thus, both linking ways permit branching of the structure and lead to the three-dimensional network structure of the micelle. α_{s1}-, α_{s2}-, and β-CN are accumulated in the core of the casein micelle. κ-CN has no phosphoseryl cluster and thus terminate a polymerization process, linking only to a hydrophobic region on another molecule. "Hairy layer" located on the micelle surface of κ-CN limits further growth and provides the stability of the micelle through a steric stabilization mechanism. The highly charged and glycosylated C-terminal end of κ-CN (glycomacropeptide, GMP, f106-169) on the surface of the casein micelle physically prevents approaching of hydrophobic regions of caseins which would lead to the aggregation and coagulation (Dalgleish, 2011; Fox et al., 2000).

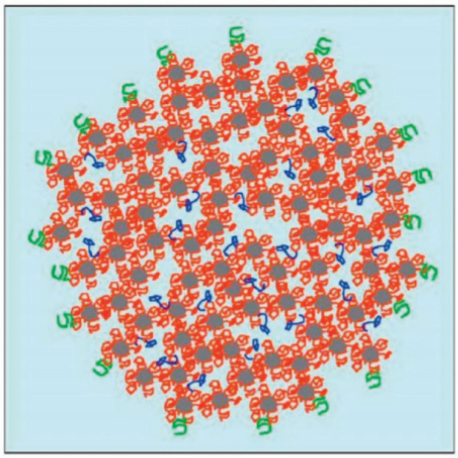


Figure 1. Schematic structure of the casein micelle. Grey: incorporated calcium phosphate nanoclusters; red: attached casein; green: the surface-located κ-casein; and blue: hydrophobically-bond β-casein (reproduced from Dalgleish (2011) with permission of The Royal Society of Chemistry).

The coagulation of caseins by the action of rennet (specific milk-clotting proteolytic enzyme) in the presence of calcium is used in the most cheese varieties to convert the milk into cheese curd.

Addition of high amounts of urea (Mcgann, 1974) and ethylenediaminetetraacetic acid (EDTA) (Griffin, Lyster, & Price, 1988) to the milk results in dissociation of the casein micelles. Also, a reducing agent dithiothreitol (DTT) can be added to reduce the disulfide bridges in casein micelle. These reagent-dependent properties of casein micelles are used in milk and cheese sample preparation for protein separation by capillary electrophoresis (CE) (Heck et al., 2008; Otte, Zakora, Kristiansen, & Qvist, 1997).

1.1.3.1 Nomenclature of caseins

Bovine caseins show wide qualitative variability caused by genetic polymorphism. A total of 35 casein genetic variants have been identified by AA and DNA sequencing and characterized within the *Bos* genus (Farrell et al., 2004). Casein genetic variants differ in one or two AA and by the deletion of few consecutive AA residues from the certain positions of the casein sequence. The major genetic variants of casein and their differences in AA sequence are shown in Table 2 (Farrell et al., 2004).

α_{s1}-CN comprises a single polypeptide chain, which counts 199 AA residues with a calculated molecular weight 23,615 Da (Figure 2, Table 2). α_{s1}-CN consist of two fractions, the major contains 8 moles P per mole casein (α_{s1}-CN-8P) and the minor – 9 moles P (α_{s1}-CN-9P) with the additional phosphorylated serine residue at position 41 (Figure 2) (Farrell et al., 2004). The reference casein is α_{s1}-CN B-8P. The location of phosphorylated AA residues divide the α_{s1}-CN into three parts: a hydrophobic N-terminal end, a hydrophilic central fragment containing phosphoseryl clusters, and a hydrophobic C-terminal end (Fox et al., 2000).

```
  1                                  10                                  20
H-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-
 21                                  30                                  40
Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-
 41                                  50                                  60
Ser-Lys-Asp-Ile-Gly-SeP-Glu-SeP-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met-
 61                                  70                                  80
Glu-Ala-Glu-SeP-Ile-SeP-SeP-SeP-Glu-Glu-Ile-Val-Pro-Asn-SeP-Val-Glu-Gln-Lys-His-
 81                                  90                                  100
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-
 101                                 110                                 120
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-SeP-Ala-Glu-Glu-Arg-Leu-
 121                                 130                                 140
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-
 141                                 150                                 160
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-
 161                                 170                                 180
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-
 181                                 190                                 199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-0H
```

*Figure 2. The structure of reference α_{s1}-CN with 8 P (α_{s1}-CN B-8P). **SeP**, sites of phosphorylation. Potentially phosphorylated AA residue is underlined (reproduced from Farrell et al. (2004) with permission of American Dairy Science Association).*

Table 2. Major genetic variants of bovine caseins. Adapted from Farrell et al. (2004).

Casein	Variant	Molecular weight, Da	Differences in AA position												
								53		59		66	192		
α_{s1}-CN	B*	23,615						Ala		Gln		SerP	Glu		
	C	23,542											Gly		
			33		47							130			
α_{s2}-CN	A*	25,226	Glu		Ala							Thr			
			18	25	35	36	37	67	72	88	93	106	122	137/138	152
β-CN	A[1]	24,023						His							
	A[2] *	23,983	SerP	Arg	SerP	Glu	Glu	Pro	Gln	Leu	Met	His	Ser	Leu/Pro	Pro
	B	24,092						His					Arg		
			10				97	104	135	136			148	155	
κ-CN	A*	19,037	Arg				Arg	Ser	Thr	Thr			Asp	Ser	
	B	19,006								Ile			Ala		

*Reference casein (CN); AA: amino acid

α_{s2}-CN has 207 AA residues with a calculated molecular weight 25,226 Da (Figure 3, Table 2). Four different phosphorylated forms of α_{s2}-CN, containing 10–13 moles P per casein, have been identified. The reference casein is genetic variant A with 11 moles P. Potential phosphorylation sites are marked on Figure 3 (Farrell et al., 2004). Similarly to the α_{s1}-CN, α_{s2}-CN can be divided into four parts: a hydrophilic N-terminal fragment with a hydrophilic phosphoseryl cluster, a hydrophobic fragment, another hydrophilic part, containing further phosphorylated AA residues, and a hydrophobic C-terminal tail of α_{s2}-CN sequence (Fox et al., 2000).

```
  1                                   10                                  20
H-Lys-Asn-Thr-Met-Glu-His-Val-SeP-SeP-SeP-Glu-Glu-Ser-Ile-Ile-SeP-Gln-Glu-Thr-Tyr-
 21                                   30                                  40
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-SeP-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-
 41                                   50                                  60
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-SeP-SeP-SeP-Glu-Glu-
 61                                   70                                  80
SeP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-
 81                                   90                                  100
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-
 101                                  110                                 120
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-
 121                                  130                                 140
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-SeP-Thr-SeP-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-
 141                                  150                                 160
Met-Glu-SeP-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-
 161                                  170                                 180
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-
 181                                  190                                 200
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-
 201                  207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu-OH
```

Figure 3. The structure of reference α_{s2}-CN with 11 moles P (α_{s2}-CN A-11P). ***SeP****, sites of phosphorylation. Potentially phosphorylated AA residues are underlined (reproduced from Farrell et al. (2004) with permission of American Dairy Science Association).*

The sequence of **β-CN** consists of 209 AA with a calculated molecular mass 23,983 Da (Figure 4, Table 2). The reference casein is β-CN A^2 with 5 moles P per casein (Farrell et al., 2004). β-CN is the most hydrophobic casein. Most of the molecule charge is accumulated to the hydrophilic N-terminal end of β-CN with cluster of phosphoseryl residues. Second part of the molecule concentrates the neutral and hydrophobic AA residues (Fox et al., 2000). This uneven distribution of the charge can lead to the leakage of the β-CN from the casein micelle in the cold (Aoki, Yamada, & Kako, 1990). The fraction of β-CN in bovine milk consists also of γ_1-, γ_2-, and γ_3-CN (β-CN f29-209, β-CN f106-209, and β-CN f108-209, respectively), which are the products of plasmin (native milk protease) activity towards the β-CN. In addition, the proteose peptones, the remaining complementary fragments of β-CN released during plasmin induced degradation, also have been identified in milk (Eigel et al., 1984).

Figure 4. The structure of reference β-CN with 5 moles P (β-CN A²-5P). ***SeP****, sites of phosphorylation. Potentially phosphorylated AA residues are underlined. Plasmin cleavage sites are marked with arrows (reproduced from Farrell et al. (2004) with permission of American Dairy Science Association).*

κ-CN consists of 169 AA residues, and reference casein is genetic variant κ-CN A with 1 mole P with a molecular weight 19,037 Da (Figure 5) (Farrell et al., 2004). An N-terminal end of κ-CN is hydrophobic, while a C-terminal tail, GMP, cleaved by chymosin at Phe_{105}-Met_{106} bond, is hydrophilic (Fox et al., 2000).

Figure 5. The structure of reference κ-CN with 1 mole P (κ-CN A-1P). ***SeP****, site of phosphorylation. Potentially phosphorylated AA residues are underlined. Chymosin cleavage cite is marked with arrow (reproduced from Farrell et al. (2004) with permission of American Dairy Science Association).*

Värv et al. (Värv, Belousova, Sild, & Viinalass, 2009) have studied genetic diversity in milk proteins among Estonian dairy breeds. There are three cattle breeds in Estonia: 75% of Estonian Holstein, 25% of Estonian Red, and less than 1% of Estonian Native.

Three allelic variants of β-CN (A1, A2, and B) and κ-CN (A, B, and E) and two for α_{s1}-CN (B and C) have been identified during the determination of casein genes polymorphism of Estonian milk (Jõudu et al., 2007; Värv et al., 2009). Various genetic variants of casein have different affinities towards rennet, effect cheesemaking quality of milk and rheological properties of the curd (Caroli, Chessa, & Erhardt, 2009).

1.1.4 Minerals

The main minerals of milk are distributed between the serum phase and casein micelles. Potassium, sodium, and chloride ions are present in the diffusible fraction, while the portion of the calcium, magnesium and inorganic phosphate are associated with CCP in the casein micelles. A small part of calcium, half of inorganic phosphate, two-thirds of magnesium and most of the citrate are in the aqueous phase of milk (Gaucheron, 2005). The calcium and phosphate have an important role in the aggregation reaction during rennet coagulation of milk and influence the structure and texture of cheese. There are several other microelements associated with milk fat globule membrane, enzymes etc. (Fox et al., 2000).

1.2 Cheese

Cheese is a fresh or matured dairy product obtained mainly by enzymatic coagulation of milk, followed by curd processing, whey separation, shaping and ripening. In cheese, milk fat and caseins are concentrated about tenfold, while most of lactose, soluble mineral salts, and whey proteins are expelled into the whey (Fox et al., 2015). Nutritionally, cheese is an important source of proteins, fatty acids, vitamins, and minerals. There is no definite classification of cheese varieties, as these vary widely in characteristics. The Cheddar, Dutch (i.e., Gouda and Edam), Swiss (e.g., Emmental), hard Italian (e.g., Parmigiano Reggiano and Grana Padano), and *pasta filata* (e.g., Mozzarella) are the most important families of cheeses. Overall, cheeses can be classified based on:

- texture and water content. Grouping the cheeses by the moisture content in non-fat substances (MNFS) from extra-hard (<40%) to soft (70-82%);
- method of coagulation. Rennet, acid, acid/heat cheeses;
- ripening indices. Classifying according to the extent of chemical breakdown of proteins and fats;
- microorganisms involved in cheesemaking and ripening. Those are mostly lactic acid bacteria (LAB), propionic acid bacteria, also moulds and yeasts in some varieties (McSweeney, Ottogalli, & Fox, 2004).

1.2.1 General steps of cheesemaking

Despite large variation, there are basic steps essential for most of cheese varieties (Figure 6). The production of cheeses can be generally divided into two phases: conversion of milk to gel and ripening of the pressed curds (Fox et al., 2015).

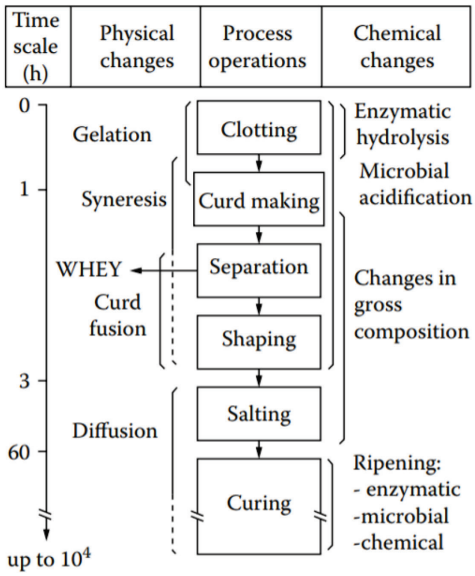
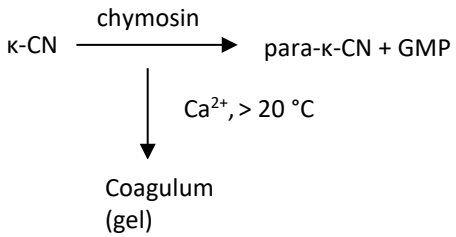


Figure 6. Schematic diagram of physical and biochemical changes essential for ripening of the most cheeses (reproduced from Walstra et al. (2006) with permission of Taylor & Francis Group).

Cheese milk is usually heat treated (low temperature short time pasteurization) to reduce the bacterial load followed by standardization to adjust the casein to fat ratio. Bactofugation and membrane filtration are also often applied to remove the microbial cells and spores (Fox et al., 2015).

Starter cultures and in some cheese varieties also adjunct starters are added to cheese milk before renneting to ensure the lactose conversion to lactic acid. The rate of acidification varies between the cheese varieties. In hard and semi-hard cheeses the pH decreases to approximately 5.0–5.3 during the first 20 h (Fox et al., 2015). The acidity, salt content, temperature and anaerobic environment inhibit the growth of most unwanted microorganisms and determine the activity of enzymes involved in cheese maturation. In addition, the lactic acid can be transformed into various compounds (e.g., acetic acid, propanoic acid), which contribute to cheese flavor (McSweeney & Sousa, 2000).

Another crucial step defining the characteristics of the final cheese is coagulation of milk induced by rennet (in most cases chymosin) (Figure 6 and 7). Chymosin initiates the enzymatic phase of coagulation by splitting the bond Phe_{105}-Met_{106} in κ-CN and cutting off the "hairy layer" stabilizing the casein micelles in milk serum (Kumar, Grover, Sharma, & Batish, 2010):



The hydrophilic GMP (κ-CN f106-169) is released from the surface of the micelles into the whey during further processing, while the para-κ-CN (κ-CN f1-105) remains attached to the casein micelle (Figure 7). Cutting the GMP from the surface of the micelle reduces its zeta potential from -20 to -10 mV as well as Van der Waals interactions and repulsive electrostatic forces. When about 85% of κ-CN is hydrolyzed, the aggregation phase of renneting commences; micelles can approach closer to each other and begin to aggregate in the presence of calcium, forming the coagulum (Figure 7) (Fox et al., 2000; Walstra et al., 2006).

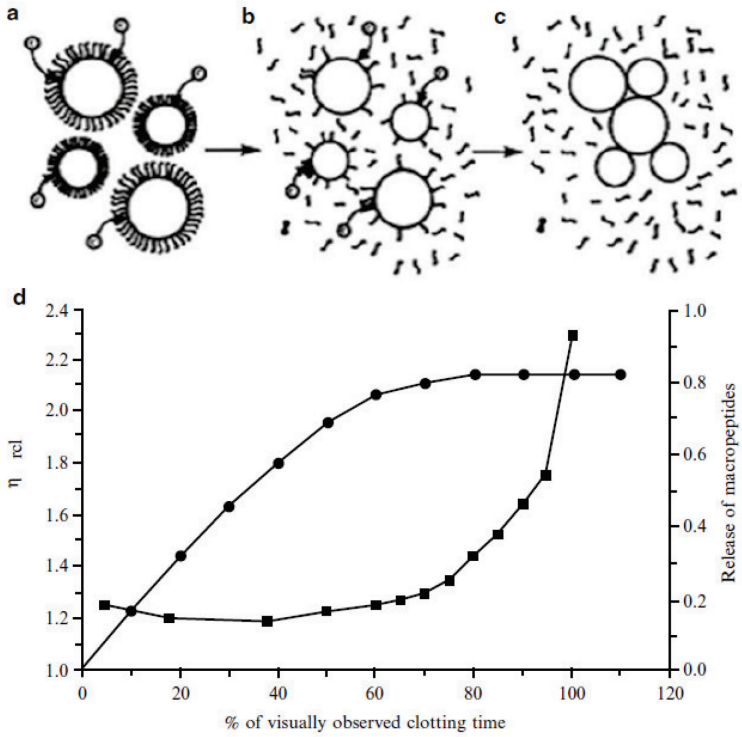


Figure 7. Scheme of coagulation of milk by chymosin. a) casein micelle with GMP on the surface being attacked by chymosin; b) splitting the GMP; c) aggregation of micelles; d) release of GMP (filled circles) and changes in relative viscosity of coagulum (filled squares) during the coagulation (reproduced from Fox et al. (2017) with permission of Springer, Boston, MA).

There are several factors affecting the rennet flocculation time (RFT) and firmness of the gel during cheesemaking. These include refrigerated storage of milk before cheesemaking (Malacarne et al., 2013), pasteurization temperature of milk (Kethireddipalli & Hill, 2015), renneting pH and temperature (Ong, Dagastine, Auty, Kentish, & Gras, 2011; Ong, Dagastine, Kentish, & Gras, 2012), protein (Amalfitano et al., 2019) and calcium content of milk (Lucey & Fox, 1993).

Once the strong coagulum is formed, cutting, stirring and scalding of the curds follow to promote syneresis and sufficient whey expelling from the grains (Figure 6). The temperature and lactose content of the whey-curd mixture is regulated by partial removal of the whey and the addition of hot water to the cheese vat. Curd particle size, temperature range and time of heating are crucial to achieve of the desired pH,

moisture, lactose and mineral contents of the final cheese and to influence the growth of starter bacteria during the vat process and cheese ripening (Fox et al., 2015).

After the washed curds achieve the desired pH and moisture content, part of the whey is drained and the curd is formed and pressed in moulds ranging from small (250 g, e.g., Camembert) to large (60−80 kg, e.g., Emmental). Curds of the *pasta filata* cheeses (e.g., Mozzarella, Halloumi) are heated in hot water (55−75 °C) to reach their characteristic fibrous structure (Fox et al., 2015). Subsequently, pressure is applied to the curd mass in the moulds to achieve a close rind of the cheese. The salting step includes the brine salting (most cheese varieties) or applying dry salt to the cheese surface.

The ripening period of cheeses ranges from a couple of weeks (mild, high-moisture, and smear surface-ripened) to over 2 years (low-moisture, hard type cheeses) (Fox et al., 2015).

1.2.2 Peculiarities of Gouda-type cheese ripening

Traditional Gouda-type cheese is a semi-hard washed-curd cheese, salted in brine and matured for 6 to 60 weeks. Gouda cheese is produced mainly from bovine milk, clotted with fermentation-produced or microbial chymosin. To obtain the desired texture and moisture content, a low scalding temperature (37−40 °C) is applied. Mixed undefined mesophilic DL-starter culture (*Lactococcus (Lc.) lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*) is used to produce acid, ferment citrate and produce CO_2. MNFS of ripened cheese is below 63% and pH ranges from 4.9 to 5.6. Gouda cheese has smooth texture and small evenly distributed round to oval holes (Düsterhöft, Engels, & Huppertz, 2004).

During ripening, the initial degradation of caseins takes place mainly by the action of rennet (chymosin), which is active on α_{s1}-CN and in lesser extent on β-CN (Walstra, Noomen, & Geurts, 1999). About 70−80% of α_{s1}-CN is hydrolyzed within two months of ripening. The activity of milk indigenous proteinases (mainly plasmin) in cheeses with low scalding temperature is low and thus only 50−60% of β-CN in Gouda cheese is degraded after six months of maturation (Düsterhöft et al., 2004). Thermophilic starter bacteria (e.g., *Streptococcus (St.) thermophilus*, *Lactobacillus (Lb.) helveticus*) could be added to the mesophilic starter to accelerate the peptidolysis and enhance further conversion of FAA to flavor compounds. Usually, the use of thermophilic starters is accompanied with the application of higher curd cooking temperatures, which contributes to the higher activity of the plasmin and lower residual activity of the chymosin toward β-CN and α_{s1}-CN, respectively. In this dissertation, the Old Saare cheese and the cheese produced from reverse osmosis-concentrated milk can be classified as Gouda-type, while in addition to mesophilic starters also thermophilic cultures were used.

1.2.3 Application of reverse osmosis (RO) in cheese technology

Concentration of milk by RO is economically more efficient (cheaper and less energy-consuming) than alternative process of thermal evaporation, that may affect the sensory properties of the concentrated product (Chemat et al., 2017). In milk concentrated by RO (RO-milk) only a part of water is removed through a RO filter, retaining all other compounds (fat, protein, lactose, and minerals) in the retentate (Mistry & Maubois, 2017). The RO technology can be used on dairy farm to pre-concentrate large volumes of milk in order to save the transportation costs.

The RFT, the rate of gel formation and the final gel firmness are of great importance in cheese manufacturing and have been shown to effect the curd moisture as well as appropriate fat and protein content (Fagan, Castillo, Payne, O'Donnell, & O'Callaghan, 2007). Sørensen et al. have investigated the changes in chymosin-induced gelation behavior and the rheology of coagulum of RO-milk. The RO-milk was shown to have longer RFT, due to the lower enzyme to substrate ratio in retentate, but at the same time the higher rate of gel formation compared to the raw unconcentrated milk. Moreover, lowering the amount of chymosin added, the RFT increased and the rate of gel formation decreased. Thus, the process of milk filtration by RO itself does not alter the gelation properties of concentrated milk (Sørensen, Larsen, & Wiking, 2019).

In cheese production, the utilization of RO-milk can potentially increase the production capacity and cheese yields by reducing at the same time the amounts of starter and rennet. Nevertheless, only in a limited number of studies the application of RO membrane processing in cheese production has been reported. Agbevavi and co-wokers were the first who made an attempt to utilize the whole RO-milk for Cheddar cheese manufacturing in a pilot plant (Agbevavi et al., 1983). Compared to the cheese made from original milk, high bacterial contamination and ununiform texture were observed in the RO-cheese. Barbano and Bynum have succeeded in the manufacture of the Cheddar RO-cheese with increased yield in a commercial cheese plant (Barbano & Bynum, 1984). However, a significantly slower casein breakdown (TN soluble in 12% TCA and TN soluble in pH 4.6 acetate buffer were used as indices of proteolysis) was noted at the later stages of Cheddar RO-cheese ripening compared to the control cheese (Bynum & Barbano, 1985). At the moment there is a lack of information about proteolysis in semi-hard and hard-type cheeses produced from RO-milk.

1.3 Proteolysis during cheese ripening

Cheese ripening is characterized by serial physico-chemical changes via proteolytic, lipolytic and glycolytic reactions. Among these processes proteolysis is the most complex and important one, which contributes to the development of texture and produces flavor precursors in cheese (McSweeney & Sousa, 2000). General mechanism of proteolysis starts from degradation of caseins to large peptides by residual coagulant (chymosin) and plasmin (Figure 8). Cell-envelope proteinases (CEP) of LAB subsequently hydrolyze the large peptides to small ones that can be transported via specific peptide transport system into the LAB cytoplasm for further degradation by various intracellular peptidases to shorter peptides and FAA. FAA can be converted to the numerous flavor compounds such as aldehydes, carboxylic acids, esters etc. (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). The characterization of the extent and pattern of proteolysis in cheese is of great interest, as it defines the index of cheese maturity, helps to optimize the cheesemaking by varying the technological parameters and proteolytic agents, and thus controls the quality of a final product.

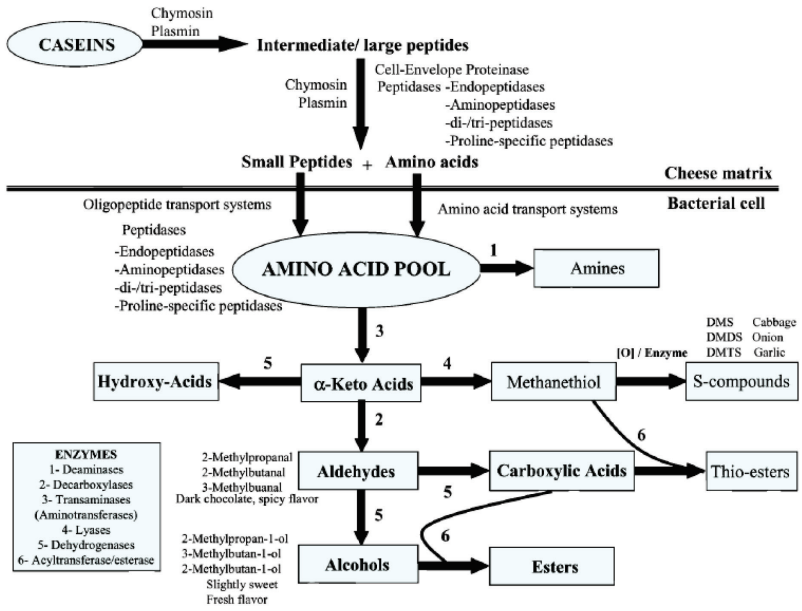


Figure 8. General scheme of proteolysis and formation of flavor compounds from free amino acids in cheese. DMS: dimethyl sulfide; DMDS: dimethyl disulfide; DMTS: dimethyl trisulfide (reproduced from Singh, Drake, & Cadwallader (2003) with permission of John Wiley and Sons).

1.3.1 Chymosin action

Chymosin is the main enzymic component of calf rennet, traditionally most frequently used in cheesemaking. Chymosin (EC 3.4.23.4; 88−94% milk clotting activity) is an aspartyl proteinase naturally produced in the stomachs of young mammals, which has activity at low pH and prefers to cleave the bonds Phe-X and Leu-X (Visser, 1993). Because of the decreasing availability of calf rennet, its high cost and ethical concerns, an alternative rennet substitutes from animal, microbial and plant sources are being tested and used (Çep & Akın, 2013; Guinee & Wilkinson, 1992).

The major role of chymosin is to destabilize the casein micelle by cleaving the Phe_{105}-Met_{106} bond in κ-CN and to coagulate the milk (Kumar et al., 2010). Most of the chymosin is removed with the whey, but some is transferred to the curd. The amount of the retained chymosin in the curd is up to 15%, depending on cheese variety, cooking temperature and the pH at the whey draining (Sousa et al., 2001).

The cooking temperature of the curds has a large effect on the activity of chymosin. Thus, in Gouda-type cheeses with cooking at 37−40 °C, the chymosin is more active compared to low or no chymosin activity in Swiss-type or hard Italian cheeses with cooking temperature 52−54 °C (Guinee & Wilkinson, 1992).

The first target of chymosin in fresh cheese is the bond Phe_{23}-Phe_{24} in α_{s1}-CN, which is not hydrolyzed during milk coagulation, but may be degraded in cheese during ripening (Upadhyay, McSweeney, Magboul, & Fox, 2004). The primary degradation product α_{s1}-I-CN (α_{s1}-CN f24−199) can be detected by CE, and small complementary peptide α_{s1}-CN f1−23 is further rapidly digested by bacterial CEP. Additionally, it was shown that in solution chymosin cleaves α_{s1}-CN at twelve sites (Fox et al., 2000). The breakdown of α_{s1}-CN by chymosin is much faster than that of β-CN, being hydrolyzed preferably at the bond Leu_{192}-Tyr_{193} (Visser, 1993). The obtained large

peptides β-CN f1−192 cannot be identified by CE. Chymosin cleaves β-CN also at six sites in the C-terminal part of the sequence. The hydrolysis of β-CN by chymosin is retarded by 5% (i.e. salt content in cheese environment) and completely inhibited by 10% NaCl. α_{s2}-CN is relatively resistant to chymosin hydrolysis due to the hydrophobic regions of the sequence (Fox et al., 2000).

1.3.2 Plasmin action

Milk contains about 60 different enzymes, most being inactivated by heat treatment and their functions are not defined (Fox et al., 2015). The dominant native proteinase in milk is plasmin (EC 3.4.21.7), which is a part of the complex of enzyme/activator/inhibitor system. Both plasmin and its inactive form plasminogen are found in bovine milk in a ratio approximately 1:9, bound to casein micelles and the fat globule membrane. The optimal conditions for plasmin are pH 7.5−8.0 and 37 °C, but it is also active within a broad range of temperatures and pH (Nielsen, 2002). The reduction of pH can release plasmin and plasminogen from the micelles (Crudden, Fox, & Kelly, 2005).

Plasmin and plasminogen are heat-stable and fully survive the pasteurization and the scalding process during cheesemaking. Plasmin activity is high in cheeses with high cooking temperature (Swiss-type), because of the thermal inactivation of inhibitors of plasminogen activators leading to the conversion of plasminogen to the active plasmin (Kaminogawa, Mizobuchi, & Yamauchi, 1972). Plasmin preferentially cuts polypeptide chain after lysine and, to a lesser extent, after arginine residue. Plasmin hydrolyses α_{s1}-CN and β-CN at the same rate, α_{s2}-CN is subjected to the slower degradation, and plasmin has a very low activity towards κ-CN (Sousa et al., 2001). The main substrate for plasmin is β-CN, resulting in the formation of β-CN f29-209 (γ_1-CN), f106-209 (γ_2-CN), and f108-209 (γ_3-CN) (Eigel et al., 1984) and corresponding N-terminal peptides β-CN f1-29, f1-105, and f1-107. The hydrolysis of β-CN starts already in milk before cheese manufacture and ripening (Upadhyay et al., 2004). In solution, plasmin hydrolyzes α_{s1}-CN at 17 cleavage sites, with the cleavage preference towards the center of the molecule and subsequent hydrolysis of the polypeptides derived (McSweeney, Olson, Fox, Healy, & Højrup, 1993). Plasmin has several cutting sites on α_{s2}-CN (Sousa et al., 2001).

Another milk indigenous proteinase is cathepsin D, which specificity is very similar to chymosin. The milk coagulation property of cathepsin D is poor, due to its low amounts in milk. In addition to cathepsin D, some minor proteolytic enzymes are present in milk such as elastase and cathepsin B (Sousa et al., 2001).

1.3.3 Proteolytic system of lactic acid bacteria (LAB)

In this dissertation, the starter culture used for cheesemaking consisted of mesophilic *Lactococcus* and *Leuconostoc* species, and thermophilic *Lactobacillus* species and *St. thermophilus*. In addition to the primary role of starters to ferment lactose to lactic acid and decrease the pH, the LAB provide a large pool of proteinases and peptidases capable to hydrolyze large peptides to smaller ones and to release FAA (Figure 8).

The proteolytic system of LAB has been studied extensively. The degradation of large peptides by LAB is initiated by extracellular CEP named lactocepin or PrtP. The intake of peptides and FAA by bacterial cells occurs via the transporters of di/tripeptides, oligopeptides (capable to transport peptides of 4 to at least 18 AA in length) and specific transporters of amino acids (e.g. the transporters of Glu/Gln, Ile/Leu/Val residues) (Detmers, Kunji, Lanfermeijer, Poolman, & Konings, 1998; Kunji et al., 1996).

Bacterial cells contain various intracellular peptidases (e.g., endopeptidases, aminopeptidases, proline-specific peptidases, di/tripeptidases) (Sousa et al., 2001). In the result of autolysis of the bacterial cell, the intracellular peptidases release to the cheese matrix during ripening (Crow et al., 1995). In *Lactococcus* no carboxypeptidase activity has been found (Sousa et al., 2001). Carboxypeptidases have been identified only in few *Lactobacillus* stains (Slattery, O'Callaghan, Fitzgerald, Beresford, & Ross, 2010).

To date, numerous *in vitro* studies have been investigated the specific action of individual peptidases and CEP of LAB on caseins (Exterkate & Alting, 1993; Miclo et al., 2012; Sadat-Mekmene et al., 2011) and in milk (Solieri, De Vero, & Tagliazucchi, 2018). Several studies have been focused on the determination of proteolytic activities of single strain starters in cheese juice (Sheehan, O'Cuinn, FitzGerald, & Wilkinson, 2006). Nevertheless, the effects of *in situ* conditions, i.e. cheese matrix containing various species of bacteria, on the activity of proteolytic system of LAB are less studied.

1.4 Methods to study proteolysis

The genetic and non-genetic polymorphism of milk proteins makes it difficult to determine the compositional changes of different caseins in milk and cheese. For evaluation of proteolysis during cheese ripening, the non-specific chemical methods based on the quantification of soluble N in consequent cheese extractions or the measurement of reactive groups (e.g., $-NH_2$) are widely used (Ardö & Polychroniadou, 1999; McSweeney & Fox, 1997) (Figure 9). All these methods provide robust information on the overall extent of proteolysis, while do not allow the determination of specific proteinaceous compounds one by one and estimate the activity of the proteolytic enzymes.

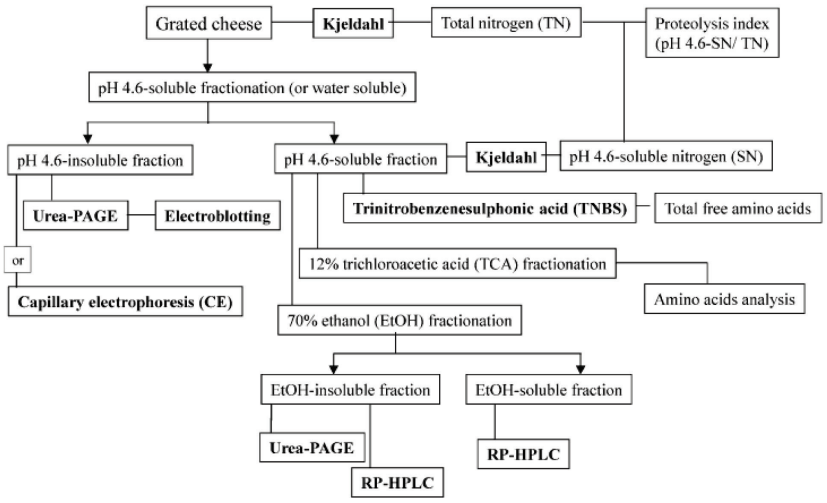


Figure 9. The main methods used for evaluation of proteolysis in cheese during ripening. Analytical methods are marked in bold (reproduced from Sousa et al. (2001) with permission of Elsevier).

1.4.1 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a better alternative to traditional PAGE and a comparable method to HPLC (Bobe, Beitz, Freeman, & Lindberg, 1998; Kinghorn, Norris, Paterson, & Otter, 1995). CE is a high-resolution, quantitative (in case of using standards), cost-effective, automated analytical technique with simple sample preparation and little waste production, that enables simultaneous determination of whey proteins and caseins in dairy products (de Jong, Visser, & Olieman, 1993; Miralles, Ramos, & Amigo, 2003).

CE is a separation technology based on the migration of charged molecules under the influence of electric filed inside a fused-silica capillary (20–200 μm i.d.) filled with a background electrolyte (Harvey, 2000). CE instrument consists of a suitable injection system, two reservoirs with buffer, two electrode assemblies, a separation fused-silica capillary column, a high-voltage power supply and a detector (Figure 10).

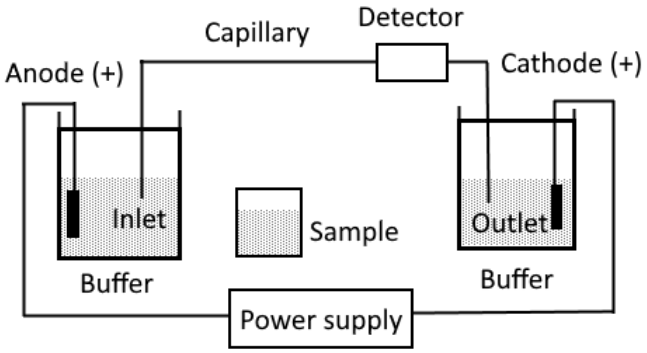


Figure 10. The principal scheme of capillary electrophoresis.

In CE, after the sample injection into the capillary, electrodes are immersed into the buffers with an appropriate buffering capacity, an electric field is applied and charged molecules begin to migrate through the capillary filled with buffer (Figure 10). When molecules pass through an optical viewing window made in the capillary external coating, the detector (ultraviolet most frequently used) response is recorded in a suitable data acquisition system. Separation of the molecules depends on their different electrophoretic mobility based on the charge, mass, size, and shape, as well as electroosmotic flow of the buffer (Mikkelsen & Cortón, 2004).

The electroosmotic flow (EOF) is the phenomenon that appears in the capillary when an electric field is applied. In the fused-silica capillary, the negatively charged silanate ions ($Si\text{-}O^-$) of the inner wall interact with positively charged ions from the buffer solution, creating an electrical double layer. With voltage, cations of the double layer migrate towards the cathode, pulling along the bulk of the buffer solution and producing EOF. At high buffer pH, the EOF is larger than the electrophoretic migration and flushes the negatively charged proteins also towards the cathode. Thus, cations, neutrals, and anions can be analyzed in a single run since they migrate in the capillary in the same direction (Mikkelsen & Cortón, 2004). Several additives can be added to the buffer to suppress EOF and alter electrophoretic mobility of molecules (Dolník, 2008).

In separation of proteins, the problem of interaction between proteins and uncoated capillary surface exists, decreasing the separation efficiency, accuracy, and repeatability. To avoid the adsorption of the proteins onto the negatively charged fused-silica capillary wall, the internal wall of the capillary is usually coated with a hydrophilic polymer (neutral capillary, commercially available) (Nowak, Woźniakiewicz, Gładysz, Janus, & Kościelniak, 2017). Permanent coating of capillaries improves separation efficiency and repeatability, while prolonging the lifetime of capillaries at the same time, increasing the analysis cost. Modifications of fused-silica capillary wall charge by different coatings can also decrease the EOF, while leaving the electrophoretic mobility of molecules unaffected (Dolník, 2008). In this study, neutral polyvinyl alcohol coated capillary (neutral PVA) was used, which provided the neutralization of EOF (Nowak et al., 2017). At acidic buffer of pH 3.0 used in this study in CE analysis, proteins were mainly positively charged and during the separation in neutral capillary migrated towards the cathode.

CE has been proved to be an efficient technique for separation of proteins in cow's (Otte et al., 1997), goat's (Recio, Amigo, & Lopez-Fandino, 1997), ewe's (Izco, Ordóñez, Torre, & Barcina, 1999), and mixed milk (de Jong et al., 1993; Molina, Martín-Álvarez, & Ramos, 1999). CE has been used for separation of different genetic variants and phosphorylation states of caseins in milk (Heck et al., 2008), as well as for evaluation of milk heat treatment (Recio et al., 1997). CE has been applied for analysis and identification of main casein fractions and primary hydrolysis products in different cheese varieties during ripening (Rehn, Petersen, Saedén, & Ardö, 2010; Rodriguez-Nogales & Vázquez, 2007).

1.4.2 Liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS)

Liquid chromatography (LC) is an analytical method that separates compounds in a complex mixture on a chromatographic column by pumping a liquid mobile phase through a stationary phase. The elution order of solutes depends on their polarity and the interaction with the stationary phase. Different flow rates of the sample components lead to the separation of analytes at different retention times (Harvey, 2000).

The combination of LC with MS is the most widely used approach in peptidomic studies of cheeses (Mentana et al., 2016; Stefano Sforza, Ferroni, Galaverna, Dossena, & Marchelli, 2003). The MS analyzes the mass-to-charge ratio (m/z) and signal intensities of the ionized peptides recording the MS spectra. In MS/MS experiments, peptide ions, in turn, are selected for further fragmentation to obtain the ion spectra (MS/MS) (Figure 11). On the basis of MS/MS spectra, the peptides of interest are identified; the quantitative information is derived from the extracted ion chromatogram (XIC).

Recently, MS/MS has emerged as a powerful tool for identification of bioactive peptides in cheese with beneficial effects on the gastrointestinal, cardiovascular, immune and nervous systems (Nielsen et al., 2017; Sánchez-Rivera et al., 2014). Several peptides have been characterized for their contribution of bitterness perception of cheeses (Karametsi et al., 2014). Moreover, the phosphopeptides that accumulate in cheese during ripening have been identified by LC-MS/MS (Ardö et al., 2007).

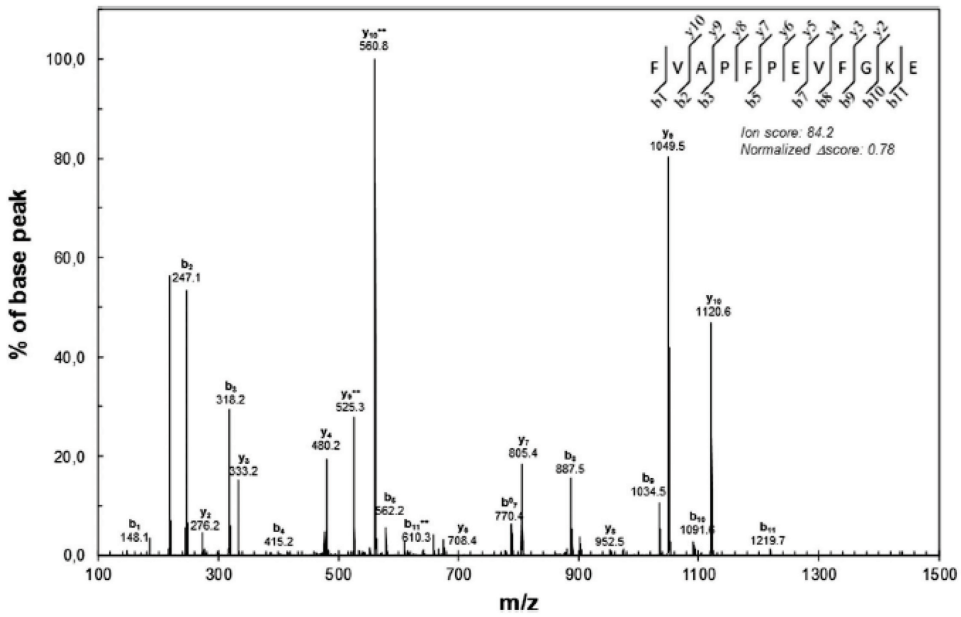


Figure 11. The representative MS/MS spectrum of ion at m/z 638.8. Peptide α_{s1}-CN f24-35 (reproduced from Mentana et al. (2016) with permission of John Wiley and Sons).

MS/MS has been successfully used for determination of 116 casein-derived peptides from Coalho cheese (Fontenele et al., 2017), 70 peptides from Camembert-type cheese in the study of the effect of milk heat treatment and addition of adjunct cultures on peptide profiles (Galli et al., 2017), and 57 most abundant oligopeptides in Parmigiano-Reggiano cheese during two years of ripening (Sforza et. Al., 2012). Hence, most of the research on cheese peptidomics has been focused on the identification of limited number of identified peptides and thus provides only scarce information on casein hydrolysis. Nevertheless, an extensive MS/MS analysis of human milk peptidome has revealed almost 700 peptides (Guerrero et al., 2014), and 682 peptides have been identified from mastitic and healthy bovine milk (Guerrero et al., 2015).

There are different types of MS instrumentation and fragmentation techniques. In our study, Orbitrap-based MS/MS system with the collision-induced dissociation (CID) fragmentation provided a high mass accuracy and an ultra-high resolution of more than 3,000 water-soluble peptides in water-soluble extracts (WSE) of cheese. The molecular masses of fragmented ions were accurately measured allowing to compare them with those predicted *in silico* for the sequences in the protein database, also checking the presence of post-translational modifications. In this study, a large number of water-soluble peptides were identified and semi-quantified by LC-MS/MS and used to characterize the changes in the proteolytic cascades in cheese during ripening.

2 The Aims of This Dissertation

The main objective of this thesis was the development of novel approach to study casein hydrolysis and to obtain the comprehensive picture of proteolysis in cheese, starting from the initial slight degradation of caseins in milk, evolving substantially during cheese manufacture and essentially taking place in full extent during ripening.

The specific goals were as follows:

- to develop a set of analytical and computational methods to obtain a comprehensive picture of cheese proteolysis;
- to determine changes in a possibly wide range of proteinaceous fractions (caseins, water-insoluble and soluble peptides, free amino acids) during cheese ripening applying up-to-date analytical methods (capillary electrophoresis, liquid chromatography and mass-spectrometry (LC-MS/MS));
- to apply Phyton-based computational tool to LC-MS/MS data to characterize formation and degradation of peptides during Old Saare cheese ripening;
- to determine the extent of proteolysis in raw milk during refrigerated transportation from farm to dairy;
- to study the impact of milk concentration by reverse osmosis (RO) on the course of primary proteolysis during RO-cheese production and ripening.

3 Materials and Methods

The following sections are provided to make the materials and methods better accessible to the reader. Detailed information is available in the publications (see Appendix).

3.1 Cheese manufacturing and sampling

In Publication I, two industrial trials (10 tons of milk each) of Old Saare cheese made in Saaremaa Dairy Plant (Estonia) were analyzed. The cheese milk was bactofuged and pasteurized at 72 °C for 23 s. After cooling to 32 °C, a DVS starter cultures (DCC-260 and EMFOUR; both from Chr. Hansen Ltd., Hørsholm, Denmark) were added 30 min prior the addition of microbial rennet (ChyMax®, Chr. Hansen Ltd.). The starter DCC-260 consisted of the following species: *Lactobacillus (Lb.) helveticus, Lb. paracasei, Lactococcus (Lc.) lactis* ssp. *cremoris, Lc. lactis* ssp. *lactis, Lc. lactis* ssp. *lactis* biovar. *diacetylactis, Leuconostoc sp., Streptococcus (St.) thermophilus*. The starter EMFOUR comprised a single species *Lb*. *helveticus*. The scalding temperature was 52 °C. After pressing, 15-kg blocks were cut and salted in brine to a final content of 1.2% salt in cheese and ripened for eight months. Samples were taken after 0 (1-day-old cheese, after pressing and salting), 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 months of ripening, and stored at -20 °C until the analyses. Cheese blocks were sampled from the interior part, and at each sampling time two samples were taken from different location from each of the two cheese blocks from two cheesemaking trials. Thus, all the results on cheese proteolysis are presented as mean values calculated from 4 replicates characterizing two cheesemaking vats analyzed at 10 ripening points.

The evaluation of the changes in protein composition of cheese milk was an additional part of the study. The composition and renneting properties of over 1000 raw bulk milk samples from 170 individual dairy farms of Estonia were analyzed and described in Publication II. In addition to the milk from cooling tanks at the farms, the samples from the corresponding truck tanks and dairy silo with pooled milk from several farms were analyzed throughout the study. To evaluate the possible changes in protein composition of milk during the refrigerated transportation from farms to the dairy plant the milk samples were collected throughout the selected milk supply chain: 36 raw bulk milk samples from farm tanks, ten samples − from the truck tanks and one pooled sample from a dairy silo were analyzed by CE.

In Publication III, hard cheese was produced from 100 L of bovine milk concentrated by RO (concentration factor of 1.9, dry matter 21.7% w/w, pH 6.44) and pasteurized at 74 °C for 15 s. After heat treatment, the RO-milk was cooled to 32 °C and inoculated with a starter culture 30 min prior to addition of microbial rennet (8 g 100 L^{-1}; 1300 IMCU g^{-1}, ChyMax®, Chr. Hansen Ltd.). The starter consisted freeze-dried DVS multiple strains of mesophilic and thermophilic LAB (CHN-11, LH-B02, and ST-B01; Chr. Hansen Ltd.). FD-DVS CHN-11 comprised the following species: *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis* biovar. *diacetylactis*, *Leuconostoc* sp.; FD-DVS LH-B02 − *Lb. helveticus* and FD-DVS ST-B01 – *St. thermophilus*. The scalding was performed at 48 °C for 30 min. After pressing (for 20 min consecutively at 1, 1.8, and 2.1 bar), cheeses were brine-salted (20% NaCl, w/v) for 16 h, coated with wax and ripened at 13 °C for 6 months. Samples were taken from the inner part of the cheeses at 0 (fresh cheese before salting), 0.5, 1, 2, 4, 6 months of ripening and stored at -20 °C until the analyses.

3.2 Compositional analyses

Moisture content of milk and cheeses was measured using Precisa XM60 (Precisa Gravimetrics AG, Dietikon, Switzerland) and Mettler-Toledo HR83 (Mettler-Toledo AG, Greifensee, Switzerland) moisture analyzers (ISO, 2004). The pH of milk and cheeses was measured with a pH meter (Mettler-Toledo Ltd., Leicester, UK) by inserting a glass electrode into the compressed grated cheese samples. Total fat content of milk and cheeses was determined by the method of the Association of Official Analytical Chemists AOAC 933.05 (AOAC, 2012). Total N (TN) content in milk, citrate dispersions (CD) and water-soluble extracts (WSE) of cheeses was determined by the Kjeldahl method, using a factor of 6.38 for conversion of TN to total protein (TP).

3.3 Determination of proteinaceous compounds

3.3.1 Protein composition by CE

Separation of intact proteins and long peptides in cheese and milk was performed with an Agilent CE System (Agilent Technologies, Waldbronn, Germany) (Publication I and III) and a Beckman P/ACE^{TM} MDQ instrument controlled by 32 Karat^{TM} version 8.0 software (Beckman Coulter, Inc., Brea, CA, USA) (Publication II) according to the method described by (Ardö & Polychroniadou, 1999).

In Publications I and III, CD of cheese samples was prepared by mixing 5 g of grated cheese with 20 mL of 0.5 M trisodium citrate solution at 50−55 °C using a combined hot-plate magnetic stirrer with multiple stirring positions (RT 15 Power IKAMAG, IKA®–Werke GmbH & Co, Germany) for approximately 60 min until complete dissolution of cheese. Thereafter, sample volume was raised to 100 mL with ultrapure laboratory grade water (Milli-Q) and centrifuged at 1,630 g at 4 °C for 30 min to remove fat. CD samples of cheeses were mixed with a sample buffer (1:1). Milk samples were prepared by mixing 300 mL of skimmed milk (3000 x g at 10 °C for 10 min) with 700 mL of sample buffer (Publication II). Both milk and cheese CD samples incubated with a sample buffer for 1 h at room temperature, filtered through a 0.45-mm pore size polyethersulfone (PES) filter (Whatman, Maidstone, UK) and loaded into the 200 mL vials for CE analysis. Sample buffer (pH 8.6) consisted of 10 M urea, 0.83 g L^{-1} MHEC, 167 mM Tris, 67 mM disodium EDTA, 42 mM 3-(N-morpholino)propanesulphonic acid, and 17 mM dithiothreitol.

Separations of proteins were performed using a neutral polyvinyl alcohol coated capillary column (Agilent Technologies Finland OY, Finland) of 56 cm (Publication I and III) and 40 cm (Publication II) effective length and 50 µm i.d., with a slit opening of 100 x 800 mm, at 45 °C with a linear voltage gradient from 0 to 25 kV in 3 min, followed by a constant voltage of 25 kV. The separation buffer (pH 3.0) contained 20 mM trisodium citrate dihydrate, 190 mM citric acid, 0.5 g L^{-1} MHEC and 6 M urea. All buffers were filtered through a 0.45 mm pore size filter (Whatman). Before each separation, the capillary was flushed for 3 min in the reverse direction with both water and separation buffer. Milk and cheese samples were injected by pressure injection at 3.4 kPa for 20 s. Milk protein fractions were detected by UV absorbance at 214 nm.

Proteins were identified by injection of milk protein standards (α-lactalbumin, β-lactoglobulin, κ-, β-, and α_s-CN; Sigma-Aldrich Co. LLC, USA) and by comparison of the electropherograms obtained with those presented in literature (Heck et al., 2008; Miralles et al., 2003; Otte et al., 1997). Integration of the peaks was carried out after setting the baseline from valley to valley (Miralles et al., 2003).

3.3.2 Determination of water-soluble peptides by LC-MS/MS

WSE of cheeses were prepared by homogenizing 2.5 g of grated cheese in 22.5 mL of Milli-Q water (12,500−13,000 rpm) using Polytron PT 2100 dispersing aggregate with a diameter of 20 mm (Kinematica AG, Switzerland), followed by heating for 10 min at 75 °C and centrifugation for 20 min at 4 °C at 13,304 g. Supernatant was frozen in 1.5-mL Eppendorf® Protein LoBind microcentrifuge tubes (Eppendorf AG, Germany) and stored at -20 °C until further purification and analysis of water-soluble peptides and FAA with LC-MS/MS and UPLC, respectively.

WSE of cheeses were purified with C18 StageTips (Thermo Fischer Scientific Inc., USA) according to (Rappsilber, Mann, & Ishihama, 2007) protocol, and analyzed by LC-MS/MS using an Agilent 1200 series nanoflow system (Agilent Technologies, USA) connected to a Thermo Scientific LTQ Orbitrap mass-spectrometer (Thermo Electron, USA) equipped with a nanoelectrospray ion source (Proxeon, Denmark). Peptides were separated with a 240 min gradient from 8 to 40% B using a flow-rate of 0.2 µL min^{-1} and sprayed directly into the mass-spectrometer operated at 220°C capillary temperature and 2.2 kV spray voltage.

Full mass spectra were acquired in a profile mode, with a mass range from m/z 300 to 1800 at the resolving power of 60,000 at m/z 400. Raw MS files from the LTQ Orbitrap were processed using the MaxQuant software package version 1.5.2.8; (Cox & Mann, 2008). MS/MS spectra were searched against a protein sequence database containing the following casein genetic variants: A, B, and C variants for α_{s1}-CN; A, C, and D variants for α_{s2}-CN; A1, A2, A3, and B variants for β-CN and A, B, E, and F1 variants for κ-CN (Caroli et al., 2009; Farrell et al., 2004) and the MaxQuant built-in common contaminants database (245 entries). The search included the following variable modifications: methionine oxidation, N-terminal acetylation, N-terminal glutamine and asparagine deamination, formation of S-S disulfide bonds and phosphorylation of Ser, Thr, and Tyr. The peptide search included peptides in the length range from 4 to 25 amino acids.

3.3.3 Determination of FAA by UPLC

FAA (Publication I) in cheeses were analyzed by UPLC system (Acquity UPLC; Waters Corp., Milford, MA, USA) controlled by Waters Empower™ 2.0 software (Waters Corp.,). Separations were performed on a 2.1 × 100 mm Waters Acquity UPLC AccQ•Tag Ultra column (Waters Corp.). WSE samples of cheeses were mixed with isopropanol (1:1) to precipitate the proteins, centrifuged for 5 min at 4 °C at 13,304 g and filtered through a 0.2 µm PTFE filter (Merck Millipore Ltd.). Prior to the injection, FAA were derivatized with AccQ•Fluor Reagent (Waters Corp.) according to the manufacturer's procedure. The absolute concentrations of amino acids were calculated using standard curves and expressed as mmol kg^{-1} of cheese for total FAA, and individual amino acids were also expressed as relative content of the total amount of amino acids (mol %). For more information see Publication I.

3.3.4 Quantification of proteins and peptides

Semi-quantification of caseins. In Publication I the estimated concentrations of casein fractions and the products of their primary hydrolysis in cheese samples were calculated as follows:

$$C_x = \frac{\frac{A_x}{t_x} \times (CD_{prot} - WSE_{prot})}{\sum_{i=1}^{n} \frac{A_i}{t_i}},$$

where C_x is the estimated concentration of fraction x (g kg^{-1} of cheese); A_x − the peak area in the electropherogram of fraction x; t_x − the migration time of fraction x; n − the total number of identified peaks; CD_{prot} – content of proteinaceous compounds determined in CD fractions by the Kjeldahl method (g kg^{-1} of cheese); WSE_{prot} – content of proteinaceous compounds determined in WSE fractions by the Kjeldahl method (g kg^{-1} of cheese).

In the calculations of the content of different proteinaceous compounds by Kjeldahl method in CD and WSE cheese fractions the conversion factor 6.38 was used, which was the best scientifically approved value not only for milk but also proposed for cheese and other dairy products (Maubois & Lorient, 2016). The calculated content of proteinaceous compounds includes the N content originating from FAA and water-soluble peptides in case of WSE_{prot}, and additionally N content of caseins in case of CD_{prot}.

Semi-quantification of water-soluble peptides. The similar approach was applied to estimate the concentrations of water-soluble peptides in Publication I. The MS intensities of small peptides were recalculated using the following equation:

$$C_y = \frac{I_y \times (WSE_{prot} - FAA)}{\sum_{i=1}^{n} I_i},$$

where C_y is the estimated concentration of the peptide *y* (g kg^{-1} of cheese); I_y – the intensity of the peptide *y*; *n* – the total number of peptides identified by MS; WSE_{prot} – content of proteinaceous compounds determined in WSE fractions by the Kjeldahl method (g kg^{-1} of cheese); *FAA* – total concentration of FAA determined by UPLC.

For better representation of the changes of different casein fractions during cheese ripening, the concentrations of casein fractions and small water-soluble peptides (expressed in g kg^{-1} of cheese) were recalculated to mol kg^{-1} of cheese using the molar weights of casein fractions and small peptides. Here should be noted, that the molar weights of the phosphate groups were not taken into account during the recalculations of phosphopeptides (determined by LC-MS/MS analysis) to mol kg^{-1} of cheese, as MaxQuant software does not allow to determine the ratio of phosphorylated and non-phosphorylated copies of the same peptides.

During the CE analysis, all peak areas were integrated, including not only peaks which could be identified based on the standards or data from the literature, but also the other small peaks not identified before. Therefore, because of the absence of the molecular weights of those unidentified fractions, the data presented in the Table 3 (Publication I) with the overall relative distribution of different casein fractions during Old Saare cheese ripening was shown in g kg^{-1} of cheese. Also, the data presented in Figure 19 was recalculated to N content in g kg^{-1} of cheese (expressed as % of total N content) to make it easier to compare the results with other studies and cheese varieties.

Relative quantification of proteins. The calculations of the protein fraction were done in two ways. To obtain the relative concentration of each protein fraction in milk and cheese in Publication II and III the following equation was used (Heck et al., 2008):

$$C_X = \frac{\frac{A_X}{t_X}}{\sum_{i-1}^{n} \frac{A_i}{t_i}} \times 100\%,$$

where C_X is the relative concentration of fraction X (%); A_X − the peak area in the electropherogram of fraction X; t_X − the migration time of fraction X; n − the total number of identified peaks that comprise 100% of the area.

In CE, the migration time (corresponding to the peak maximum) is the time that takes each component to move from the beginning of the capillary to the detector window (Mikkelsen & Cortón, 2004). During electrophoretic separations, the peak area of the protein fraction depends on the migration time (Beckers & Boček, 2004). Moreover, there is often some fluctuation in the migration time of protein fraction under identical conditions, which makes difficult the comparison of the protein peak areas of different samples. Therefore, in the calculations of protein concentration the ratio of the peak area to the corresponding migration time was used to compensate the differences in migration time from run to run.

3.4 Data processing and statistical analysis

In Publication I, calculations and visualization of the results obtained from MaxQuant processing of the LC-MS/MS data were performed using in-house data analysis routines implemented in Python programming language (Python Software Foundation, version 3.5, available at http://www.python.org) taking into account all 3,040 water-soluble peptides.

In Publication I, for more detailed analysis of the dynamics of the peptides found in cheese during ripening, the most abundant ones, that accounted altogether at least 50% of all small peptides by estimated molar abundance were sorted out at each time point (Publication I). Altogether 169 most abundant peptides (48 from α_{s1}-, 20 from α_{s2}-, and 101 from β-CN) were further subjected to Principle component analysis (PCA) and clustering analysis for better understanding the dynamics of peptide formation throughout cheese ripening. For PCA analysis, data set of 169 peptides was centered, autoscaled, and four PCs were selected describing >90% of the variance. In clustering analysis (separates n samples into k groups) k-means clustering was applied. In the result of grouping of the peptides into several groups, four groups were chosen as optimal, as the average deviance of individual peptides profiles from the groups mean was the smallest. Four groups of most abundant peptides followed the specific patterns during Old Saare cheese ripening (Figure 17).

In Publication II, Student's t-test was used to verify whether the mean characteristics of milk from individual farms and pooled milk from tank trucks and dairy silos were different. The differences were considered significant if *p* values were less than 0.05.

4 Results and Discussion

A summary of the findings is presented in this section. More detailed discussions are available in Publications I–III in the appendix.

4.1 Comprehensive approach to follow proteolysis in cheese during ripening (Publication I)

Publication I describes a comprehensive study of the changes in the content of different proteinaceous fractions in hard Old Saare cheese (mesophilic and thermophilic starters, scalding temperature 52 °C) during eight months of ripening. Intact caseins and their degradation products (water-insoluble peptides) were measured by capillary electrophoresis (CE), water-soluble peptides – by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), free amino acids (FAA) were analyzed using ultra-performance liquid chromatography (UPLC), and N content in casein fractions was determined by Kjeldahl method.

4.1.1 Primary proteolysis

The characteristic pattern of primary casein degradation during Old Saare cheese maturation determined by the activities of residual chymosin and native plasmin is presented on the electropherograms on Figure 12. The precise identification of intact caseins and their degradation products in milk and cheese by CE, the issues associated with application of commercial standards and quantification of caseins as well as the order of the migration of different fractions are described in detail in the Section 4.2 (Proteolysis in milk during refrigerated transportation).

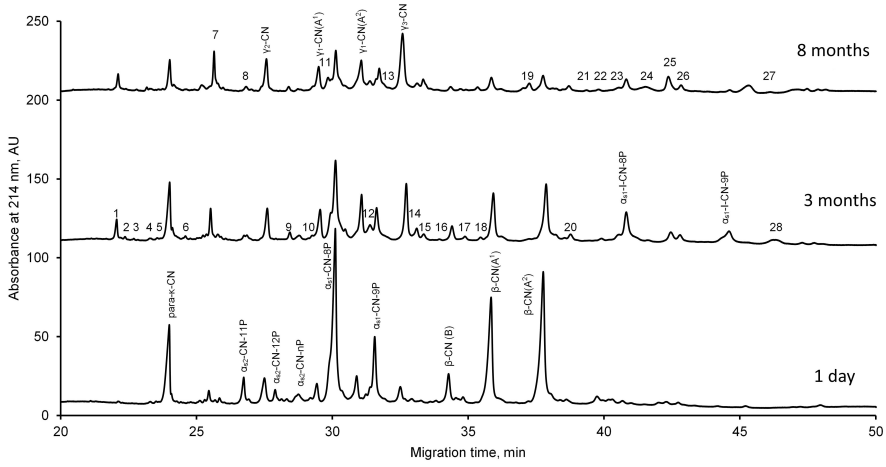


Figure 12. Capillary electropherograms of Old Saare cheese at the first day, 3, and 8 months, showing the hydrolysis profile of caseins during ripening. CN: casein; para-κ-CN: κ-CN f1–105; γ_1-CN: β-CN f29–109; γ_2-CN: β-CN f106–209; γ_3-CN: β-CN f107–209; α_{s1}-I-CN-8P: α_{s1}-CN f24–199; α_{s1}-I-CN-9P: α_{s1}-CN f24–199 9P; A^1, A^2, and B: genetic variants of caseins; xP: number of phosphate groups in casein; AU: arbitrary unit. Non-identified peaks are marked with numbers 1-28.

The substantial changes in the content of the main casein fractions were observed over eight months of Old Saare cheese maturation. The hydrolysis of α_{s1}-CN (8P and 9P) at Phe_{23}-Phe_{24} by chymosin was observed with concomitant appearance of α_{s1}-CN-I (8P and 9P) (α_{s1}-CN f24-199; approximately at 41.0 and 45.0 min). The breakdown of β-CN (A^1, A^2, and B) by plasmin at Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, and Lys_{107}-Glu_{108} resulted in the formation of γ_1-CN (A^1 and A^2) (β-CN f29-209; at 29.5 and 31 min), γ_2-CN (β-CN f106-209; at 27.5 min), and γ_3-CN (β-CN f108-209; at 32.5 min). Other minor peaks of intact caseins identified in cheese were: α_{s2}-CN (11P, 12P, and nP) and κ-CN (Figure 12). The areas of non-identified peaks (marked with numbers 1-28 on Figure 12) were also integrated and taken into account in the further calculations of the content of the main casein fractions in order to obtain the integral picture of proteolysis (Section 4.1.3). The non-identified peaks were considered as degradation products due to their mainly increasing behavior during cheese ripening and, obviously, represent large-sized peptides released from the primary breakdown of α_{s2}-CN or some other insoluble peptides derived from α_{s1}-CN and β-CN produced by the action of proteinases other than chymosin and plasmin.

4.1.2 Characterization of peptide profiles

The formation of water-soluble peptides by the activities of bacterial proteinases and peptidases during ripening of Old Saare cheese was monitored by the analysis of the WSE by LC-MS/MS. While a large number of studies focus on the detection of 100−150 most abundant peptides, we aimed to identify a possibly wide range of peptides of different sizes. The application of the in-house developed Python-based computational tool to analyze and visualize the results obtained by LC-MS/MS as well as the identification of cutting sites of the enzymes and clustering analysis of the peptides, enabled a new approach for detailed characterization of cheese ripening. In total, 3,266 small peptides of 4 to 25 AA in length (226 peptides of non-casein origin) were identified by MS/MS, thus also the less abundant peptides were included in further analysis embracing nearly the whole peptidome of water-soluble fraction.

4.1.2.1 LC-MS/MS data analysis

The m/z values and the MS/MS spectra obtained from Thermo Scientific LTQ Orbitrap system were further analyzed with MaxQuant using the integrated peptide database search engine Andromeda (Cox et al., 2011). The .fasta files configured with Andromeda provide the specific protein amino acid sequences, which allow the high throughput sequence assignment of a large number of peptides. MaxQuant is a continuously developing and updating platform for MS-based peptidomics data analysis, which supports the identification of post-translational modifications (e.g., phosphorylation) (Tyanova, Temu, & Cox, 2016). Another advantageous option in MaxQuant is the “Match between runs”, which is especially useful in the identification of low-abundant peptides. When the measured MS/MS spectrum is not quite sufficient to assign the peptide, the identification from another LC-MS run is transferred by matching the mass and the retention time. This option allows to improve the identification and leads to more complete peptides profile of the sample (Tyanova et al., 2016).

A large data volume received from the analysis of MS/MS spectra by MaxQuant (3,040 water-soluble casein-derived peptides) was subsequently treated by Python Software using the in-house script to facilitate the calculations and perform the visualizations of the peptide profiles. Python uses the list of identified peptides and their corresponding intensities as an input to localize the position of each peptide on

the respective protein sequence. Using the figures drawn by Python it is possible to follow the changes in the content of peptides in cheese during ripening and to assign the cutting sites of peptides to the specific enzymes taking part in casein degradation. On these figures, the activity of bacterial peptidases on oligopeptides, produced by plasmin and chymosin action on intact caseins, can be seen.

The label-free quantification (extraction of peptide signal intensity to compare the peptide abundances across samples) have been used for *Lc. lactis* and *Escherichia coli* proteome studies (Arike et al., 2012; Lahtvee et al., 2011; Wiśniewski & Rakus, 2014). Similar approach was used for semi-quantification of the peptides in our study. The estimated concentrations in mmol/kg of cheese were calculated using the intensities, molecular weights of peptides reported by MaxQuant and N content of proteinaceous compounds in WSE fraction determined by Kjeldahl (see Quantification of proteins and peptides in Materials and Methods section). The use of the estimated concentrations made the comparison and combination of the results obtained by different instrumental methods (CE, LC-MS/MS and UPLC) easier and more appropriate to follow the full course of proteolysis.

4.1.2.2 Peptide coverage profiles

The list of all peptides (in total 3,266) identified by MS/MS in this study is available online in Supplementary Material of Publication I (http://www.journalofdairyscience.org; Suppl. Table S1). Nine hundred forty four water-soluble peptides originated from α_{s1}-CN (21-29% of all peptides depending on the ripening point), 813 − from α_{s2}-CN (8-17%), 1,049 − from β-CN (56-69%), 234 − from κ-CN (0.1-3%), and 226 peptides from miscellaneous milk-derived proteins (under 0.3% of total MS/MS intensity).

Figure 13 displays the β-CN sequence coverage profiles throughout the eight months of Old Saare cheese maturation obtained by the processing of MaxQuant data by the script written by Python. The sequence of β-CN from the N-terminal to the C-terminal end was plotted on the horizontal axis. The sequences of peptides originated from the corresponding protein were aligned and plotted on the β-CN sequence. The estimated concentrations of the peptides were presented by different colors according to the scale on the right. The most abundant peptides were colored in dark, whereas the less abundant ones in light colors. The peptide coverage profiles of α_{s1}-CN, α_{s2}-CN, and κ-CN were obtained in similar ways (Publication I, Suppl. Figure S2).

Mapping all the detected peptides on the corresponding protein sequence aids the visualization of the proteolysis and provides a good overview of the regions of each casein molecule, that are better accessible to proteolytic enzymes or remain intact. Such peptide coverage profiles provide a perception of the whole complexity of the data set comprised of 3,040 peptides. This underlines the needs for seeking new ways for representation of the data to better follow the progress of proteolysis in cheese.

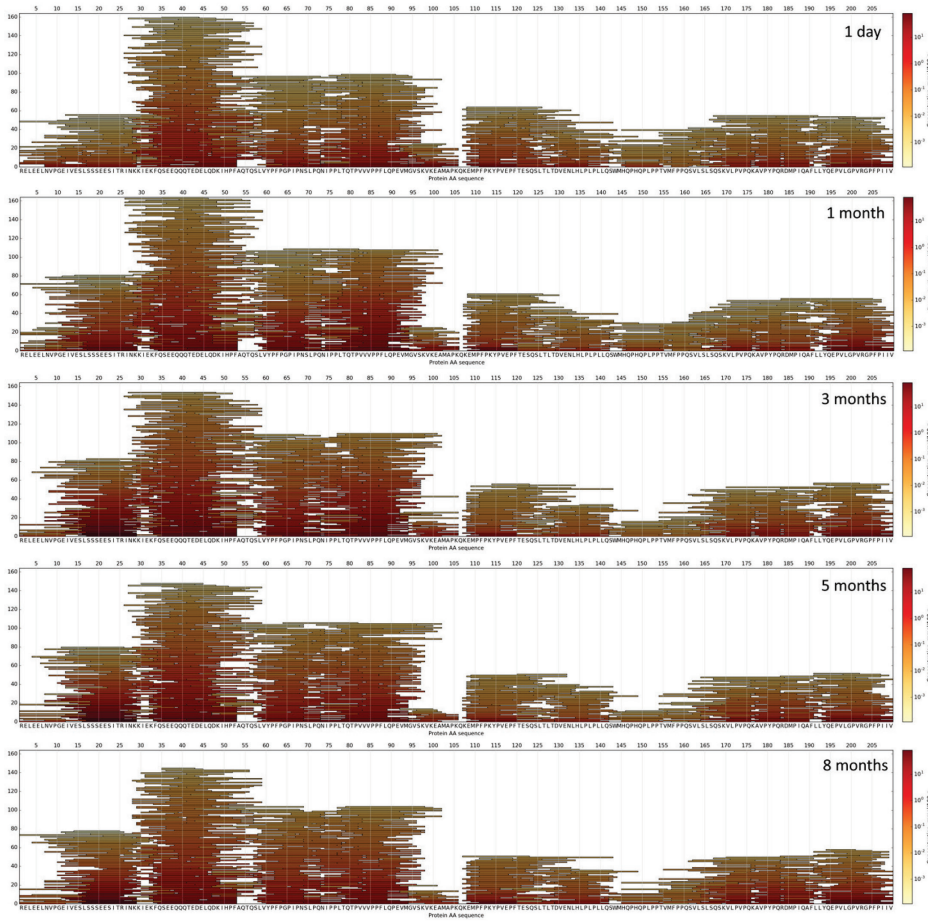


Figure 13. An example of peptide coverage profiles of β-CN (1,049 small water-soluble peptides) in 1-day-, 1-month-, 3-month-, 5-month-, 8-month-old Old Saare cheese. The sequence of β-CN is plotted on the horizontal axis. The estimated concentrations (mmol kg^{-1} of cheese; mean values of four measurements) of peptides are shown in colors according to the color scale displayed on the right.

To distinguish the casein regions that are highly degraded (with high number of peptides) and ones with low abundance of peptides, and to compare the degradation patterns of all caseins, the cumulative casein sequence coverage maps were introduced (Figure 14). The summed estimated concentrations of every amino acid located at the same position along the protein sequence were plotted on the corresponding casein sequences.

The cumulative proteolytic patterns allow to visualize and monitor the changes in the content of peptides originating from particular parts of casein sequences during cheese maturation. Each color corresponds to a particular ripening point. As the concentrations of peptides released from different caseins varied in a large range, the data for each casein were plotted on y-axis with different scale.

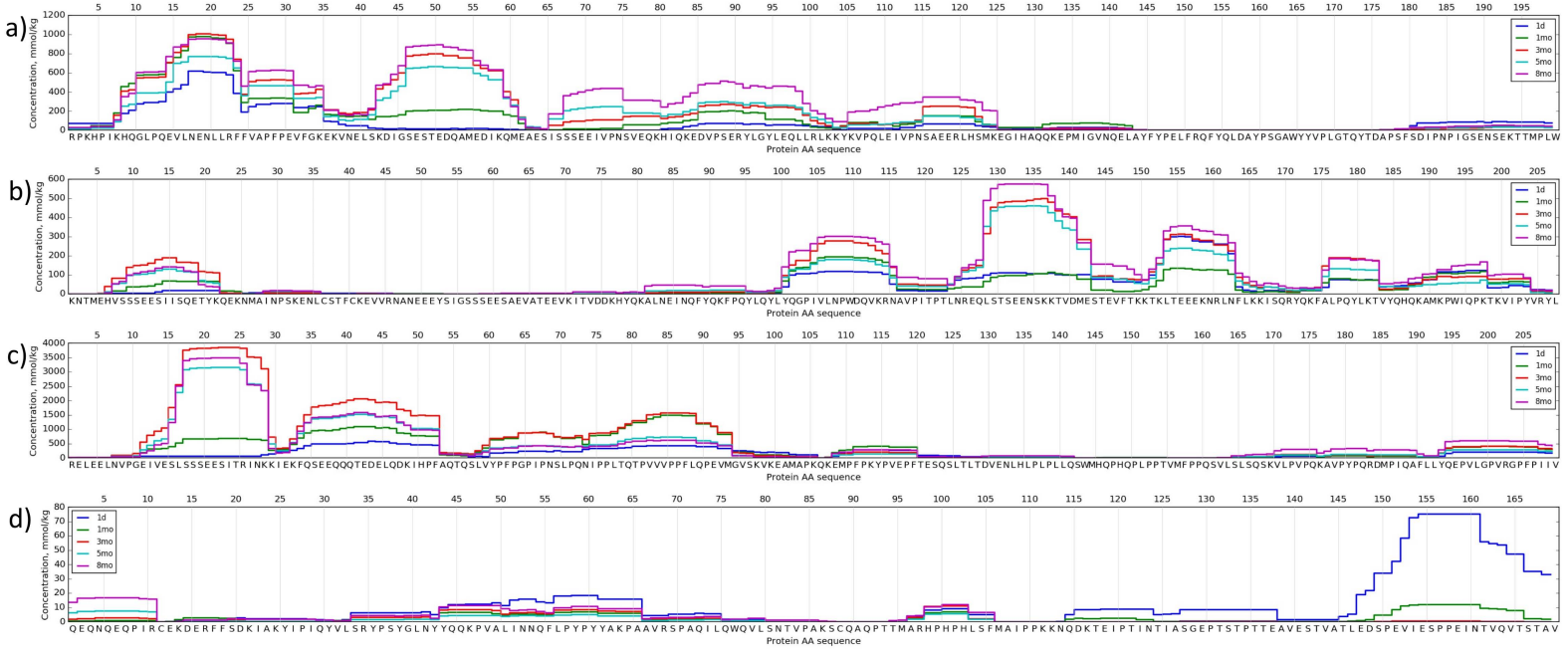


Figure 14. The regions of α_{s1}-CN (a), α_{s2}-CN (b), β-CN (c), and κ-CN (d) with the identified peptides (based on all 3,040 water-soluble peptides) in 1-day- (1d, blue line), 1-month- (1mo, green), 3-month- (3mo, red), 5-month- (5mo, dark blue), 8-month-old (8mo, purple) Old Saare cheese. On the horizontal axis, the sequences of the caseins are plotted. On the vertical axis, cumulative estimated concentrations (mmol kg^{-1} of cheese) of the amino acids in the identified peptides. The results are mean values of four measurements.

The cumulative proteolytic patterns showed clear difference between hydrolysis patterns of different caseins. Among all caseins, the certain β-CN regions were hydrolyzed most intensively and thus had the highest concentrations of peptides (Figure 14c). The concentrations of peptides from most digested α_{s1}-CN regions (Figure 14a) were two to four times lower than that of β-CN. A lower overall content of peptides derived from α_{s2}-CN (Figure 14b) correlates well with a lower abundance of this casein in cheese (Farrell et al., 2004). The peptides from κ-CN were found at lowest concentrations compared to peptides from other caseins (Figure 14d).

Going through the concentration curves of different colors one can follow an increase, decrease or more or less constant trend in the hydrolysis of peptides in particular casein regions along the whole sequence. As seen from Figure 14, the release of peptides was highly specific to the regions of the caseins. The N-terminal part of the sequence of β-CN, f6-93, was the most extensively hydrolyzed region. A significant number of peptides derived also from the regions f106-120 and f170-209, whereas the lowest proteolytic activity was observed against the sequence f120-170 showed a very low proteolytic activity. Similarly, the majority of peptides from α_{s1}-CN originated from the N-terminal part of sequence f1-124, whereas the C-terminal part f125-199 was degraded less to the low-molecular weight peptides. In case of α_{s2}-CN, low amounts of peptides were produced mostly form the C-terminal region of the sequence, and some peptides were released from f1-24. In addition to very low hydrolysis of *para*-κ-CN part (f1-105) of κ-CN, some dominating peptides were released from GMP part (f106-169), which were quickly degraded during the first month of ripening.

When looking at the most peptide providing regions of the casein sequences on Figure 14, three different contours of proteolytic cascades can be seen, suggesting involvement of the specific types of enzymes in the degradation of those regions. The proteolytic cascades with a ladder at the N-terminal end indicate that peptides differ by one or two amino acid residues in length at their N-terminal end, while C-terminal end of peptides remains more or less intact at the same time (Figure 14). Such ladders reveal the activity of LAB aminopeptidases released during cell lysis (Tjwan Tan, Poolman, & Konings, 1993). Thus, for instance, the plasmin-derived oligopeptides from f7-28 and f29-52 of β-CN were successively degraded with the formation of peptides f11/12/13/15/16/17-28 and f29/30/32/33/34/35/40/41-52 (Figure 14c; the list of peptides is available in Publication I, Table 4). The involvement of bacterial carboxypeptidases into cheese ripening was obvious. For instance, the accumulation of the peptides f128-137/138/140/142 of α_{s2}-CN was observed, those N-terminal end remained the same and C-terminal part was consecutively cleaved by one or two amino acid residues (Figure 14b). The third contour of the cutting pattern involves both amino- and carboxypeptidases activities (e.g., f41-64 of α_{s1}-CN), although the random activity of LAB endopeptidases can also be suggested (Tjwan Tan et al., 1993).

4.1.2.3 Enzyme cleavage sites

The proteolytic maps with peptides and cleavage sites alignment over the corresponding protein sequences using Python scripts have been proposed in the MS-based peptidomic analysis of human milk (Guerrero et al., 2014) as well as healthy and mastitic bovine milk (Guerrero et al., 2015). In our study similar data visualization was applied for the first time for the interpretation of the results of the peptidomic analysis of cheese samples.

The cleavage sites of the enzymes were plotted on the casein sequences by summing up the estimated concentrations of N-terminal and C-terminal amino acids of all identified water-soluble peptides. Figure 15 shows the abundances of the enzymatic cleavages of β-CN throughout Old Saare cheese maturation. Similar cleavage site patterns were constructed for α_{s1}-CN, α_{s2}-CN, and κ-CN for all ripening points (Publication I, Suppl. Figure S3).

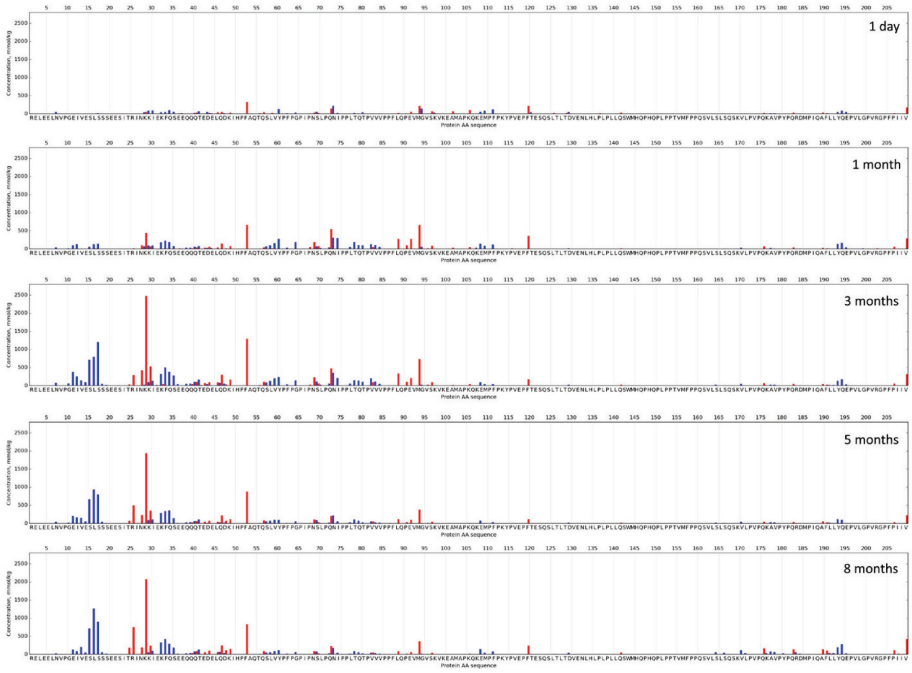


Figure 15. An example of the enzyme cleavage sites of β-CN based on all 1,049 water-soluble peptides identified in 1-day-, 1-month-, 3-month-, 5-month-, 8-month-old Old Saare cheese. Blue bars: sum of estimated concentrations (mmol kg^{-1} of cheese) of the N-terminal amino acids of peptides. Red bars: sum of estimated concentrations (mmol kg^{-1} of cheese) of the C-terminal amino acids of peptides. On the horizontal axis, the sequence of the β-CN is plotted. The results are mean values of four measurements.

In the case of β-CN, a low activity of enzymes was evident along the casein sequence in the beginning of ripening (Figure 15). As expected, with the progress of ripening the cleavage pattern covers with the plasmin-preferred sites (the bonds Lys_{28}-Lys_{29}, Lys_{105}-Gln_{106}, and Lys_{107}-Glu_{108}; (Upadhyay et al., 2004)) and the subsequent cleavages of bacterial CEP (e.g., the bonds Leu_6-Asn_7, Leu_{16}-Ser_{17}, Asn_{27}-Lys_{28}, Phe_{52}-Ala_{53}, Glu_{88}-Val_{89}, and Met_{93}-Gly_{94}; (Kunji et al., 1996; Miclo et al., 2012; Sadat-Mekmene et al., 2013)). Eventually, the proteolytic pattern of β-CN is accomplished by the action of numerous intracellular peptidases of both mesophilic and thermophilic starters on the longer peptides released in the result of the abovementioned cleavages (Figure 15).

The activity of the enzymes towards different caseins was compared at the particular stage of ripening. Figure 16 shows the enzyme cleavage sites of all caseins based on peptides identified in 8-month-old Old Saare cheese. The input data included all 3,040 water-soluble peptides, thus even less abundant ones that usually have been omitted in previously published studies on cheese peptidomics were involved to obtain a

complete picture of the casein hydrolysis. The arrows at the bottom of each casein sequence with cutting sites pattern indicate the cleavages that were assigned to the activities of the important milk indigenous enzymes as well as rennet and specific microflora enzymes used in Old Saare cheese.

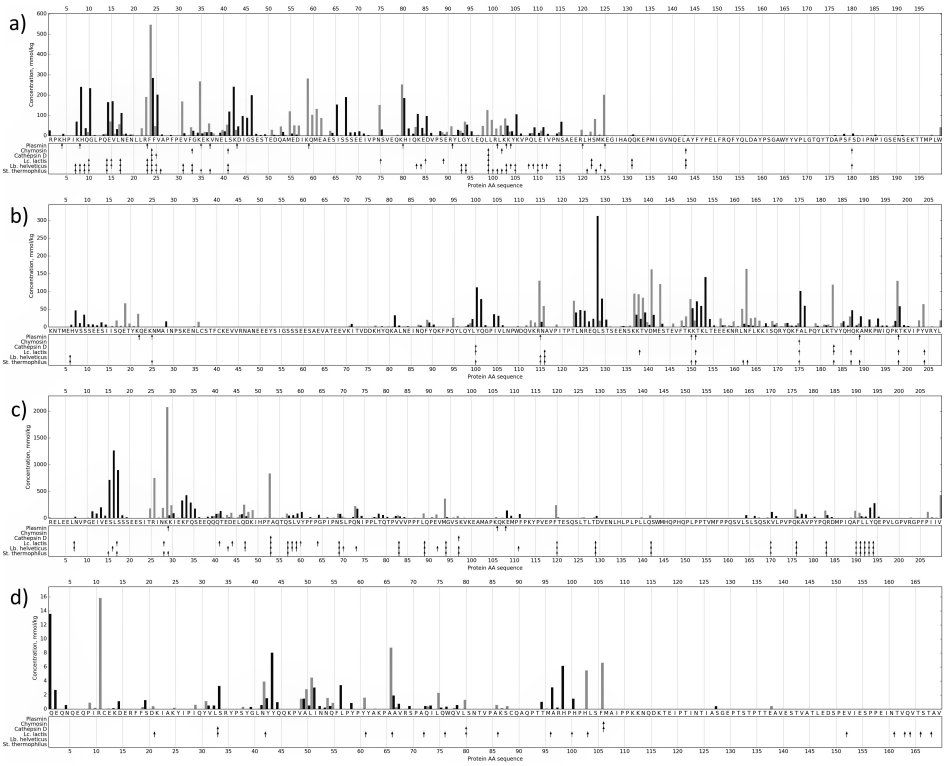


Figure 16. The enzyme cleavage sites of α_{s1}-CN (a), α_{s2}-CN (b), β-CN (c), and κ-CN (d) based on all 3,040 water-soluble peptides identified in 8-month-old Old Saare cheese. Black bars: sum of estimated concentrations (mmol kg^{-1} of cheese) of the N-terminal amino acids of all peptides. Grey bars: sum of estimated concentrations (mmol kg^{-1} of cheese) of the C-terminal amino acids of all peptides. On the horizontal axis, the sequences of the caseins are plotted. Important cleavage sites of plasmin, chymosin, cathepsin D, CEP of Lc. Lactis, CEP of Lb. helveticus and proteolytic system of St. thermophilus (in order from upper to the bottom lines) known from the literature are marked with arrows below the casein sequence. The arrows shown in the C-terminal end of κ-CN (f106-169) indicate the main cleavage sites in the beginning of ripening. The results are mean values of four measurements.

The patterns of the cleavage sites of different caseins correlate well with corresponding peptide coverage profiles. Most of the important cleavages were characterized by the initial activity of the indigenous milk proteinases plasmin (Bars & Gripon, 1989; McSweeney, Olson, Fox, Healy, & Højrup, 1993; Upadhyay et al., 2004) and cathepsin D (Larsen, Benfeldt, Rasmussen, & Petersen, 1996), chymosin (Exterkate, Lagerwerf, Haverkamp, & Van Schalkwijk, 1997; McSweeney, Olson, Fox, Healy, & HØjrup, 1993), CEP of *Lc. Lactis* (Kunji et al., 1996; Monnet, Ley, & GonzÀlez, 1992), CEP of *Lb. helveticus* (Kunji et al., 1996; Sadat-Mekmene et al., 2011) and the proteolytic system of *St. thermophilus* (Miclo et al., 2012) according to the information available in the literature (Figure 16). It should be noted that a large share of the

cleavage sites was assigned simultaneously to the actions of more than one proteolytic agent. A very detailed description of the possible mechanisms of release and breakdown of peptides to smaller fragments in Old Saare cheese, related with the potential activity of the enzymes responsible for hydrolysis of corresponding cutting sites, is provided in Publication I.

4.1.2.4 Phosphopeptides

In this study, the possible phosphorylation sites (P-sites) were automatically found by MaxQuant database search. Among all casein-derived identified peptides, 886 were found to be potentially phosphorylated. MaxQuant detected 12 potential P-sites in peptides from α_{s1}-CN, 21 – from α_{s2}-CN, 8 – from β-CN, and 6 – from κ-CN.

Most of the P-sites at Ser residue were also reported previously in plasmin-mediated peptides in Grana Padano cheese (Ferranti et al., 1997) and semi-hard Herrgård cheese (Lund & Ardö, 2004). All P-sites coincided with the experimentally determined and potential ones reported in the review of the nomenclature of caseins (Farrell et al., 2004). Additionally, 3–5 potential new P-sites in each casein were tentatively detected in this study.

Phosphorylation of caseins is a reversible post-translational modification occurring in the mammary epithelial cells, which is regulated by the action of protein kinases (Holland & Boland, 2014). Moreover, in cheese, the acid phosphatase originating from bovine milk or bacteria can modify the degree of phosphorylation of peptides (Akuzawa & Fox, 2004). In order to obtain more precise identification of phosphopeptidome from WSE of cheese, a purification procedure allowing the selective isolation of phosphopeptides (e.g., the cation exchange chromatography and immobilized metal affinity chromatography) should be used before separation by MS analysis (Dupas et al., 2009; Lund & Ardö, 2004).

Nevertheless, looking at the formation of the phosphopeptides during Old Saare cheese maturation, some interesting facts were noted. Phosphopeptides formed from plasmin-derived peptides β-CN f1-28 and f29-52 by the action of LAB enzyme activity accumulated very fast and resulted in high concentrations at the end of ripening (Figure 14; Table 4 in Publication I). This is in agreement with the observation that phosphorylated peptides are resistant to degradation by LAB peptidases (Hynek, Zuzalkova, Sikac, & Kas, 2002) and their accumulation in cheese has been also observed in other studies (Deutsch et al., 2000; Ferranti et al., 1997; Lund & Ardö, 2004). The content of phosphopeptides from the regions f41-63 and f65-79 of α_{s1}-CN as well as from f125-142 of α_{s2}-CN also increased during the eight months of ripening. In contrast to that, the phosphopeptides from region f25-80 of α_{s2}-CN showed very low concentrations throughout the whole ripening. This could be explained by the limited accessibility of the plasmin and chymosin during primary hydrolysis to this region of α_{s2}-CN. The most intense degradation of the regions of C-terminal end of α_{s2}-CN was in accordance with the literature (Farrell et al., 2004).

4.1.2.5 Principal component analysis and clustering analysis

Principal component analysis (PCA) and clustering analysis applied to 169 most abundant peptides (the list of the peptides is available in Publication I, Table 4) showed that peptides clustered into four groups were discriminated on the PCA plot according to Old Saare cheese age (Figure 17). Clusters of the peptides were obtained in terms of the complex evolution of their profiles during cheese ripening. Based on literature data, only manual clusterization of peptides has been performed previously based on the 57 most abundant peptides in hard Parmigiano-Reggiano cheese during 2 years of ripening (Sforza et al., 2012).

It is observed from the PCA and clustering analysis that the first group of peptides were characteristic to the beginning of ripening, 1-day- and 2-week-old cheese, the second group associated to the 1-3-month-old, the third – to the 3-5-month-old, and fourth – to the 6-8-month-old Old Saare cheese. A precise description of the changes in the concentrations of peptides from each group during ripening, as well as their allocation into different groups, is accessible in the Publication I.

In this study the PCA and clustering analysis were performed only on the selection of 169 most abundant peptides, but the analysis can be also easily applied to the large-scale data supplied by MS/MS analysis. This would provide an alternative and very handy way to follow the complex dynamics of peptides in cheese during ripening after their robust allocation into groups.

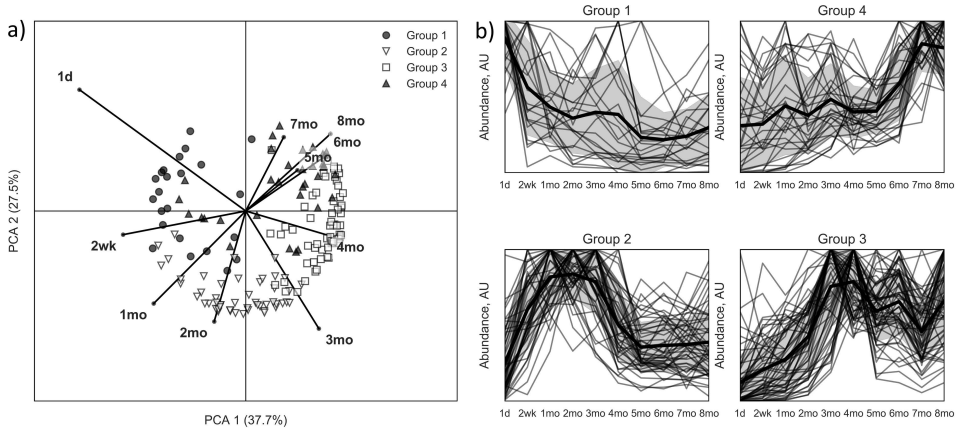


Figure 17. Principal component analysis (a) and clustering analysis (b) of 169 most abundant water-soluble peptides determined by LC-MS/MS in Old Saare cheese. The results are mean values of four measurements. d: day; wk: week; mo: month.

4.1.3 Peculiarities of casein hydrolysis in Old Saare cheese

For detailed characterization of the changes in the content of different casein fractions and small-water soluble peptides, use of molar concentrations is the most appropriate way. Therefore, in Publication I, we calculated the estimated concentrations of casein fractions using the normalized area of CE peaks and N content of proteinaceous fractions determined by Kjeldahl (see Quantification of proteins and peptides in Materials and Methods section). Taking into account the molecular weights of the known casein fractions, the values were further converted from g kg^{-1} of cheese to mmol kg^{-1} of cheese. The values in Table 3 are presented in g kg^{-1} of cheese because of

the presence of non-identified casein fractions determined by CE (presumably the insoluble degradation products of caseins), their molecular weights are not known.

As expected from CE analysis of the casein fractions, the content of β-CN (hereinafter refers to the sum of normalized areas of β-CN (A^1, A^2, and B)) was significantly reduced during cheese maturation by the action of plasmin with the concomitant production of γ_1-CN (sum of normalized areas of γ_1-CN (A^1 and A^2)), γ_2-CN, and γ_3-CN. Compared to the 1-day-old cheese, 80% of β-CN was hydrolyzed after eight months of ripening (Table 3). The strong contribution of plasmin to the hydrolysis of β-CN was evident already in 1-day-old cheese, where the fractions of γ-CN formed 21% of β-CN water-insoluble fraction and 8.2% of the total proteinaceous fraction.

Extensive degradation of α_{s1}-CN (sum of normalized areas of α_{s1}-CN-8P and α_{s1}-CN-9P) was also observed as ripening progressed, which was evidenced by the production of chymosin-derived peptides α_{s1}-CN-I (sum of normalized areas of α_{s1}-CN-I-8P and α_{s1}-CN-I-9P), yielding in 65% degradation to the end of ripening (Table 3). However, in the 1-day-old cheese in contrast to the β-CN, α_{s1}-CN-I formed only 1.4% of α_{S1}-CN water-insoluble fraction (0.4% of a total proteinaceous fraction). This is in accordance with the observation, that α_{s1}-CN is not hydrolyzed by chymosin during vat process, while the proteolysis of β-CN is initiated by plasmin already in milk before cheesemaking process (Upadhyay et al., 2004). In Publication II, we also indicated some hydrolysis of β-CN during raw milk transportation to the dairy, whereas no α_{S1}-CN proteolysis was detected. A considerable degradation of α_{s1}-CN by the end of ripening can be explained by the reversible thermal inactivation of chymosin by high temperature during curd cooking (Hayes, Oliveira, McSweeney, & Kelly, 2002).

The fractions that correspond to α_{s2}-CN (11P, 12P, nP) were also extensively degraded by plasmin resulting in 78% hydrolysis by the end of ripening (Table 3). The rate of α_{s2}-CN breakdown slowed down after the third month of ripening. Unfortunately, the breakdown products of α_{s2}-CN could not be identified by CE. In this study, the para-κ-CN was regarded as intact, supposing that the lion's share of κ-CN was hydrolyzed to para-κ-CN and GMP already during the coagulation of milk before the cheese ripening has started. Hence, the corresponding value of para-κ-CN degradation by the end of ripening was 37% (Table 3).

Despite the large differences in the levels of γ-CN and α_{s1}-I-CN in the beginning of cheese maturation, the trends and the rates of α_{s1}-CN and β-CN degradation were similar, showing the higher values up to the fourth month of ripening (Figure 18a). The content of intact α_{s1}-CN and β-CN decreased with the rate approximately 0.6 mmol and 0.7 mmol per kg of cheese per month, respectively, while after the fourth month the degradation was insignificant. The content of α_{s1}-CN and β-CN degradation products, α_{s1}-I-CN and γ-CN, increased up to the third and fourth months of ripening, respectively (Figure 18b).

The changes in the content of water-soluble peptides were also in accordance with the hydrolysis of intact caseins – the fastest formation of peptides occurred during the first 3–4 months of ripening (Table 3). Further formation and degradation of peptides became more balanced. The peptides of β-CN comprised the largest part (56–69%) of all water-soluble peptides depending on the ripening phase, while peptides from α_{s1}-CN, α_{s2}-CN and κ-CN formed 21–29%, 8–17% and 0.1–3%, respectively.

Table 3. Changes in the estimated concentrations of the main casein fractions (g kg^{-1} of cheese) during Old Saare cheese ripening[1]

Fraction	Ripening time									
	1 day	2 weeks	1 month	2 months	3 months	4 months	5 months	6 months	7 months	8 months
Intact caseins	218.8 ± 9.3	195.5 ± 11.5	190.3 ± 6.3	151.7 ± 8.1	105.9 ± 8.0	76.6 ± 11.9	74.2 ± 4.2	65.7 ± 4.9	57.8 ± 4.1	69.4 ± 6.2
α_{s1}-CN	84.9 ± 5.2	80.4 ± 7.4	77.4 ± 4.3	64.7 ± 5.2	46.2 ± 7.0	32.6 ± 6.0	30.6 ± 2.0	28.3 ± 3.4	23.3 ± 1.5	29.8 ± 3.8
α_{s2}-CN	14.7 ± 2.4	9.8 ± 2.2	10.2 ± 0.9	7.4 ± 0.7	4.2 ± 0.4	3.8 ± 0.6	3.1 ± 0.2	2.9 ± 0.2	2.7 ± 0.2	3.3 ± 0.4
β-CN	89.5 ± 7.2	78.0 ± 7.7	78.3 ± 3.1	56.6 ± 3.0	38.1 ± 1.5	22.0 ± 9.8	24.2 ± 2.1	19.3 ± 0.8	17.7 ± 1.1	17.7 ± 2.8
para-κ-CN	29.7 ± 1.1	27.4 ± 3.4	24.4 ± 3.1	23.0 ± 5.3	17.4 ± 3.4	18.3 ± 2.9	16.3 ± 2.9	15.1 ± 3.3	14.1 ± 3.6	18.6 ± 4.0
Long water-insoluble peptides	54.0 ± 6.0	72.4 ± 8.4	69.1 ± 5.1	93.2 ± 2.1	118.2 ± 4.2	130.7 ± 4.9	133.9 ± 3.2	133.4 ± 4.5	138.8 ± 4.8	130.3 ± 6.5
α_{s1}-I-CN	1.2 ± 1.0	4.0 ± 0.8	7.4 ± 1.0	11.3 ± 0.5	15.1 ± 3.0	14.3 ± 3.6	17.0 ± 1.7	14.9 ± 2.0	16.9 ± 1.1	13.1 ± 2.2
γ-CN	23.2 ± 2.3	27.0 ± 3.6	22.1 ± 1.4	35.8 ± 1.8	43.0 ± 1.9	53.3 ± 4.1	50.8 ± 1.5	52.2 ± 2.8	54.2 ± 2.0	54.2 ± 2.7
γ_1-CN	11.0 ± 2.0	12.2 ± 2.7	9.8 ± 1.0	17.3 ± 1.3	20.0 ± 1.3	22.6 ± 1.0	22.3 ± 1.0	20.5 ± 1.4	21.2 ± 1.2	21.6 ± 1.4
γ_2-CN	8.5 ± 0.5	9.1 ± 2.0	6.9 ± 0.7	8.3 ± 0.5	9.4 ± 0.3	11.0 ± 1.3	11.0 ± 0.5	11.6 ± 0.8	12.0 ± 0.7	11.2 ± 1.1
γ_3-CN	3.8 ± 1.0	5.8 ± 1.4	5.4 ± 0.6	10.1 ± 1.1	13.6 ± 1.4	19.7 ± 3.7	17.5 ± 0.9	20.1 ± 2.3	21.1 ± 1.5	21.4 ± 2.1
Unidentified	29.7 ± 5.5	41.4 ± 7.6	39.5 ± 4.8	46.2 ± 1.3	60.1 ± 2.4	63.1 ± 2.8	66.1 ± 2.4	66.3 ± 3.7	67.6 ± 4.4	63.0 ± 5.7
Small water-soluble peptides (derived from)	7.4 ± 0.4	10.5 ± 0.9	13.7 ± 1.4	16.1 ± 0.5	23.4 ± 1.8	22.8 ± 3.0	17.5 ± 1.1	19.8 ± 1.6	18.4 ± 2.4	21.9 ± 2.1
α_{s1}-CN	1.8 ± 0.1	2.7 ± 0.1	3.2 ± 0.1	4.1 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	4.2 ± 0.3	5.2 ± 0.3	5.3 ± 0.3	5.3 ± 0.3
α_{s2}-CN	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	2.0 ± 0.2	2.0 ± 0.1
β-CN	4.1 ± 0.2	6.5 ± 0.2	9.3 ± 0.3	10.3 ± 0.4	16.1 ± 1.0	15.1 ± 0.8	11.2 ± 0.8	12.1 ± 1.1	10.9 ± 0.8	10.9 ± 0.9
κ-CN	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.0	0.1 ± 0.0	<0.1
Free amino acids	1.2 ± 0.1	3.1 ± 0.2	8.4 ± 0.4	20.5 ± 0.6	34.0 ± 1.7	51.4 ± 3.2	55.9 ± 1.0	62.6 ± 1.3	66.5 ± 1.7	59.9 ± 1.1

[1] Values are means of the four measurements (mean ± SD, n = 4)

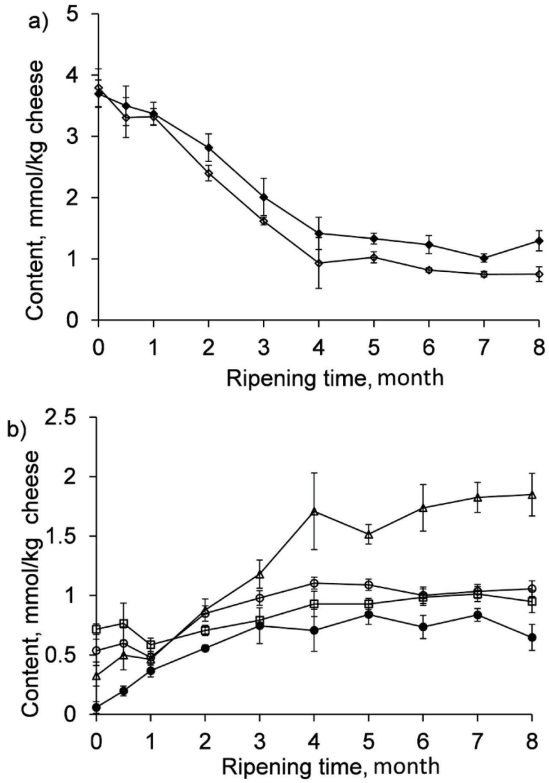


Figure 18. Casein breakdown in Old Saare cheese over eight months of ripening (a): α_{s1}-CN (♦) and β-CN (◊) and their primary degradation products (b): α_{s1}-I-CN (●), γ_1-CN (○), γ_2-CN (□), γ_3-CN (Δ). Results represent the mean estimated concentrations of the four measurements (mean ± SD, n=4).

The concentrations on the individual proteinaceous compounds were expressed in mmol kg^{-1} of cheese. To show the overall changes of all proteinaceous fractions throughout the cheese ripening we presented the content of each fraction as the percent of the total N content (Figure 19). Such presentation of the data enables to compare the extent of the proteolysis in Old Saare cheese with other varieties studied by others, using the determination of N content in different cheese fractions for evaluation of proteolysis (Ardö & Polychroniadou, 1999). For this purpose, the absolute concentrations of FAA, measured by UPLC in this study, were also converted into amino acid N content.

Total N content in Old Saare cheese (42.1 ± 0.8 g kg^{-1} of cheese) did not change significantly during the maturation ($P < 0.05$). In general, there was a 3-fold decrease in the content of intact caseins and 2.5-fold, 3-fold, and 50-fold increase in the content of water-insoluble long, water-soluble small peptides and FAA, respectively. After eight months of ripening, the N content of intact casein fraction decreased from 78 to 25% (Figure 19).

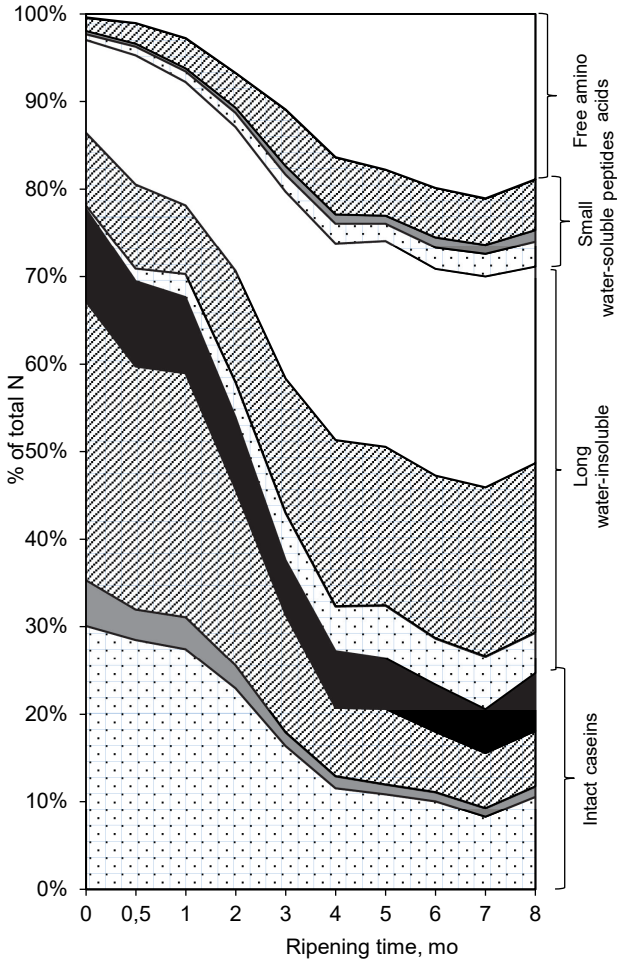


Figure 19. The changes of the intact caseins, long water-insoluble peptides, and small water-soluble peptides as proportions of total N content in Old Saare cheese during eight months of ripening. Dotted areas represent N content from α_{s1}-, grey areas – from α_{s2}-, striped areas – from β-, and black areas – from κ-CN. Twenty eight non-identified long peptides determined by CE and FAA are represented by white areas. The results are mean values of four measurements.

The content of the WSN fraction, which includes fractions of water-soluble peptides and FAA, increased from 1.3 ± 0.0 to 12.8 ± 0.3 g per kg of cheese and thus comprised 29% of TN in the 8-month-old cheese. An overall change of the content of intact casein fraction and accumulation of WSN fraction was the fastest up to the fourth month of maturation. During this period, the content of WSN increased by 2.5 g per kg of cheese monthly. In the second part of ripening, the rate of the change of proteinaceous compounds slowed down, indicating the equilibration of the rates of their formation and degradation.

The WSN content in Old Saare cheese (29% of TN) was somewhere between the ones reported for 6-month-old Cheddar (24-25%) and 24-month-old Parmesan (32-36%) (Barrett, Kelly, McSweeney, & Fox, 1999; Careri, Spagnoli, Panari, Zannoni, & Barbieri, 1996), indicating rather high extent of proteolysis in Old Saare cheese.

Based on the data obtained by CE, LC-MS/MS, UPLC and Kjeldahl analyses we constructed an overall picture of proteolysis, which allows to follow the changes in the content of different proteinaceous fractions in Old Saare cheese (Figure 19).

4.2 Proteolysis during milk refrigerated transportation (Publication II)

High microbiological quality, good chemical composition and renneting properties are the most important features of the cheese milk. Proteolysis in raw milk influences rennet coagulation of milk and can affect the whole cheesemaking process as well as the quality of the final product. In Publication II, the changes in protein composition throughout the refrigerated supply chain of raw bulk milk were analyzed. Milk samples were taken from the cooling tanks of individual dairy farms (before pumping the milk into tank trucks), from the dairy trucks containing mixed farm milk before the milk was pumped into dairy silos, and from the dairy silo containing the pooled milk from all trucks.

Figure 20 shows the representative electrophoretic profiles of main protein fractions obtained by CE (Beckman P/ACE^{TM} MDQ instrument) during the analysis of milk and cheese samples and protein standards.

The identification and absolute quantification of caseins and their hydrolysis products based on the commercial standards has been and remains quite challenging task due to 1) the complexity and impurity of the protein standards available and 2) the lack of the commercial standards of the casein breakdown products. As seen from the Figure 20, each of the available commercial casein standards used for identification of CNs in analyzed samples represent at least several peaks on the CE pattern, due to genetic polymorphism and different post-translational modifications (phosphorylation, glycosylation) intrinsic to caseins and their hydrolysis products (Farrell et al., 2004).

The main protein fractions resolve on the electropherograms into numerous peaks, which have certain migration order as well as characteristic profile of the main fractions in milk and cheese samples, which helps to compare the peaks from different electropherograms and assists identification. Based on the milk protein standards analyzed and electropherograms available in the literature (Heck et al., 2008; Miralles et al., 2003; Otte et al., 1997), the following main peaks could be identified:

- **intact caseins**: α_{s1}-CN (8P and 9P), α_{s2}-CN (11P, 12P, and nP), β-CN (with genetic variants A^1, A^2, and B), and κ-CN;
- **degradation products** of α_{s1}-CN: α_{s1}-I-CN (8P and 9P), of β-CN: γ_1-CN (A^1 and A^2), γ_2-CN, and γ_3-CN;
- **whey proteins**: α-LA and β-LG.

In Publication II, only the main protein fractions were considered. It can be seen from CE traces, that β-CN standard separates into three peaks, A^1 and A^2 genetic variants being dominating (Figure 20). The available standard of α_s-CN, including both α_{s1}- and α_{s2}-CN (Otte et al., 1997), separates into more than six components on the electropherogram, containing α_{s1}-CN with eight and nine phosphate groups as well as α_{s2}-CN with 11, 12, and n phosphate groups.

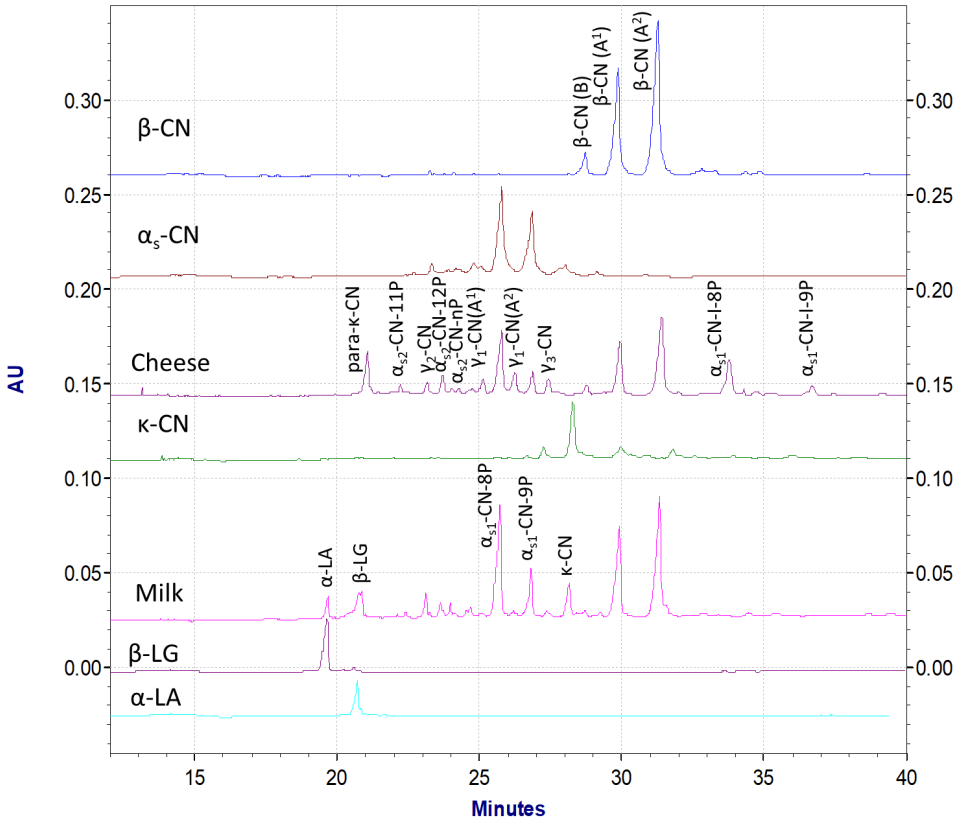


Figure 20. Capillary electropherograms of raw bulk milk and cheese samples. CN: casein; para-κ-CN: κ-CN f1−105; γ_1-CN: β-CN f29−109; γ_2-CN: β-CN f106−209; γ_3-CN: β-CN f107−209; α_{s1}-I-CN-8P: α_{s1}-CN f24−199; α_{s1}-I-CN-9P: α_{s1}-CN f24−199 9P; A^1, A^2, and B: genetic variants of β-CN; xP: number of phosphate groups in casein; AU: arbitrary unit.

κ-CN also resolves into more than six peaks with dominating component κ-CN A with one phosphate group (Farrell et al., 2004) migrating at 28.5 min (Figure 20). Taking into account, that the dominant peaks of α_{s2}-CN-11P- and κ-CN (A)-1P comprise about 50% of total α_{s2}- and κ-CN (Heck et al., 2008; Otte et al., 1997), respectively, the relative content of these CNs was calculated by multiplying their integrated areas by two. Minor peaks of α_{s2}- and κ-CN fractions may overlap with other caseins or their hydrolysis products and were not considered. Thus, only the areas of the major peaks of α_{s2}- and κ-CN were used for quantification of respective CNs in milk samples.

Taking the above mentioned into consideration, along with the commercial standards the electropherograms presented in the literature were used for precise identification of the protein peaks on the electropherograms. This was also the reason why the calculation of the relative concentration − protein normalized area to the total protein normalized area − of the protein fractions in milk and cheese samples was used in Publications II and III. The same approaches have been applied for the estimation of the protein concentrations in milk samples in other studies (Ardö, Lindblad, & Qvist, 1999; Heck et al., 2008).

The results of CE analysis of milk samples showed that the relative concentrations of β-LG, α_{s2}-, κ-, and β-CN were significantly different in milk sampled from the truck tanks and dairy silo compared to the milk sampled from individual farms before transportation (Figure 21).

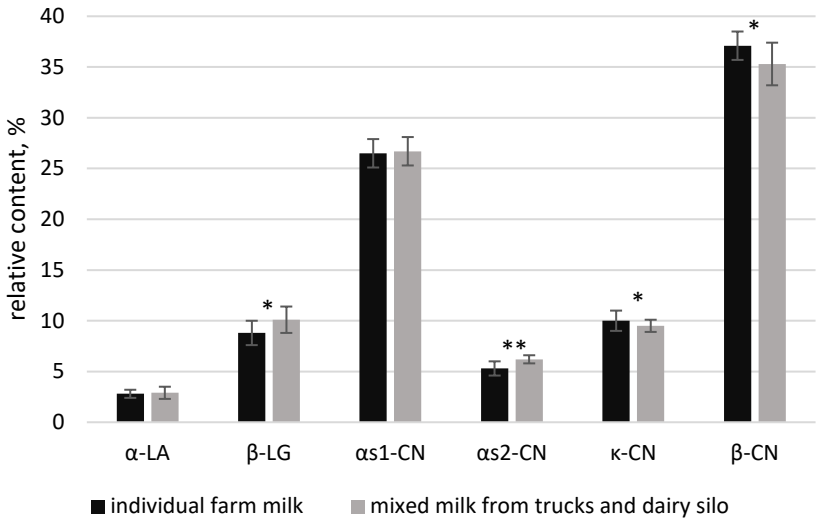


*Figure 21. The mean relative protein composition of individual raw milk sampled from at dairy farms (n=36) and mixed milk sampled from truck tanks (n=10) and dairy silo after transportation to the dairy plant (n=1). Asterisk indicates a significant difference according to Student's t-test: * $p < 0.05$, ** $p < 0.001$*

Several factors could be related to these observations. The lower relative content of κ- and β-CN ($p < 0.05$) in pooled milk after transportation to the dairy could have been caused by proteolytic activity of enzymes of psychrotrophic bacteria (Kohlmann, Nielsen, & Ladisch, 1991), which are the predominant species in Estonian raw milk (Stulova et al., 2010). Although the cold storage conditions are far from optimal for the native milk proteinase plasmin, it still could have some hydrolytic activity on β-CN in cold transportation conditions. Crudden et al. have also reported a plasmin-induced hydrolysis of β-CN during the 5 d of storage of milk at 5 °C (Crudden et al., 2005). Moreover, the proteases from psychrotrophic bacteria can activate the plasminogen, thus contributing to the activity of plasmin towards the β-CN (Frohbieter, Ismail, Nielsen, & Hayes, 2005).

On the other hand, the apparently higher content of whey protein, β-LG, was noted in the pooled milk sampled after transportation ($p < 0.05$) (Figure 21). We assume, that the relative content of β-LG in milk subjected to refrigerated storage or transportation was overestimated due to the coelution of para-κ-CN peak with the β-LG. The migration time of β-LG on the electropherogram is very close to the migration time of para-κ-CN (Otte et al., 1997), the well-known product of κ-CN degradation by rennet added during cheesemaking (Fox et al., 2000), but in our case most probably produced by the action of bacterial proteinases present in refrigerated milk (Kohlmann et al., 1991). The concomitant decrease of relative κ-CN content ($p < 0.05$) together with the apparent increase of β-LG reveals this assumption. Similar observations were described

by Miralles et al., who used CE for the determination of the degree of proteolysis in milk caused by refrigerated storage at 6 °C during 4 days (Miralles et al., 2003).

CE was shown as a good method to determine the initial proteolysis of κ- and β-CN, taking place during short-term refrigerated transportation of raw milk from farms to the dairy plant. In addition, a positive correlation between the relative κ-CN content of milk and curd firmness (Pearson's correlation coefficient r = 0.45, $p < 0.01$) was shown in Publication II. Hence, the initial proteolysis of caseins in cheese milk can lead to the formation of a softer rennet gel and the decreased cheese yield because of the release of casein degradation products (water-soluble peptides) into the whey instead of forming a part of the cheese body.

4.3 Primary proteolysis during the RO-cheese ripening (Publication III)

Numerous studies have dealt with the investigation of the primary proteolysis in traditionally produced cheeses, while studies on the primary degradation of caseins in RO-cheeses are lacking. In Publication III, hard Gouda-type cheese (RO-cheese) was produced from milk concentrated by reverse osmosis (RO) using both mesophilic and thermophilic starters and studied the impact of milk concentration on primary proteolysis during six months of ripening.

Electrophoretic profiles of 1-day-old cheese are very similar to the profiles of milk casein fraction. As can be seen from the electropherograms of the RO-cheese (Figure 22), the main difference compared to these of milk is in the disappearance of κ-CN peak (migrating between the peaks of γ_3- and β-CN(B), Figure 20) and concomitant appearance of the peak of κ-CN degradation product, para-κ-CN (κ-CN f1-105; migrating approximately at 22.0 min). The cutting of κ-CN at the peptide bond Phe_{105}-Met_{106} is induced by chymosin during the enzymatic phase of milk coagulation in the beginning of the cheesemaking procedure (Fox et al., 2000). It is known from the literature that the aggregation of casein micelles during renneting of RO milk starts at a lower degree of κ-CN hydrolysis (Lauzin, Bérubé, Britten, & Pouliot, 2019). Another study on rennet coagulation kinetics of raw milk RO retentates has shown that the rate of gel formation in RO-milk increases along with the concentration factor of RO-milk (Dussault-Chouinard, Britten, & Pouliot, 2019). The accelerated aggregation of the casein micelles is caused by reduced electrostatic repulsion between the casein molecule, induced by lower pH of RO-milk (Liu & Guo, 2008). Most likely decrease of milk pH during the RO concentration occurs due to the dissociation of phosphoric acid with the liberating of protons induced by precipitation of calcium phosphate in milk during the concentration (Gaucheron, 2005). In our study, RO concentration of milk changed the pH from 6.69 to 6.47, which is in agreement with other studies on raw milk RO retentates (Dussault-Chouinard et al., 2019; Sørensen et al., 2019). Despite of the peculiarities of RO-milk coagulation described above, the capillary electrophoretic analysis of 1-day-old RO-cheese indicated a complete degradation of κ-CN to the para-κ-CN (Figure 22, the bottom electropherogram).

The electrophoretic profiles of the intact casein fractions and their primary proteolysis products of the RO-cheese after one day, one, three and six months of ripening are shown in Figure 22. A decrease in the β-CN peaks and an increase in the area of the corresponding peaks of γ-CNs by the six months of ripening can be observed on the electropherograms. The relative concentrations of casein fractions were used to follow the course of initial casein degradation during the RO-cheese maturation.

As shown in Figure 22, the hydrolysis of α_{s1}-CN was significantly higher than that of β-CN: about 70% of α_{s1}-CN was hydrolyzed during six months of the RO-cheese ripening, whereas the degree of β-CNs hydrolysis was around 45% of its initial content.

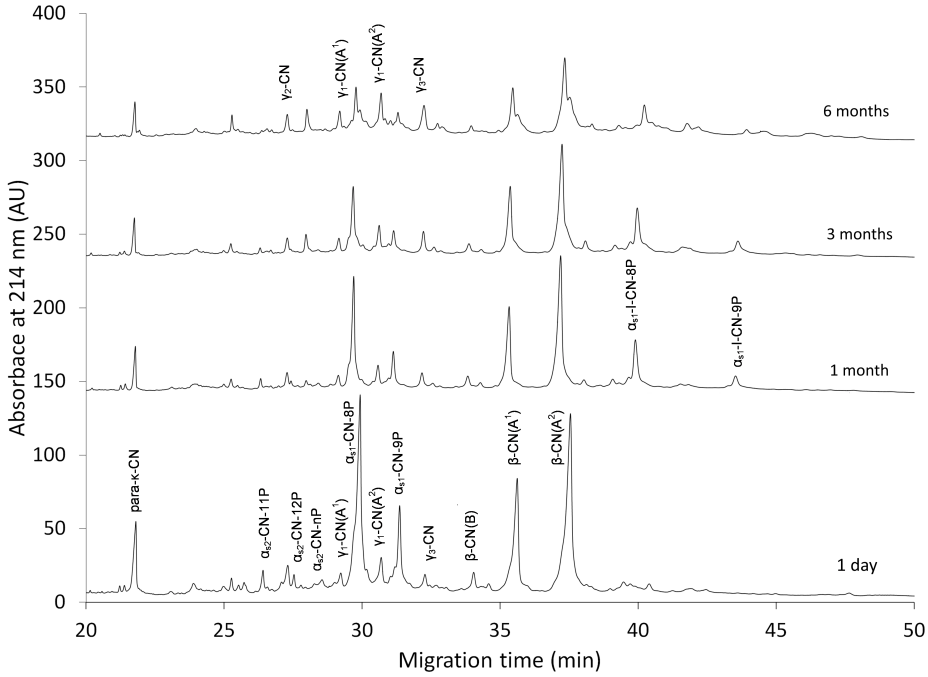


Figure 22. Capillary electropherograms of the RO-cheese obtained at 1 day, 1, 3, and 6 months, showing the hydrolysis profile of caseins during ripening. CN: casein; para-κ-CN: κ-CN f1−105; γ_1-CN: β-CN f29−109; γ_2-CN: β-CN f106−209; γ_3-CN: β-CN f107−209; α_{s1}-I-CN-8P: α_{s1}-CN f24−199; α_{s1}-I-CN-9P: α_{s1}-CN f24−199 9P; A^1, A^2, and B: genetic variants of caseins; xP: number of phosphate groups in casein; AU: arbitrary unit.

To evaluate the course of primary proteolysis in the RO-cheese, the traditional cheeses with similar manufacturing technology were chosen to compare the breakdown of the main casein fractions. In traditional cheese, the primary proteolysis is determined by a rapid degradation of intact caseins to a number of various sized polypeptides by activities of both rennet and plasmin.

In our study, the amount of rennet based on the initial amount of milk used for RO-milk production was sufficient for adequate rate of α_{s1}-CN hydrolysis in RO-cheese. The same amount of rennet based on the original unconcentrated milk volume have been used to produce Cheddar RO-cheese (Bynum & Barbano, 1985). Our findings on α_{s1}-CN degradation in the RO-cheese are in agreement with the primary proteolysis in a typical Gouda-type cheese made with mesophilic starter displaying a 70−80% decrease in α_{s1}-CN content within the first month of maturation (Schroën, Van Der Sman, & Boom, 2004; Upadhyay et al., 2004; Walstra, Noomen, & Geurts, 1999). In Old Saare cheese, made also with both mesophilic and thermophilic LAB and high scalding temperature, about 70% of α_{s1}-CN, was hydrolyzed during six months of ripening (Publication I). CE analysis of another extra-hard Västerbottenost cheese, manufactured with high scalding temperature and mesophilic starter, showed almost complete

degradation of α_{s1}- and β-CN to the end of 26 weeks of ripening (Rehn et al., 2010). Although chymosin, responsible for the initial α_{s1}-CN cleavage, has been reported to be inactivated by the high scalding temperature (Sheehan, Oliveira, Kelly, & Mc Sweeney, 2007; Upadhyay et al., 2004), the above cited studies together with this work reveal the occurrence of rather extensive α_{s1}-CN hydrolysis in cheeses produced with high scalding temperature, indicating only partial inactivation of chymosin or reactivation during the further cheese maturation (Hayes et al., 2002). Another crucial parameter that influences the activity of the enzymes is the pH of cheese. Taking into account that the pH of the RO-cheese during maturation was lower (in the range 5.1−5.2) compared to that in traditional Old Saare (pH 5.3−5.5) (Publication I) and Västerbottenost cheese (pH 5.3−5.6) (Rehn et al., 2010) and is more favorable to the proteolytic activity of residual chymosin (Sheehan et al., 2007), somewhat more extensive primary proteolysis of α_{s1}-CN could be expected in RO-cheese.

The substantial contribution of plasmin to the primary proteolysis is evident especially in hard cheeses made with high scalding temperature (Sousa et al., 2001), due to the heat-stability of plasmin and plasminogen activators (Nielsen, 2002) as well as inactivation of the heat-labile plasmin inhibitor and plasminogen activator inhibitor (Prado, Sombers, Ismail, & Hayes, 2006). CE analysis of primary proteolysis in the RO-cheese showed that significantly less β-CN, 45% of intact β-CN, was hydrolyzed compared to the levels reported in Old Saare (78%) (Publication I) and even in Gouda-type cheeses with low scalding temperature (60−50% of β-CN) (Upadhyay et al., 2004; Van den Berg, Meijer, Düsterhöft, & Smit, 2004; Walstra, Noomen, & Geurts, 1999). Considerably low pH of the RO-cheese especially in the beginning of ripening was obviously, in contrast to the hydrolysis of α_{s1}-CN, a limiting factor for hydrolysis of β-CN.

Different formation rates of chymosin- and plasmin-derived peptides were observed during primary proteolysis of RO-cheese according to the electrophoretic profiles. The chymosin-derived peptides α_{s1}-I-CNs exhibited the highest rate of formation during the first two months of maturation, while during the next four months the peaks of α_{s1}-I-CN were smaller suggesting that the rate of degradation by the action of different enzymes from LAB was higher than that of formation by residual chymosin (Figure 22). In contrast, the plasmin-derived peptides γ-CNs fairly decreased during the first month of cheese ripening, which might have been caused by their rapid degradation to the small peptides and FAA. During the further ageing months of RO-cheese, γ-CNs tended to accumulate, showing an increase in the production rate (Figure 22), possibly due to the slight increase of pH of cheese environment favouring plasmin action.

The products of primary hydrolysis are further cleaved by the action of a complex proteolytic system of LAB to generate the huge pool of small peptides and FAA (Upadhyay et al., 2004). Further proteolysis continues resulting in the formation of a number of different volatile compounds. The important pathways of production and transformation of volatile compounds as well as their correlation with RO-cheese flavor are described in detail in Publication III.

This dissertation provided novel information about the course of primary proteolysis during RO-cheese ripening. Current results provide an indication that RO-milk can be presumably used for the production of hard cheese and may encourage further research (e.g. detailed analysis of the dynamics of water-soluble peptides) for conscious application of RO technology for cheese milk concentration.

5 Conclusions

The main objective of this thesis was the application of up-to-date analytical methods for comprehensive characterization of the proteolysis in cheese, starting from minor degradation of caseins in milk and essentially taking place during cheese manufacture and ripening.

The following conclusions can be drawn from the study:

- A combined set of analytical and computational methods was developed and applied to describe the proteolytic events at the molecular level in Old Saare cheese during ripening.
    - The application of CE, LC-MS/MS, and UPLC allowed to determine the changes in the contents of a wide range of the specific proteinaceous compounds (caseins, water-insoluble and soluble peptides, free amino acids) in cheese.
    - A novel approach for treatment and visualization of the cheese peptidomic data was introduced by applying in-house developed Python-based computational tool. This enabled the first comprehensive casein sequence-based analysis of proteolytic cascades and determination of the cleavage sites of the proteolytic enzymes potentially involved in ripening of Old Saare cheese.
    - The principal component analysis applied to the cheese peptidomic data followed by grading of peptides into four groups using clustering analysis provided an alternative way for overall assessment of the complex dynamics of peptides with similarly changing trends during cheese ripening.
    - The combination of the data obtained from the up-to-date analytical methods (CE, LC-MS/MS, and UPLC) with robust determination of N content in different fractions allowed to compose a comprehensive semi-quantitative picture of casein hydrolysis in Old Saare cheese.
- Decrease of the relative amount of κ-CN ($p < 0.05$) and β-CN ($p < 0.05$) was detected in raw milk during refrigerated transportation from the farm to the dairy factory indicating that significant degree of proteolysis in cheese milk may take place before the cheese manufacturing.
- The initial casein breakdown in the cheese manufactured from the reverse osmosis-concentrated milk showed no remarkable differences compared to primary proteolysis in hard cheeses manufactured from unconcentrated milk.

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Abstract

Comprehensive Study of Proteolysis During Cheese Ripening

Proteolysis is regarded as the most complex and important process during cheese ripening contributing to the development of characteristic texture and flavour of cheese. Intact caseins undergo gradual degradation to peptides and free amino acids by residual coagulant, indigenous milk protease plasmin, cell-envelope and intracellular proteinases and peptidases of lactic acid bacteria.

Quantification of the nitrogen (N) content in various fractions of cheese extracts carried out by non-specific chemical methods is commonly used for the evaluation of the extent of proteolysis in cheese. However, this does not allow to determine the content and changes of the separate proteinaceous compounds specifically, neither to estimate the activities of the proteolytic enzymes. For a detailed elucidation of the proteolysis, tandem mass-spectrometry (MS/MS) is increasingly applied to characterize the peptides during proteolysis in cheese. However, the research is mostly focused on limited number of identified peptides and thus provide only partial information on the course of the proteolytic cascades.

A novel comprehensive approach to study the casein hydrolysis in cheese was developed in the current thesis. Capillary electrophoresis (CE), liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS), ultra-performance liquid chromatography (UPLC), and Kjeldahl methods were simultaneously used in for the quantification of the intact caseins, long and small peptides, free amino acids and N content of the different proteinaceous fractions during eight months of ripening of hard Old Saare cheese produced in Saaremaa Dairy, Estonia. The combination of up-to-date analytical methods made it possible to envisage the comprehensive process of proteolysis during cheese ripening.

To analyze and visualize the cheese peptidome, a Python-based computational tool was developed. Identification and quantification of more than 3,000 small water-soluble peptides by LC-MS/MS and subsequent allocation of all the peptides on the corresponding casein sequences allowed to create the proteolytic coverage maps that characterize the formation and degradation of the peptides throughout cheese ripening. The casein regions most susceptible to the action of proteolytic enzymes were identified. Moreover, the enzyme cleavages profiles were elucidated, and most essential cleavages were assigned to the action of known proteolytic agents: plasmin, cathepsin D, CEP of *Lc. lactis* and *Lb. helveticus*, and proteolytic system of *St. thermophilus.* Furthermore, the PCA followed by clustering analysis was applied to peptide data after robust allocation of the peptides into groups with similarly changing profiles. The proteolytic maps composed in this study can help to select the starters with desired proteolytic properties and fine-tune the cheese manufacturing process in the future.

Some proteolytic activities may take place already in raw milk, which may exert deleterious effects on the renneting properties of milk and whole cheesemaking process. Refrigerated storage conditions on farm may support the growth of psychrotrophic bacteria that possess various proteolytic enzymes. Yet, the knowledge on the changes in milk protein composition through a delivery in the refrigerated tank-trucks is scarce. Therefore, the compositional changes of caseins during refrigerated transportation of raw milk from farms to dairies were studied by CE. The study revealed significant differences in the relative content of α_{s2}-, κ-, and β-CNs in

milk before and after transportation. These changes can be attributed directly to the proteolytic activity of enzymes of psychrotrophic bacteria towards caseins or indirectly to the activation of the plasminogen activators by proteases of these bacteria. Based on the results, the shortest storage times and delivery routes for raw milk are recommended to produce cheese with the highest yield and quality.

The application of membrane processing (reverse osmosis (RO), ultrafiltration) for the concentration of cheese milk has attracted much attention to optimize the production costs and to increase the plant capacities and cheese yields. The general pathways of proteolysis in cheese made from unconcentrated milk are well characterized, while there are only few studies on the effect of milk concentration by RO. Thus, hard Gouda-type experimental cheese (RO-cheese) was manufactured from milk concentrated by RO using mesophilic and thermophilic starter cultures, and the effect of milk concentration on primary proteolysis during six months of ripening was investigated. we showed that the level of degradation of the main casein fractions by chymosin and plasmin in RO-cheese was comparable with those of the traditionally produced cheeses with similar manufacturing and ripening conditions. The less intensive hydrolysis of β-casein in the RO-cheese compared to the conventional Gouda-type cheeses could be explained by considerably lower pH of the RO-cheese, which could reduce the activity of plasmin. Findings from this study increase our knowledge of the RO-cheese manufacturing processes and may encourage the cheese producers to implement RO technology in industrial cheese manufacturing.

Lühikokkuvõte

Proteolüüsi detailne iseloomustamine juustu valmimise käigus

Proteolüüs on kõige tähtsam ja keerulisem biokeemiline protsess juustu iseloomuliku tekstuuri ja maitse kujunemisel. Proteolüüsil toimub kaseiinide järk-järguline lagundamine suurematekс ja väiksemateks peptiidideks ja vabadeks aminohapeteks, mida viivad läbi jääkkoagulant, plasmiin, piimhappebakterite rakuseinaga seotud proteaasid ning rakusisesed proteinaasid ja peptidaasid.

Proteolüüsi kulgu saab hinnata mittespetsiifiliste keemiliste meetoditega lämmastiku sisalduse kvantitatiivse määramise teel. Paraku ei võimalda need meetodid määrata üksikute valgufraktsioonide sisalduste muutusi ega tuvastada spetsiifliste proteolüütiliste ensüümide aktiivsusi juustu valmimisel. Mitmetes uuringutes on kasutatud mass-spektromeetrilisi meetodeid proteolüüsi jooksul tekkivate peptiidide iseloomustamiseks. Siiski keskendub enamus uuringuid piiratud arvu peptiidide identifitseerimisele ja ei taga seega terviklikku infot juustu proteolüüsi kohta.

Antud doktoritöös arendati välja kompleksne metoodika kaseiinide hüdrolüüsi uurimiseks juustu valmimise käigus. Kasutades kapillaarelektroforeesi (CE), vedelikkromatograafia tandem mass-spektromeetriat (LC-MS/MS) ja ülikõrgsurve vedelikkromatograafiat (UPLC) kvantifitseeriti kaseiine, nende lagundamisel tekkivaid suuri ja väikesi peptiide ning vabu aminohappeid kõva Old Saare juustu (Saaremaa) valmimisel kaheksa kuu vältel. Lisaks määrati Kjeldahli meetodiga ka üldlämmastiku sisaldus erinevates valgufraktsioonides. Kombineerides kaasaegseid analüütilisi meetodeid mittespetsiifilise üldlämmastiku määramisega kirjeldati põhjalikult juustu valmimisel toimuvat proteolüüsi.

Käesolevas töös tuvastati LC-MS/MS meetodiga üle 3000 veeslahustuva peptiidi juustu valmimise jooksul ja töötati välja Python programmil põhinev andmetöötlusmeetod, mille abil visualiseeriti saadud peptidoomi andmed. Identifitseeritud ja poolkvantifitseeritud peptiidid paigutati vastavate kaseiinide aminohappelistele järjestustele, mis võimaldas koostada kaseiinide proteolüütilisi diagramme ning iseloomustada peptiidide tekkimise ja lagunemise skeeme juustu valmimise käigus. Selliselt visualiseeritud andmed võimaldasid detekteerida proteaaside suhtes vastuvõtlikumad ja vähem hüdrolüüsitud kaseiinide piirkonnad. Samuti leiti ensüümide lõikamisprofiilid kaseiinides ning ühildati need teadaolevate proteolüütiliste ensüümide (plasmiin, katepsiin D, bakterite *Lc. Lactis* ja *Lb. helveticus* rakuseinaga seotud proteaasid, bakteri *St. thermophilus* proteolüütiline süsteem) lõikekohtadega. Lisaks rakendati peptiidide andmetele peakomponentide analüüs ja viidi läbi klasteranalüüs, mille abil jaotati tuvastatud peptiidid grupiti vastavalt nende kontsentratsioonide muutustele ajas. See aitas iseloomustada peptiidide keerulist dünaamikat jaotatuna gruppidesse jälgides nelja iseloomuliku peptiidide profiili juustu valmimise jooksul. Saadud teadmised juustu proteolüütiliste kaskaadide kohta aitavad valida juustu tootmiseks soovitud proteolüütiliste omadustega juuretisebaktereid ja teadlikult suunata juustu valmimisprotsesse.

Kaseiini osaline hüdrolüüs võib toimuda juba toorpiimas, mis võib negatiivselt mõjutada piima kalgendumisomadusi ning kogu juustu valmistamise protsessi. Farmis kiirelt jahutatud toorpiim on soodne keskkond proteolüütiliste psührotroofsete bakterite kasvuks. Samas on vähe andmeid toorpiimas valkudega toimuvate muutuste

kohta lühiajalisel jahutatud transpordil farmist piimatööstusesse. Käesolevas doktoritöös näidati, et α_{s2}-, κ-, and β-kaseiinide suhteline sisaldus enne ja pärast piima jahutatud transporti erines märkimisväärselt. Muutused kaseiinide koostises võivad tuleneda kas otseselt psührotroofsete bakterite proteaaside aktiivsusest või plasminogeeni aktivaatorite aktiveerimisest bakterite proteaaside poolt. Töö tulemustest järeldub, et parima kvaliteediga ja kõrgeima saagisega juustu saab võimalikult värskest (lühike säilimisaeg enne juustutootmist) ja minimaalselt transporditud piimast.

Membraantehnoloogiate (pöördosmoos (PO), ultrafiltreerimine) rakendamine juustupiima kontsentreerimiseks võib vähendada tööstuse tootmiskulusid ja potentsiaalselt suurendada tootmisvõimsust ja juustusaagist. Proteolüüsi eripära traditsioonilistes ehk kontsentreerimata piimast valmistatud juustudes on uuritud aastaid, kuid juustupiima PO-kontsentreerimise mõju juustus toimuvale proteolüüsile on seni vähe uuritud. Käesolevas töös valmistati PO-kontsentreeritud piimast kõva Gouda-tüüpi juust kasutades nii mesofiilset kui ka termofiilset juuretist, ning uuriti piima kontsentreerimise mõju juustu primaarsele proteolüüsile kuue kuu valmimise jooksul. Leiti, et PO-juustus toimus põhiliste kaseiini fraktsioonide hüdrolüüs kümosiini ja plasmiini toimel sarnaselt traditsioonilise tehnoloogia järgi valmistatud juustudele. Võrreldes tavalise Gouda-tüüpi juustuga oli PO-juustus vähem intensiivne β-kaseiini lagunemine, mis võib olla tingitud märkimisväärselt madalamast pH-st PO-juustus ja sellest tulenevalt madalamast plasmiini aktiivsusest. Antud doktoritöö annab uusi teadmisi sellest, kuidas mõjutab juustus toimuvat proteolüüsi piima kontsentreerimine pöördosmoosi teel ning julgustab juustutootjaid rakendama PO-kontsentreerimist juustupiima eeltöötlemisel.

Appendix 1

Publication I

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Comprehensive analysis of proteolysis during 8 months of ripening of high-cooked Old Saare cheese

A. Taivosalo,*†[1] T. Kriščiunaite,* A. Seiman,*† N. Part,* I. Stulova,* and R. Vilu*†
*Center of Food and Fermentation Technologies, Akadeemia tee 15A, 12618 Tallinn, Estonia
†Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

ABSTRACT

We applied capillary electrophoresis, liquid chromatography coupled with tandem mass-spectrometry (MS/MS), and ultra-performance liquid chromatography to determine the composition of water-insoluble and water-soluble proteinaceous fractions of the cheese and to study in detail the degradation of caseins during 8 mo of ripening of Estonian high-temperature cooked hard cheese Old Saare. The application of high-resolution and high-accuracy MS/MS enabled identification of more than 3,000 small peptides, representing a fairly full casein peptidome containing peptides of 4 to 25 AA in length: 1,049 from β-casein (CN), 944 from α_{S1}-CN, 813 from α_{S2}-CN, and 234 from κ-CN. The majority of β-CN- and α_{S1}-CN-derived peptides originated from the N-terminal parts of the molecule, f6-93 and f1-124, respectively; peptides from α_{S2}-CN arose predominantly from the C-terminal end f100-162. At the beginning of ripening, we found a relatively high amount of peptides originating from the glycomacropeptide part of κ-CN, whereas peptides from *para*-κ-CN prevailed during the later stages of ripening of the cheese. The cleavage patterns of β-CN, α_{S2}-CN, as well as α_{S1}-CN, showed that primary proteolysis was started mainly by plasmin, although a low proteolytic activity of chymosin was also evident. Based on the analysis of cleavage sites, we observed a significant participation of proteolytic enzymes, including amino- and carboxypeptidases, of both mesophilic and thermophilic starter bacteria in further hydrolysis of oligopeptides during the ripening. Several new phosphopeptides were detected in the result of MS/MS data analysis. The profiles of the estimated concentrations of phosphopeptides revealed that those originating from β-CN and α_{S1}-CN accumulated during cheese maturation. In contrast, we did not notice any generation of phosphopeptides from the highly phosphorylated part of α_{S2}-CN, f25-80, presumably due to the inaccessibility of this region to the action of plasmin and chymosin. The analysis of cleavage sites and the combination of principal component and clustering analyses provided a characterization of the complex dynamics of formation and degradation of peptides during cheese maturation. We made an attempt to obtain a comprehensive picture of proteolysis during Old Saare cheese ripening on the basis of the detailed peptidomic data, including also the less abundant peptides determined by MS/MS, and complemented by the data on intact caseins and free AA and reported the results in the paper.
Key words: high-cooked cheese, cheese ripening, proteolysis, cheese peptidomics

INTRODUCTION

Proteolysis is one of the most important biochemical processes during cheese ripening, manifesting itself as a continuous accumulation of small peptides and free amino acids (**FAA**), leading to the formation of aroma compounds and contributing to both flavor and texture of cheeses (Fox and McSweeney, 1996). In the high-temperature cooked cheeses, plasmin is the primary proteolytic agent (Upadhyay et al., 2004), which hydrolyzes intact caseins into large (water-insoluble) and intermediate-sized (water-soluble) peptides (Sousa et al., 2001), whereas chymosin is largely thermally inactivated (Hayes et al., 2002; Sheehan et al., 2007). Cell envelope proteinases (**CEP**) of lactic acid bacteria (**LAB**) act subsequently on primary hydrolysis products of casein, and after peptides of appropriate size have been formed, bacterial transporters transfer them into cytoplasms of LAB for further degradation by intracellular peptidases into smaller peptides and FAA (Kunji et al., 1996). The release of peptides is further facilitated by the lysis of starter and nonstarter LAB releasing their intracellular peptidases into cheese environment (Wilkinson and Kilcawley, 2005). From the initial steps of cheesemaking up to the end of ripening, the starter survival and enzymatic activities after lysis of cells are determined by temperature, NaCl concentration, and pH (Gatti et al., 2008).

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[1]Corresponding author: anastassia@tftak.eu

In high-temperature cooked cheeses, thermophilic LAB *Lactobacillus helveticus* is a major proteolytic agent, which produces a large amount of casein degradation products during cheese ripening (Hynes et al., 2003; Milesi et al., 2011). *Streptococcus thermophilus* is known to have lesser proteolytic activity (Callanan and Ross, 2004), but has been also shown to generate numerous peptides from different caseins (Miclo et al., 2012). Although the protease activity of LAB appears to be strain dependent, the common cleavage sites have been identified for some of the strains in vitro conditions (Kunji et al., 1996; Scolari et al., 2006; Jensen et al., 2009; Miclo et al., 2012; Sadat-Mekmene et al., 2013). The detailed information available on proteolytic systems of LAB allows to make an attempt to assign the enzyme activities to the origin of peptides in cheese.

Proteolysis in cheese during ripening has been extensively studied. Characterization of overall extent of proteolysis is often limited to unspecific determination of N content of the particular protein degradation fractions in cheese (Ardö, 1999; Milesi et al., 2011). Chromatographic methods have been widely used for the separation of casein molecules and their degradation products, for the dynamics of general profiles of water-soluble peptides, and for the quantification of FAA (Ardö and Gripon, 1995). More recently, capillary electrophoresis (**CE**) has been applied for more efficient separation of the water-insoluble fraction and identification of the products of primary proteolysis in different cheese varieties during ripening (Albillos et al., 2005; Rodriguez-Nogales and Vázquez, 2007; Rehn et al., 2010). The application of MS in the analysis of proteolysis has contributed to the identification of most abundant water-soluble peptides or particular peptides of interest (Gagnaire et al., 2001; Ardö et al., 2007; Piraino et al., 2007; Sforza et al., 2012; Sánchez-Rivera et al., 2014). These data have been further used to deduce the relationship between specific starter cultures added to cheese and the formation of certain casein hydrolysis products. Much less is known, however, how parallel hydrolysis cascades of peptides take place during ripening. The application of high-resolution and high-accuracy MS instruments developed for protein chemistry enables simultaneous study of the thousands of peptide components (Hu et al., 2005) and can assist in increasing the understanding of proteolytic events in cheese.

In this study, we analyzed proteolysis in Old Saare cheese, a high-temperature (52°C) cooked variety manufactured in Estonia from pasteurized bovine milk coagulated by rennet and containing both mesophilic and thermophilic LAB and ripened for 8 mo. We monitored industrial production of Old Saare cheese throughout ripening applying CE, liquid chromatography coupled with tandem mass spectrometry (**LC-MS/MS**), and ultra-performance liquid chromatography (**UPLC**). The aim of this study was to identify a possibly wide range of the proteolysis products (intact caseins, long water-insoluble and water-soluble peptides, and FAA) and to show their changes in the content during ripening of Old Saare cheese.

MATERIALS AND METHODS

Cheese Manufacture and Sampling

Two controlled industrial trials of Old Saare cheese manufacturing were carried out in the Saaremaa Dairy Plant (Estonia) at the same day. Cow milk for cheeses was collected either from 1 (first trial) or 3 farms (second trial) on the island of Saaremaa. The cheese milk was bactofuged and pasteurized at 72°C for 23 s. Chy-Max rennet and DVS starter cultures DCC-260 and EMFOUR (both from Chr. Hansen Ltd., Hørsholm, Denmark) were used. According to the manufacturer information, the starter cultures used contained strains of mesophilic and thermophilic LAB of the following species: *Lactococcus lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lb. acidophilus*, and *Lb. helveticus* in unspecified proportions. Cheeses were produced in 15-kg blocks, salted in brine to a content of 1.2% in the cheese, and ripened for 8 mo.

Cheese blocks were analyzed after pressing (1-d-old cheese), after 2 wk of ripening, and subsequently once per month during the ripening period of 8 mo. Cheese blocks were sampled from the interior part, and at each sampling time 2 pieces of cheese were taken from each of both cheese blocks obtained from 2 cheesemaking trials. Thus, all data presented in our work (except moisture content and pH of cheese) show mean values calculated from 4 replicates characterizing 2 cheesemaking trials analyzed at 10 ripening points. Moisture content and pH of cheeses were measured at the dairy using a moisture analyzer Precisa XM60 (Precisa Gravimetrics AG, Dietikon, Switzerland; ISO, 2004) and a pH meter (Mettler-Toledo Ltd., Leicester, UK), respectively.

Sample Preparation for CE, LC-MS/MS, UPLC, and Kjeldahl Analyses

Citrate dispersions (**CD**) of cheeses were prepared by mixing 5 g of grated cheese with 20 mL of 0.5 *M* trisodium citrate solution at 50 to 55°C using a combined hot-plate magnetic stirrer with multiple stirring positions (RT 15 Power IKAMAG, IKA-Werke GmbH & Co, Staufen im Breisgau, Germany) for approximately

60 min until complete dissolution of cheese. Thereafter, sample volume was raised to 100 mL with ultrapure laboratory-grade water (Milli-Q) and centrifuged at 1,630 × *g* at 4°C for 30 min to remove fat. Samples were stored at −20°C until further analysis with CE.

Water-soluble extracts (**WSE**) of cheeses were prepared by homogenizing 2.5 g of grated cheese in 22.5 mL of Milli-Q water (12,500–13,000 rpm) using Polytron PT 2100 dispersing aggregate with a diameter of 20 mm (Kinematica AG, Luzern, Switzerland), followed by heating for 10 min at 75°C and centrifugation for 20 min at 4°C at 13,304 × *g*. Supernatant was frozen in 1.5-mL Eppendorf Protein LoBind microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and stored at −20°C until further purification and analysis with LC-MS/MS and UPLC.

Total N (**TN**) content in CD and WSE of cheeses was determined by the Kjeldahl method. To obtain a total content of different fractions needed for further estimation of the concentrations of individual proteins and peptides determined by CE and LC-MS/MS analyses, a factor of 6.38 was applied for conversion of N to the content of proteinaceous compounds. We used the value of the conversion factor of 6.38 determined for milk which was also proposed to be used for cheese and other dairy products (Maubois and Lorient, 2016).

Analysis of Intact Caseins and Long Peptides

Intact caseins and primary proteolysis products in cheeses were analyzed by CE according to the method described by Ardö and Polychroniadou (1999). The CD samples of cheeses were mixed with a sample buffer (1:1), incubated for 1 h at room temperature, filtered through a 0.45-μm polyvinylidene fluoride (PVDF) filter (Merck Millipore Ltd., Tullagreen, Carrigtwohill, County Cork, Ireland), and loaded into 100-μL vials for CE analysis. Sample buffer (pH 8.6) consisted of 10 *M* urea, 0.83 g/L of methylhydroxyethylcellulose, 167 m*M* Tris, 67 m*M* disodium EDTA, 42 m*M* 3-(*N*-morpholino) propanesulfonic acid, and 17 m*M* dithiothreitol.

The CE analyses were carried out with the Agilent CE System (Agilent Technologies, Waldbronn, Germany) with diode-array detection. Separations were performed using a neutral polyvinyl alcohol coated capillary column (Agilent Technologies Finland Oy, Espoo, Finland) of 56 cm effective length and 50 μm i.d., at a temperature of 45°C with a linear voltage gradient from 0 to 25 kV in 3 min, followed by a constant voltage of 25 kV. Separation buffer (pH 3.0) contained 20 m*M* trisodium citrate, 190 m*M* citric acid, 0.5 g/L methylhydroxyethylcellulose, and 6 *M* urea. The samples were injected by pressure at 34.0 mbar for 20 s. Proteinaceous fractions were detected by UV absorbance at 214 nm.

Proteinaceous fractions were identified by injection of milk protein standards (α-LA, β-LG, κ-CN, β-CN, and α_S-CN; Sigma-Aldrich Co. LLC, St. Louis, MO) and by comparison of obtained electropherograms with those presented in literature (Otte et al., 1997; Miralles et al., 2003; Albillos et al., 2007; Heck et al., 2008). The peaks were integrated after setting the baseline from valley to valley (Miralles et al., 2003).

Analysis of Water-Soluble Peptides

LC-MS/MS Analysis. The WSE samples of cheeses were purified with C18 StageTips (Thermo Fisher Scientific Inc., Waltham, MA) according to Rappsilber et al. (2007) protocol, and analyzed by LC-MS/MS using an Agilent 1200 series nanoflow system (Agilent Technologies, Santa Clara, CA) connected to a Thermo Scientific LTQ Orbitrap MS equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark). Purified peptides were dissolved in 0.5% trifluoroacetic acid, and 2 μg of peptide fractions were loaded on a self-packed fused silica emitter (150 mm × 0.075 mm, Proxeon) packed with ReproSil-Pur C18-AQ 3 μm particles (Dr. Maisch, Ammerbuch, Germany) using a flow rate of 0.7 μL/min. Peptides were separated with a 240 min gradient from 8 to 40% B (A: 0.5% acetic acid, B: 0.5% acetic acid/80% acetonitrile) using a flow rate of 0.2 μL/min and sprayed directly into the MS operated at 220°C capillary temperature and 2.2 kV spray voltage.

Full mass spectra were acquired in a profile mode, with a mass range from m/z 300 to 1,800 at the resolving power of 60,000 at m/z 400. Up to five data-dependent MS/MS spectra were acquired in centroid in the linear ion trap for each FTMS full-scan spectrum, and each fragmented ion was dynamically excluded for 80 s. Full scans were performed after the accumulation of 1,000,000 charges, whereas fragmentation scans required the accumulation of 5,000 charges, and ion injection times were 500 and 150 ms, respectively. Monoisotopic precursor selection was enabled, ions with a charge state of ≥1+ were selected for fragmentation, and unassigned charge states were rejected.

Database Search. Raw MS/MS files from the LTQ Orbitrap were analyzed with MaxQuant software package (version 1.5.2.8; Cox and Mann, 2008). The MS/MS spectra were searched against a protein sequence database containing the following casein genetic variants: A, B, and C variants for α_{S1}-CN; A and C for α_{S2}-CN; A^1, A^2, A^3, and B variants for β-CN; and A, B, E, and F^1 variants for κ-CN (Farrell et al., 2004; Caroli et al., 2009) and the MaxQuant built-in common contaminants database (245 entries). The search was done against both forward and reverse protein sequences by

the Andromeda search engine (Cox et al., 2011). Parent mass and fragment ions were searched with a maximal initial mass deviation of 7 mg/kg and 0.5 Da, respectively. The search included the following variable modifications: methionine oxidation, N-terminal acetylation, N-terminal glutamine and asparagine deamination, formation of S-S disulfide bonds, and phosphorylation of Ser, Thr, and Tyr. The peptide search included peptides in the length range from 4 to 25 AA. For peptide identification, the false discovery rate was set to 0.01, and the "match between runs" search function was used to improve peptide quantification coverage.

Analysis of FAA

Analysis of FAA was performed by a UPLC system (Acquity UPLC, Waters Corp., Milford, MA), including a binary solvent manager, a sample manager, and a photodiode array detector, controlled by Waters Empower 2.0 software. Separations were performed on a 2.1 × 100 mm Waters Acquity UPLC AccQ·Tag Ultra Column operated at 55°C and equilibrated with AccQ·Tag Ultra eluent A (Waters Corp.). The WSE samples of cheeses were mixed with isopropanol (1:1) to precipitate the proteins, centrifuged for 5 min at 4°C at 13,304 × *g*, and filtered through a 0.2-μm PTFE filter (Merck Millipore Ltd.). Before the injection, FAA were derivatized with AccQ·Fluor Reagent (Waters Corp.) according to the manufacturer's procedure. The injection volume was 1.0 μL, the AA were eluted at a flow rate of 0.3 mL/min using a linear gradient of 0 to 100% of AccQ·Tag Ultra eluent B (Waters Corp.), and the absorbance was recorded at 260 nm. Empower software (Waters Corp.) was used for data processing. The absolute concentrations of AA were calculated using standard curves, and individual AA were also expressed as a relative content of the total amount of AA (mol %).

Data Analysis

Semi-Quantification of Proteins and Peptides. For calculation of the estimated concentrations of protein and peptide components identified by CE and LC-MS/MS analyses, the peak areas from electropherograms and the MS/MS intensities were normalized and recalculated in terms of grams per kilogram of cheese, using the content of proteinaceous compounds determined in CD (CD_{prot}) and WSE (WSE_{prot}) fractions by the Kjeldahl method, and total concentration of FAA, as presented below.

The intact caseins and the products of primary hydrolysis determined by CE were recalculated as follows:

$$C_x = \frac{\frac{A_x}{t_x} \times \left(CD_{prot} - WSE_{prot}\right)}{\sum_{i=1}^{n} \frac{A_i}{t_i}},$$

where C_x is the estimated concentration of fraction x (g/kg of cheese); A_x is the area in the electropherogram of fraction x; t_x is the migration time of fraction x; and n is the total number of the peaks.

The MS/MS intensities of small peptides were recalculated using the following equation:

$$C_y = \frac{I_y \times \left(WSE_{prot} - FAA\right)}{\sum_{i=1}^{n} I_i},$$

where C_y is the estimated concentration of the peptide y (g/kg of cheese); I_y is the intensity of the peptide y; n is the total number of peptides identified by MS/MS; and FAA is the total concentration of FAA determined by UPLC.

Further the concentrations of identified CE fractions and small peptides were recalculated to mol/kg of cheese using molar weights. As we could not estimate a true ratio between fully, partially, and nonphosphorylated copies of phosphopeptides, the molar weights of the phosphate groups were not taken into account in the recalculation of phosphopeptides to mol/kg of cheese.

For the calculations we assumed that the larger part of peptides longer than 25 AA and not covered by MS/MS analysis has been eluted in the CE electropherograms as unidentified peaks, those areas were also taken into account in all calculations. Also we assumed that di- and tripeptides were rapidly degraded into FAA by the enzymes of LAB, and hardly accumulated in the cheese mass in significant amounts. Urea and ammonia were not measured in the fraction either, which might have led to some overestimation of the total amount of small water-soluble peptides in the WSE.

Treatment of MS/MS Data. Calculations and visualizations were performed using in-house data analysis routines implemented in Python programming language (Python Software Foundation, version 3.5, available at http://www.python.org) based on all 3,040 casein-derived small peptides identified by LC-MS/MS.

To look in more detail at the most abundant peptides present in cheese during ripening, peptides were selected based on their estimated molar concentrations and subjected to further analysis. At each time point, the most abundant peptides that accounted for altogether at least 50% of all small peptides by molar abundance were sorted out. At each ripening time, 54

to 83 peptides met this criterion; altogether, 169 peptides (48 from α_{S1}-CN, 20 from α_{S2}-CN, and 101 from β-CN) were selected and subjected to principal component analysis (**PCA**) and clustering analysis. The data set corresponding to 169 individual peptides was first preprocessed using centering and autoscaling, followed by PCA. This approach has 2 advantages. Firstly, after normalization, the clustering is based on the peptide concentration profiles rather than on their estimated concentration. Secondly, by selecting a limited number of principal components (**PC**), the clustering is based only on most obvious profile features while reducing the noise in individual profiles. Explained variance and eigenvalues of individual PC of PCA were used to select the appropriate number of PC. Four PC described >90% of the variance. After that, additional PC added a little to total explained variance. Therefore, data corresponding to the first 4 PC of individual peptides were chosen for clustering analysis. In clustering analysis, k-means clustering was applied. The algorithm aims to separate n samples into k groups, in which each observation belongs to the group with the nearest mean. Therefore, k-means clustering takes the total number of groups as the input parameter. Several values for the total number of groups were tested. In total, 4 groups were chosen as optimal, as the average deviance of individual peptides profiles from the groups mean was the smallest. When using less than 4 groups, the deviance increased dramatically. When more groups than 4 were formed, the profiles of several groups looked very similar, indicating that these peptides had similar behavior.

RESULTS AND DISCUSSION

Gross Composition of Cheese and Overall Distribution of Proteolysis Fractions

The moisture content decreased from 38.5 to 35.5% (wt/wt) and pH increased approximately from 5.3 to 5.7 during 8 mo of the cheese ripening (Table 1).

The mean value of TN content in Old Saare cheese was 42.1 ± 0.8 g/kg of cheese and did not differ significantly during the ripening ($P < 0.05$). Figure 1 shows the changes in overall estimated composition of caseins and their degradation products throughout the ripening process. In 1-d-old cheese, intact caseins comprised approximately 78% of TN and decreased to 25% of total TN in the 8-mo-old cheese (Figure 1). The content of water-soluble N (**WSN**), including water-soluble peptides and FAA, increased almost 10-fold from 1.3 ± 0.0 to 12.8 ± 0.3 g/kg, and comprised about 29% of TN by the end of the ripening. Hydrolysis of full-length caseins and accumulation of WSN fraction was particularly pronounced during the first 4 mo; the concentration of WSN increased by approximately 2.5 g/kg of cheese per mo. In general, the ripening of Old Saare was characterized by a high extent and depth of proteolysis. The ratio of WSN to TN in the 8-mo-old Old Saare was somewhere in between that observed in 24-mo ripened Parmesan (32–36%) and 6-mo-old Cheddar (24–25%; Careri et al., 1996; Barrett et al., 1999).

Primary Proteolysis

The CD fraction of Old Saare cheese resolved into 43 prominent peaks on the CE curves (Figure 2). From these, we identified 15 peaks either as intact caseins: α_{S1}-CN (8P and 9P), α_{S2}-CN (11P, 12P, and nP), β-CN (with genetic variants A^1, A^2, and B), and para-κ-CN [regarded as intact in this study as the vast majority of κ-CN is hydrolyzed to para-κ-CN and glycomacropeptide (**GMP**) already during the vat process before ripening], or their breakdown products: α_{S1}-I-CN (8P and 9P), γ_1-CN (A^1 and A^2), γ_2-CN, and γ_3-CN. Twenty-eight prominent peaks could not be assigned to any specific casein fraction (marked with numbers on sample electropherograms in Figure 2), but were considered as casein degradation products due to their mainly increasing behavior during cheese ripening (an overall change of this fraction during the ripening is shown in Figure 1). Unidentified peaks can refer presumably to primary hydrolysis products of α_{S2}-CN or to some water-insoluble large peptides derived from α_{S1}-CN and β-CN produced by the action of proteinases other than chymosin and plasmin.

Among all caseins, β-CN was subjected to most extensive degradation; compared to the 1-d-old cheese we found about 80% of β-CN hydrolyzed during 8 mo of

Table 1. Moisture content and pH of Old Saare cheese during ripening[1]

	Ripening time				
Item	1 d	1 mo	3 mo	5 mo	8 mo
Moisture content, %	38.5 ± 0.1	36.5 ± 0.0	36.1 ± 0.5	35.8 ± 0.2	35.5 ± 0.0
pH	5.27 ± 0.04	5.30 ± 0.00	5.44 ± 0.01	5.40 ± 0.00	5.67 ± 0.04

[1]Values are calculated based on the data of the 2 cheesemaking trials provided by the dairy (mean ± SD, n = 2).

ripening (Table 2). The hydrolysis of intact α_{S2}-CN was also very high (78%), the corresponding value for α_{S1}-CN was 65%, and for *para*-κ-CN only 37% (Table 2).

We observed a strong contribution of plasmin to the hydrolysis of β-CN already in the 1-d-old cheese: γ-CN formed 21% of water-insoluble β-CN-derived fraction, and 8.2% of total proteinaceous fraction (Table 2). In contrast, α_{S1}-I-CN, the primary hydrolysis product of α_{S1}-CN generated by chymosin, formed only 1.4% of α_{S1}-CN water-insoluble fraction (0.4% of total proteinaceous fraction) in the 1-d-old cheese. This is in agreement with the fact that β-CN hydrolysis by plasmin starts already in milk, while α_{S1}-CN is not hydrolyzed by chymosin during milk coagulation, but is degraded in cheese during ripening (Upadhyay et al., 2004). On the other hand, despite the large differences in the initial levels of γ-CN and α_{S1}-I-CN at the beginning of the ripening, the hydrolysis of α_{S1}-CN and β-CN showed similar trends and degradation rates, which were the highest within the first 4 mo of cheese ripening (Figure 3a); the content of intact β-CN decreased approximately 0.7 mmol/kg of cheese per month and that of α_{S1}-CN decreased 0.6 mmol/kg of cheese per month. During the remaining period, the breakdown of β-CN and α_{S1}-CN was insignificant or very modest. Likewise, the content of α_{S2}-CN decreased rapidly during the first 3 mo of ripening (Table 2), the degradation rate slowing during further ripening. In parallel with the degradation of β-CN and α_{S1}-CN intact fractions, γ-CN content increased steadily up to 4 mo, and α_{S1}-I-CN content, up to 3 mo of ripening (Figure 3b). Although Old Saare cheese is produced using quite high scalding temperature (52°C), the considerable degradation of intact α_{S1}-CN in parallel with the production of α_{S1}-I-CN may indicate a substantial residual activity of chymosin in Old Saare cheese. Chymosin is known to be quite susceptible to irreversible thermal denaturation in liquid environment, but has been shown to be only partially and reversibly inactivated when bound to casein matrix during curd cooking at high temperatures and can even regain its activity after initial inactivation (Hayes et al., 2002; Sheehan et al., 2007). On the contrary, the contribution of plasmin to primary proteolysis is well defined in cheeses cooked at higher temperatures (Kaminogawa et al., 1972; Sousa et al., 2001; Sheehan et al., 2007), probably due to inactivation of plasminogen activator inhibitors (Farkye and Fox, 1990). Our results on β-CN degradation in the high-temperature cooked Old Saare cheese are consistent with these observations, even though the initial pH of the fresh cheese (pH 5.3) has been rather low for optimal plasmin action (Table 1). It is noteworthy that the dynamics of content of individual γ-CN was quite different throughout the ripening: the rates of γ_1-CN and γ_3-CN accumulation during the first 4 mo were much higher than that of γ_2-CN, content of which changed very slowly during the entire ripening.

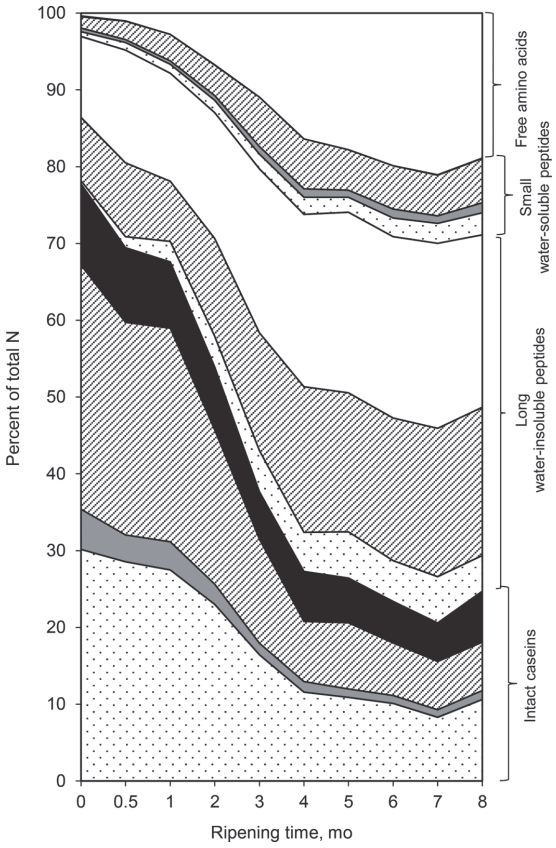

Figure 1. Estimated distribution of the main casein fractions during the ripening of Old Saare cheese within the intact caseins, long water-soluble peptides, and small water-soluble peptides: dotted areas represent α_{S1}-CN, gray areas represent α_{S2}-CN, striped areas represent β-CN, and black areas represent κ-CN and their degradation products. Unidentified long peptides determined by capillary electrophoresis and free AA are represented by white areas. Results represent the mean values of duplicated samples from each of 2 cheesemaking trials (n = 4).

Dynamics of Small Peptides

In total, we identified 3,266 unique water-soluble peptides (4–25 AA in length) by LC-MS/MS analysis (Supplemental Table S1; https://doi.org/10.3168/jds.2017-12944). From those, 944 arose from α_{S1}-CN, 813 from α_{S2}-CN, 1,049 from β-CN, 234 from κ-CN, and 226 from miscellaneous milk-derived proteins (under

0.3% of total MS/MS intensity): mainly from osteopontin (177 peptides), also from β-LG (17 peptides), and few peptides from bovine fibrinogen and complement component C7. The total intensity of peptides from osteopontin obtained by MS/MS in 1-d-old cheese was comparable with the intensity of peptides from GMP part of κ-CN. Most of the peptides identified from osteopontin had maximum intensities in 1-d-old cheese, showing a rapid decrease already in 1- to 2-mo-old cheese and remaining low until the end of ripening. Our results indicated that osteopontin was somehow subjected to proteolysis during ripening of the cheese, but its impact to cheese characteristics is unclear and demands further investigation. Thus, we focused our study on the characterization of the proteolysis of casein components in particular.

Eight hundred eighty-six of all casein-derived peptides identified by MS/MS were found to be potentially phosphorylated. The quantitative proteomics software MaxQuant detected the phosphorylation of casein peptides at the following positions: Ser_{41}, Ser_{46}, Ser_{48}, Thr_{49}, Ser_{64}, Ser_{66}, Ser_{67}, Ser_{68}, Ser_{75}, Ser_{115}, Ser_{122}, Ser_{188} in α_{S1}-CN; Thr_3, Ser_8, Ser_9, Ser_{10}, Ser_{13}, Ser_{16}, Thr_{19}, Ser_{31}, Ser_{52}, Ser_{56}, Ser_{57}, Ser_{58}, Ser_{61}, Thr_{66}, Ser_{129}, Thr_{130}, Ser_{131}, Ser_{135}, Thr_{138}, Ser_{143}, Thr_{144} in α_{S2}-CN; Ser_{15}, Ser_{17}, Ser_{18}, Ser_{19}, Ser_{22}, Thr_{24}, Ser_{35}, Thr_{41} in β-CN; and Ser_{127}, Thr_{133}, Thr_{145}, Ser_{149}, Ser_{166}, Thr_{167} in κ-CN sequence. These phosphorylation positions included all Ser-P sites identified in plasmin-released phosphopeptides of Grana Padano cheese (Ferranti et al., 1997a) and phosphopeptides isolated from semi-hard Herrgård cheese made with mesophilic DL-starter (Lund and Ardö, 2004). In addition, LC-MS/MS technique used in our study allowed to detect phosphopeptides with phosphate groups at all experimentally determined as well as potential phosphorylation sites in caseins reviewed by Farrell et al. (2004), including Thr residues. Also, some new phosphopeptides were found with phosphate groups at sites not previously reported in the literature (Thr_{49}, Ser_{122}, Ser_{188} in α_{S1}-CN; Ser_{13}, Thr_{19}, Ser_{52}, Ser_{135}, Thr_{138}, Thr_{144} in α_{S2}-CN; Ser_{22}, Thr_{24}, Thr_{41} in β-CN; and Thr_{133}, Ser_{166}, and Thr_{167} in κ-CN). Phosphorylation of caseins is a complicated posttranslational modification, which could be influenced by many factors, including genetic expression of protein kinases, substrate availability, and accessibility of the modification site of casein (Holland and Boland, 2014). Some studies have shown the activity of phosphatases from milk or bacteria present in cheeses modifying phosphorylation of peptides (Akuzawa and Fox, 2004). Therefore, the

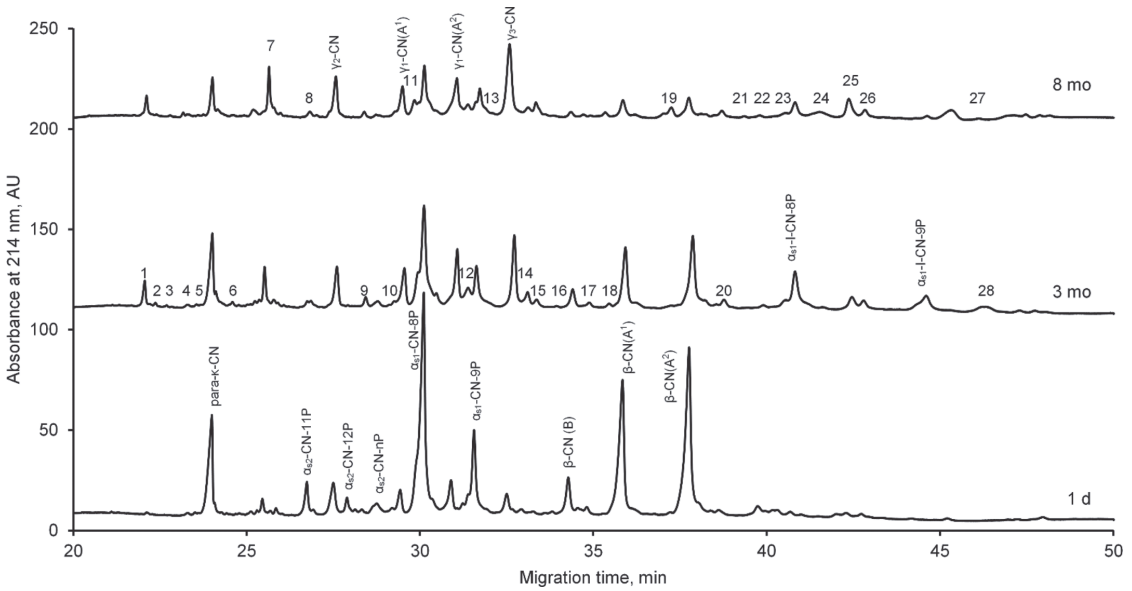

Figure 2. The representative electropherograms of Old Saare cheese obtained by capillary electrophoresis at 1 d, 3 mo, and 8 mo of ripening. The peaks were identified by injection of milk protein standards and comparison with the profiles presented in literature (Otte et al., 1997; Miralles et al., 2003; Albillos et al., 2007; Heck et al., 2008). Unidentified peaks are marked with numbers 1 to 28. AU = arbitrary units.

characterization of actual phosphorylation sites of peptides and the degree of modification needs an additional deep analysis of individual phosphopeptides.

An overall change of the content of small peptides was the fastest during the first 3 to 4 mo of ripening (Figure 1, Table 2) and slowed down during the later stages of the ripening, which indicated that the rates of formation and degradation of small peptides became more balanced. Peptides originating from β-CN comprised the largest part (56–69%) of the small peptides depending on the ripening time, followed by the peptides derived from α_{S1}-CN (21–29%), from α_{S2}-CN (8–17%), and finally from κ-CN forming 0.1 to 3% of the whole fraction of small water-soluble peptides.

For a general review of the patterns of small peptides throughout 8-mo ripening of Old Saare cheese, we

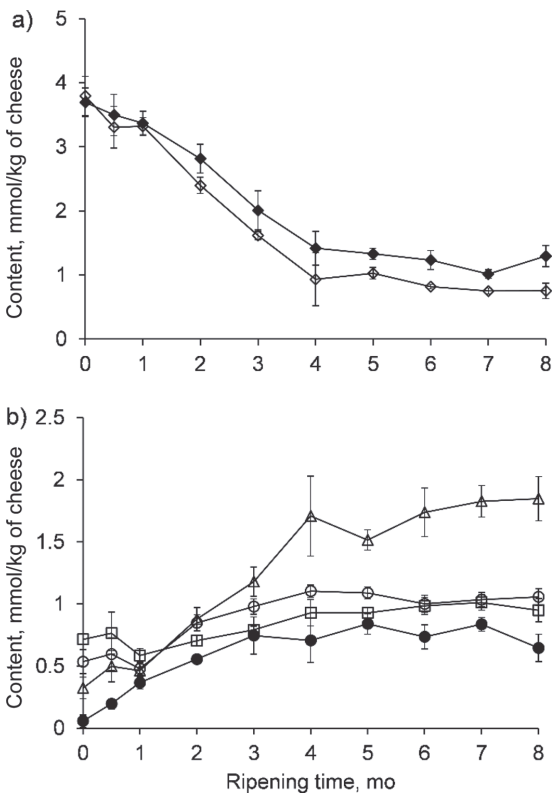

Figure 3. Time course of casein hydrolysis: (a) α_{S1}-CN (◆) and β-CN (◊) and their primary degradation products; (b) α_{S1}-I-CN (●), γ_1-CN (○), γ_2-CN (□), γ_3-CN (Δ) during the ripening of Old Saare cheese. Results represent the mean estimated concentrations of duplicated samples from each of 2 cheesemaking trials (n = 4); vertical bars represent SD.

Table 2. Changes in the estimated concentrations of the main casein derived fractions (g/kg of cheese) during the ripening of Old Saare cheese[1]

	Ripening time									
Fraction	1 d	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
Intact caseins	218.8 ± 9.3	195.5 ± 11.5	190.3 ± 6.3	151.7 ± 8.1	105.9 ± 8.0	76.6 ± 11.9	74.2 ± 4.2	65.7 ± 4.9	57.8 ± 4.1	69.4 ± 6.2
α_{S1}-CN	84.9 ± 5.2	80.4 ± 7.4	77.4 ± 4.3	64.7 ± 5.2	46.2 ± 7.0	32.6 ± 6.0	30.6 ± 2.0	28.3 ± 3.4	23.3 ± 1.5	29.8 ± 3.8
α_{S2}-CN	14.7 ± 2.4	9.8 ± 2.2	10.2 ± 0.9	7.4 ± 0.7	4.2 ± 0.4	3.8 ± 0.6	3.1 ± 0.2	2.9 ± 0.2	2.7 ± 0.2	3.3 ± 0.4
β-CN	89.5 ± 7.2	78.0 ± 7.7	78.3 ± 3.1	56.6 ± 3.0	38.1 ± 1.5	22.0 ± 9.8	24.2 ± 2.1	19.3 ± 0.8	17.7 ± 1.1	17.7 ± 2.8
para-κ-CN	29.7 ± 1.1	27.4 ± 3.4	24.4 ± 3.1	23.0 ± 5.3	17.4 ± 3.4	18.3 ± 2.9	16.3 ± 2.9	15.1 ± 3.3	14.1 ± 3.6	18.6 ± 4.0
Long water-insoluble peptides	54.0 ± 6.0	72.4 ± 8.4	69.1 ± 5.1	93.2 ± 2.1	118.2 ± 4.2	130.7 ± 4.9	133.9 ± 3.2	133.4 ± 4.5	138.8 ± 4.8	130.3 ± 6.5
α_{S1}-I-CN	1.2 ± 1.0	4.0 ± 0.8	7.4 ± 1.0	11.3 ± 0.5	15.1 ± 3.0	14.3 ± 3.6	17.0 ± 1.7	14.9 ± 2.0	16.9 ± 1.1	13.1 ± 2.2
γ-CN	23.2 ± 2.3	27.0 ± 3.6	22.1 ± 1.4	35.8 ± 1.8	43.0 ± 1.9	53.3 ± 4.1	50.8 ± 1.5	52.2 ± 2.8	54.2 ± 2.0	54.2 ± 2.7
γ_1-CN	11.0 ± 2.0	12.2 ± 2.7	9.8 ± 1.0	17.3 ± 1.3	20.0 ± 1.3	22.6 ± 1.0	22.3 ± 1.0	20.5 ± 1.4	21.2 ± 1.2	21.6 ± 1.4
γ_2-CN	8.5 ± 0.5	9.1 ± 2.0	6.9 ± 0.7	8.3 ± 0.5	09.4 ± 0.3	11.0 ± 1.3	11.0 ± 0.5	11.6 ± 0.8	12.0 ± 0.7	11.2 ± 1.1
γ_3-CN	3.8 ± 1.0	5.8 ± 1.4	5.4 ± 0.6	10.1 ± 1.1	13.6 ± 1.4	19.7 ± 3.7	17.5 ± 0.9	20.1 ± 2.3	21.1 ± 1.5	21.4 ± 2.1
Unidentified products	29.7 ± 5.5	41.4 ± 7.6	39.5 ± 4.8	46.2 ± 1.3	60.1 ± 2.4	63.1 ± 2.8	66.1 ± 2.4	66.3 ± 3.7	67.6 ± 4.4	63.0 ± 5.7
Small water-soluble peptides	7.4 ± 0.4	10.5 ± 0.9	13.7 ± 1.4	16.1 ± 0.5	23.4 ± 1.8	22.8 ± 3.0	17.5 ± 1.1	19.8 ± 1.6	18.4 ± 2.4	21.9 ± 2.1
α_{S1}-CN	1.8 ± 0.1	2.7 ± 0.1	3.2 ± 0.1	4.1 ± 0.2	4.8 ± 0.2	5.2 ± 0.2	4.2 ± 0.3	5.2 ± 0.3	5.3 ± 0.3	5.3 ± 0.3
α_{S2}-CN	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	2.4 ± 0.1	2.0 ± 0.2	2.0 ± 0.1
β-CN	4.1 ± 0.2	6.5 ± 0.2	9.3 ± 0.3	10.3 ± 0.4	16.1 ± 1.0	15.1 ± 0.8	11.2 ± 0.8	12.1 ± 1.1	10.9 ± 0.8	10.9 ± 0.9
κ-CN	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.0	0.1 ± 0.0	<0.1
Free AA	1.2 ± 0.1	3.1 ± 0.2	8.4 ± 0.4	20.5 ± 0.6	34.0 ± 1.7	51.4 ± 3.2	55.9 ± 1.0	62.6 ± 1.3	66.5 ± 1.7	59.9 ± 1.1

[1]Values are the means of duplicated samples from each of 2 cheesemaking trials (mean ± SD, n = 4).

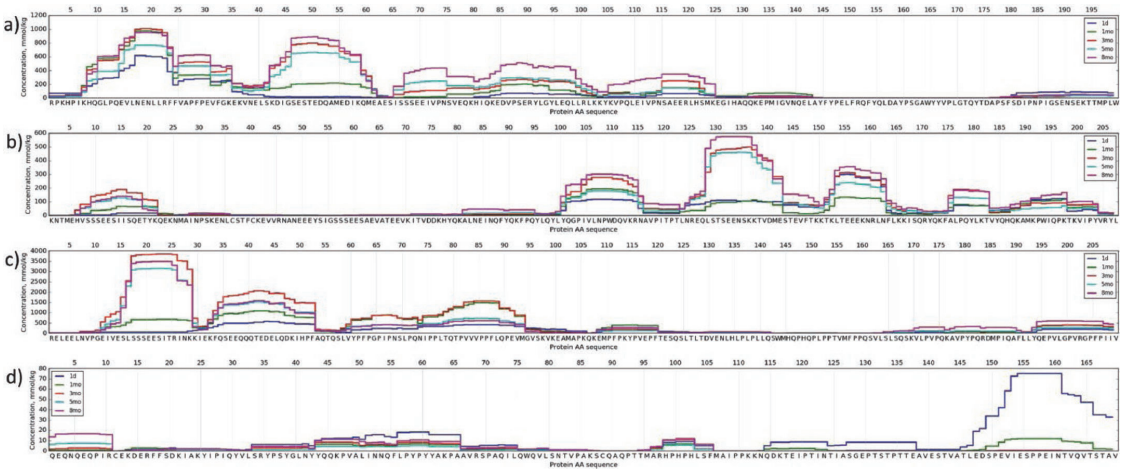

Figure 4. Regions on α_{S1}-CN (a), α_{S2}-CN (b), β-CN (c), and κ-CN (d) sequences where the identified small peptides derive from (based on all 3,040 water-soluble peptides determined by liquid chromatography-MS/MS). Cumulative concentrations of the AA in the identified peptides are plotted on the casein sequences. Results represent the mean estimated concentrations of duplicated samples from each of 2 cheesemaking trials (n = 4).

aligned the identified 3,040 peptides on the corresponding casein sequences and plotted them considering their estimated concentrations in the samples (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-12944). We summed the estimated concentrations of all AA present in identified peptides and located in the same positions along particular protein sequence, and plotted the cumulative concentrations on corresponding sequences to get an overview of the preferable regions of caseins from where the majority of small peptides were derived (Figure 4). Further, to facilitate the analysis of cleavage sites, we also summed the estimated concentrations of all AA corresponding to the N-terminal and C-terminal ends of identified peptides and plotted them on the entire protein sequences (Supplemental Figure S2, https://doi.org/10.3168/jds.2017-12944, and Figure 5).

β-CN-Derived Small Peptides. The majority of β-CN-derived water-soluble peptides arose from the N-terminal part of the sequence, f6-93 (Figure 4c). We identified also a significant fraction of peptides from the regions f106-120 and f170-209. However, we observed a very low proteolytic activity on the β-CN sequence f120-170 (Supplemental Figure S1c, https://doi.org/10.3168/jds.2017-12944, and Figure 4c). It should be noted also that the amounts of the peptides of β-CN were significantly higher that the amounts from other casein variants (Figure 4).

Most of the peptides from the region β-CN f1–28 (Figure 4c) were formed presumably due to the preferred action of plasmin on the bond Lys_{28}-Lys_{29} (Fox and McSweeney, 1996) and subsequent activity of bacterial proteinases and peptidases on that fragment. The relative share of these peptides increased from approximately 3.6% in the 1-d-old cheese up to 40% of all β-CN derived peptides by the end of ripening indicating their clear accumulation. A multiphosphorylated peptide β-CN f7–28 formed from f1–28 by the cleavage of the bond Leu_6-Asn_7 possibly by CEP of *Lc. lactic* and by *Lb. helveticus* (Kunji et al., 1996), was abundant already in the 1-d-old cheese and remained at high concentrations until the end of the ripening (Table 3). Furthermore, 2 peptides, β-CN f7–16 and f17–28, the potential degradation products of β-CN f7–28 by the action of CEP of lactococci and proteolytic system of *St. thermophilus* on the bond Leu_{16}-Ser_{17} (Kunji et al., 1996; Miclo et al., 2012), were also present in comparable and substantial amounts in the 1-d-old cheese. A significant decrease in relative amounts of mesophilic lactococci during the vat process of Old Saare cheese has been indicated by pyrosequencing of 16S cDNA [L. Blank, Department of Chemistry and Biotechnology, Tallinn University of Technology (**TUT**), Tallinn, Estonia, personal communication]. Nevertheless, the significant production of peptides β-CN f7–28, f7–16, and f17–28 already in fresh cheese may indicate a high activity of CEP of mesophilic starter bacteria before an actual cheese ripening began. Pyrosequencing of 16S cDNA has also shown that during the first month of ripening *Lb. helveticus* and *St. thermophilus* were the

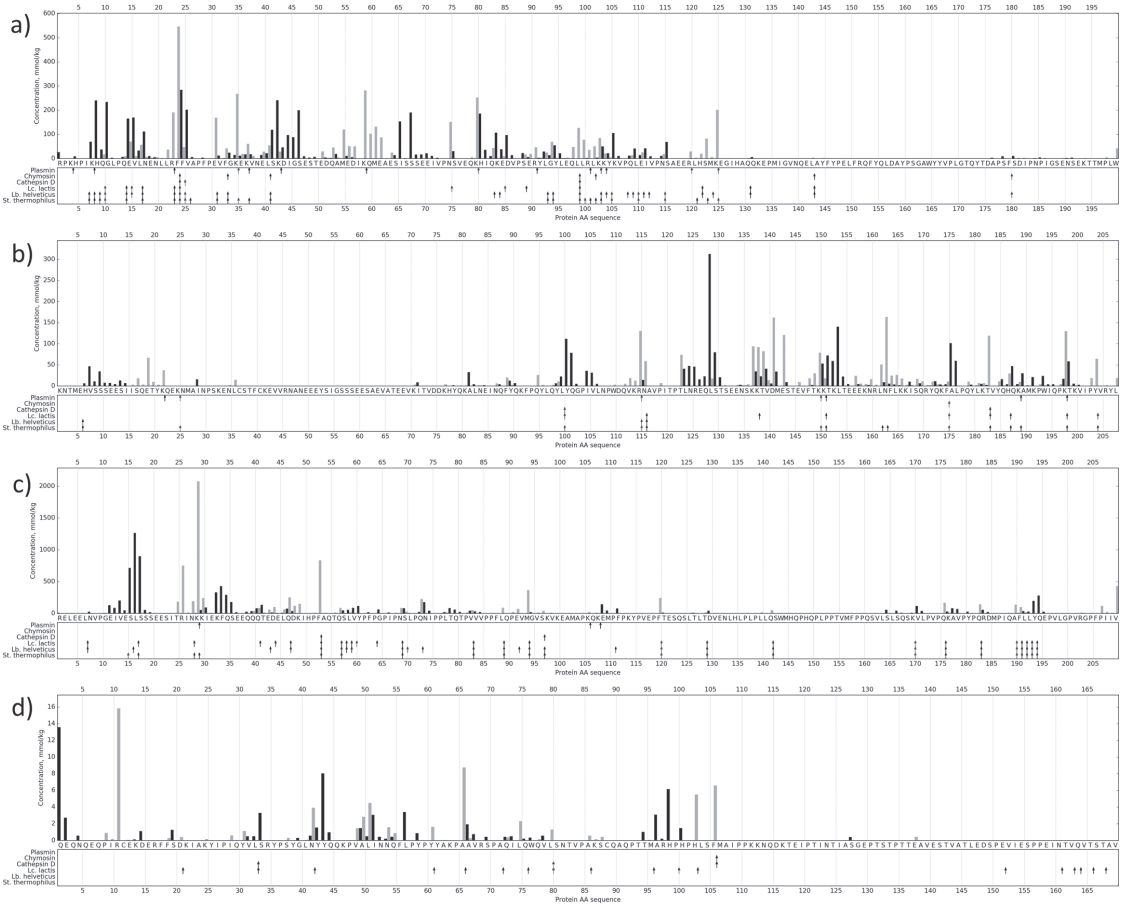

Figure 5. Cleavage sites plotted on the α_{S1}-CN (a), α_{S2}-CN (b), β-CN (c), and κ-CN (d) sequences in the 8-mo-old Old Saare cheese (based on all 3,040 water-soluble peptides determined by liquid chromatography-MS/MS). Black bars indicate summed estimated molar concentrations of the AA at the N-terminal and grey bars at the C-terminal ends of all identified peptides. Important cleavages found in this study and assigned to known proteolytic agents: plasmin (Le Bars and Gripon, 1989; McSweeney et al., 1993b; Fox and McSweeney, 1996), chymosin (McSweeney et al., 1993a; Exterkate et al., 1997), cathepsin D (Larsen et al., 1996), cell envelope proteinases of *Lactococcus lactis* (Monnet et al., 1992; Exterkate and Alting, 1993; Kunji et al., 1996), CEP of *Lactobacillus helveticus* (Kunji et al., 1996; Sadat-Mekmene et al., 2011), and the proteolytic system of *Streptococcus thermophilus* (Miclo et al., 2012) are marked with the arrows. The degradation of the C-terminal part of κ-CN f106–169 can be clearly seen only during the first month of ripening; accordingly, the arrows shown in the C-terminus of the protein indicate prominent cleavage sites determined at the beginning of the ripening. Results represent the mean values of duplicated samples from each of 2 cheesemaking trials (n = 4).

main metabolically active species in Old Saare cheese (L. Blank, TUT, Tallinn, Estonia, personal communication). Thus, the thermophilic bacteria were likely to play an essential role in the hydrolysis of the β-CN during the ripening of Old Saare cheese. Three phosphopeptides, β-CN f15/16/17–28, accumulated very fast during the ripening and comprised in terms of their molar abundance approximately 20% of β-CN-derived water-soluble peptides in cheese samples ripened for 8 mo (Table 3). These peptides could be generated from β-CN f1/7–28 by the action of both mesophilic and thermophilic starters on Glu_{14}-Ser_{15} (*St. thermophilus*; Miclo et al., 2012), Ser_{15}-Leu_{16} (*Lb. helveticus*; Kunji et al., 1996; Sadat-Mekmene et al., 2011), and Leu_{16}-Ser_{17} (*Lc. lactis* and *St. thermophilus*; Kunji et al., 1996; Miclo et al., 2012). Besides, we observed a substantial cleavage of the bond Asn_{27}-Lys_{28} (Figure 5c), which has been previously reported to be susceptible for

splitting by CEP of *Lc. lactis* (Kunji et al., 1996) as well as *St. thermophilus* proteolytic system (Miclo et al., 2012). The noticeable formation of several peptides (f11/12/13/15/16/17-28) differing in length by 1 or 2 AA residues in the N-terminal end and produced by successive degradation of β-CN f7–28, whereas the C-terminus remains the same (Table 3), may indicate significant activity of aminopeptidases in the cheese, though the endopeptidases of LAB may also be involved (Tan et al., 1993). However, the cleavage patterns of β-CN, showing that the N-terminal AA of peptides were in the consecutive positions in the region f11–17, and lacking the C-terminal AA of other (complementary) peptides in the same positions (Figure 5c), clearly supported the assumption of the action of aminopeptidases in the formation of these peptides. In addition, we observed a significant cleavage of Arg_{25}-Ile_{26} bond (Figure 5c); unfortunately, we did not find the data on the origin of this cleavage in the literature. It is noteworthy that among different peptides located in the region β-CN f1–28, especially phosphorylated ones with the phosphate groups at positions Ser_{15}, Ser_{17}, Ser_{18}, Ser_{19}, Ser_{22}, and Thr_{24}, appeared in the highest amounts during the ripening as noted above. Low activity of acid phosphatase (an enzyme that determines the degree of phosphorylation of caseins and their hydrolysis products) has been shown to inhibit degradation of phosphopeptides in cheese (Hynek et al., 2002). Hence, the high abundance of phosphopeptides in Old Saare is in accordance with the observations made in earlier studies that multiphosphorylated casein peptides are much more resistant to further hydrolysis by LAB peptidases than other fragments (Hynek et al., 2002), and that especially the phosphopeptides arising from β-CN phosphoserine-rich region f15–22 tend to accumulate during cheese ripening (Ferranti et al., 1997a; Deutsch et al., 2000; Lund and Ardö, 2004).

The peptides from the strongly negatively charged hydrophilic region of β-CN f29–52 were also present in substantial amounts in Old Saare cheese (Figure 4c, Table 3), comprising approximately 16 to 24% of β-CN derived water-soluble peptides depending on the ripening time. Most of the peptides were present in the highest amount at 3 to 4 mo of ripening. The N-terminus of these peptides supposedly was the result of plasmin cleavage followed by a sequential action of aminopeptidases released from the lyzing LAB. The C-terminus of the peptides from this region was determined presumably by the hydrolysis of the bond Phe_{52}-Ala_{53} either by CEP of *Lc. lactis* (Kunji et al., 1996) and *Lb. helveticus* strains (Sadat-Mekmene et al., 2011), by proteolytic system of some *St. thermophilus* strains (Miclo et al., 2012), or by indigenous milk proteinase cathepsin D (Larsen et al., 1996). We also detected extensive cleavage at other known preferable in vitro sites of CEP of lactococci (Gln_{40}-Thr_{41}, Asp_{43}-Glu_{44}, Gln_{46}-Asp_{47}; Kunji et al., 1996) and *Lb. helveticus* (Glu_{42}-Asp_{43}, Gln_{46}-Asp_{47}; Kunji et al., 1996; Sadat-Mekmene et al., 2011; Figure 5c). Similarly to the case of previous region β-CN f1–28, the peptides from β-CN f29–52 detected at the highest amounts (i.e., peptides f29/30/32/33/34/35/40/41–52), belonged to potential phosphopeptides with phosphorylated Ser_{35} and Thr_{41} (Table 3).

The third group of peptides generated from β-CN at high amounts, especially in the 1- to 4-mo-old cheese, arose from a hydrophobic region f53–93 (Figure 4c, Table 3). The region β-CN f53–93 contained numerous cleavage sites of CEP of lactococci, overlapping with the preferable hydrolysis sites of the enzymes of *Lb. helveticus* and *St. thermophilus* reported in the literature [e.g., Gln_{56}-Ser_{57}, Asn_{68}-Ser_{69}, Val_{82}-Val_{83}, Glu_{88}-Val_{89}, Met_{93}-Gly_{94} (Kunji et al., 1996; Sadat-Mekmene et al., 2011; Miclo et al., 2012)], which we found significantly cleaved in our study (Figure 5c). We observed a number of additional possible cleavages of *Lb. helveticus* [i.e., Gln_{72}-Asn_{73} and Glu_{91}-Val_{92} (Sadat-Mekmene et al., 2011)]; peptides originating from the hydrolysis of respective bonds were present in notable amounts throughout Old Saare ripening (Table 3). Simultaneous accompanying activity of aminopeptidases on the regions β-CN f57–60 and f77–80 was also observed (Figure 5c). Many peptides resulting from aforementioned cleavages (β-CN f60–72, f69–93, and f73–91/93/96) were present in high concentrations in Old Saare cheese from the very beginning of ripening (Table 3).

The C-terminal part of β-CN released a relatively low amount of peptides (Figure 4c). However, the cumulative concentrations of peptides from the regions β-CN f94–120 and f170–209 were similar to those from α_{S1}-CN and α_{S2}-CN parts of the sequences with the highest abundance of peptides (Figure 4). Proteolytic profiles of the regions β-CN f94–120 and f170–209 may be characterized by the primary action of cathepsin D supported by a possible action of proteolytic enzymes of *Lb. helveticus* and *St. thermophilus* on the bond Ser_{96}-Lys_{97} (Larsen et al., 1996; Sadat-Mekmene et al., 2011; Miclo et al., 2012), as well as by the initial activity of plasmin on the bonds Lys_{105}-Gln_{106} and Lys_{107}-Glu_{108} (Fox and McSweeney, 1996). We also found a pronounced cleavage at numerous in vitro indicated hydrolysis sites of the proteolytic enzymes of both mesophilic and thermophilic starters, Phe_{110}-Pro_{111}, Phe_{119}-Thr_{120}, Lys_{169}-Val_{170}, Gln_{175}-Lys_{176}, Gln_{182}-Arg_{183}, Ala_{189}-Phe_{190}, Phe_{190}-Leu_{191}, Leu_{191}-Leu_{192}, Leu_{192}-Tyr_{193}, and Tyr_{193}-Gln_{194} (Kunji et al., 1996; Sadat-Mekmene et al., 2011; Miclo et al., 2012; Figure 5c). The abovementioned cleavages led to the formation of the follow-

Table 3. List of the most abundant peptides (μmol/kg of cheese) derived from β-CN, α_{S1}-CN, and α_{S2}-CN detected in water-soluble extract of Old Saare by liquid chromatography-MS/MS[1]

Fragment	Ripening time					Groups
	1 d	1 mo	3 mo	5 mo	8 mo	
β-CN f7–16	16.1 ± 2.9	9.3 ± 1.9	6.1 ± 1.1	2.2 ± 1.3	1.5 ± 0.5	1
β-CN f7–28	26.8 ± 16.0	20.2 ± 12.1	44.0 ± 21.6	34.1 ± 16.3	20.4 ± 7.8	3
β-CN f11–28	0.6 ± 0.4	70.4 ± 39.2	261.6 ± 178.6	130.8 ± 63.9	99.8 ± 18.5	3
β-CN f11–29	0.2 ± 0.2	10.3 ± 5.0	89.3 ± 108.5	61.7 ± 37.4	22.8 ± 12.3	3
β-CN f12–28	1.0 ± 0.7	111.3 ± 40.2	180.2 ± 49.6	120.4 ± 82.5	59.9 ± 22.1	3
β-CN f12–29	0.3 ± 0.3	13.2 ± 6.2	54.5 ± 55.5	36.0 ± 19.2	18.6 ± 11.1	3
β-CN f13–28	0.0 ± 0.0	9.6 ± 4.7	122.1 ± 46.4	134.9 ± 47.0	188.2 ± 56.8	4
β-CN f14–29	0.0 ± 0.0	0.5 ± 0.8	72.7 ± 62.2	55.9 ± 21.7	44.9 ± 27.2	3
β-CN f15–25	0.2 ± 0.2	3.2 ± 1.1	45.4 ± 23.4	79.4 ± 58.2	119.5 ± 83.0	3
β-CN f15–27	0.8 ± 0.4	6.3 ± 2.5	30.7 ± 23.5	28.2 ± 30.1	20.2 ± 20.9	3
β-CN f15–28	2.4 ± 2.2	51.4 ± 41.5	499.5 ± 393.9	444.6 ± 424.6	464.0 ± 420.8	3
β-CN f15–29	0.1 ± 0.1	6.2 ± 5.7	129.6 ± 139.0	111.8 ± 125.4	107.5 ± 117.2	3
β-CN f16–24	0.1 ± 0.1	1.2 ± 0.1	34.7 ± 7.3	70.7 ± 25.9	181.8 ± 54.1	3
β-CN f16–25	0.3 ± 0.3	16.4 ± 2.4	151.8 ± 40.1	258.7 ± 16.1	382.0 ± 26.9	3
β-CN f16–27	1.5 ± 0.5	37.9 ± 13.4	112.1 ± 47.6	74.1 ± 25.4	63.0 ± 18.9	3
β-CN f16–28	3.0 ± 1.8	74.0 ± 22.1	477.6 ± 149.1	522.1 ± 83.3	631.8 ± 178.4	3
β-CN f17–25	0.1 ± 0.2	2.5 ± 1.8	76.3 ± 37.0	125.6 ± 58.9	200.6 ± 54.4	3
β-CN f17–27	3.1 ± 1.6	45.9 ± 9.2	240.3 ± 23.0	117.2 ± 30.9	94.7 ± 14.7	3
β-CN f17–28	17.4 ± 9.7	85.2 ± 58.3	783.6 ± 100.5	513.0 ± 41.1	579.6 ± 258.8	3
β-CN f17–29	0.4 ± 0.5	16.2 ± 9.2	95.9 ± 28.2	39.0 ± 8.0	21.7 ± 5.6	3
β-CN f18–25	0.0 ± 0.0	0.7 ± 0.1	12.9 ± 7.0	28.8 ± 15.4	39.9 ± 20.1	3
β-CN f29–46	8.3 ± 2.7	6.2 ± 2.5	17.7 ± 6.5	10.4 ± 2.7	8.6 ± 2.5	3
β-CN f29–52	42.7 ± 26.1	51.2 ± 31.1	53.0 ± 24.5	55.7 ± 36.4	23.8 ± 22.0	1
β-CN f30–36	16.9 ± 7.4	6.7 ± 4.5	2.2 ± 1.5	0.3 ± 0.7	1.3 ± 0.8	1
β-CN f30–40	20.2 ± 3.9	4.6 ± 0.5	9.2 ± 3.0	6.8 ± 5.1	6.1 ± 2.9	1
β-CN f30–46	7.9 ± 3.4	7.2 ± 3.3	14.2 ± 5.3	9.6 ± 1.1	10.9 ± 1.8	3
β-CN f30–52	17.2 ± 8.6	54.6 ± 16.3	77.7 ± 20.3	68.4 ± 19.8	47.4 ± 28.5	3
β-CN f32–40	4.3 ± 4.9	17.7 ± 6.7	38.4 ± 15.0	24.3 ± 15.9	27.2 ± 17.0	2
β-CN f32–43	5.9 ± 2.6	21.0 ± 3.2	27.2 ± 9.9	20.1 ± 1.7	30.0 ± 19.9	3
β-CN f32–46	5.7 ± 2.5	27.7 ± 15.0	68.3 ± 18.6	53.0 ± 22.1	54.2 ± 12.3	3
β-CN f32–52	10.7 ± 2.3	52.5 ± 9.3	68.4 ± 50.2	87.1 ± 32.1	78.8 ± 58.9	3
β-CN f33–40	6.2 ± 2.3	16.6 ± 3.6	62.8 ± 16.1	35.8 ± 28.5	51.6 ± 24.3	3
β-CN f33–42	5.1 ± 2.5	15.9 ± 4.8	43.0 ± 6.1	28.9 ± 7.8	39.1 ± 6.8	3
β-CN f33–43	7.4 ± 3.1	25.2 ± 5.1	52.1 ± 11.4	36.3 ± 9.0	54.0 ± 22.8	3
β-CN f33–46	5.6 ± 1.9	42.4 ± 3.6	66.6 ± 9.8	44.0 ± 28.4	72.4 ± 14.1	3
β-CN f33–52	7.5 ± 9.1	74.3 ± 27.0	151.0 ± 73.9	99.1 ± 54.8	82.3 ± 15.1	3
β-CN f34–46	4.0 ± 2.9	25.7 ± 3.4	52.2 ± 14.5	47.6 ± 18.1	49.4 ± 11.3	3
β-CN f34–52	54.9 ± 20.0	93.6 ± 42.4	219.2 ± 102.0	210.4 ± 78.4	136.2 ± 49.2	3
β-CN f34–56	15.8 ± 5.2	24.0 ± 2.1	36.7 ± 10.4	28.8 ± 12.4	19.9 ± 7.7	3
β-CN f35–52	24.7 ± 30.8	43.6 ± 18.4	166.1 ± 71.0	89.8 ± 36.4	104.8 ± 66.3	3
β-CN f39–52	2.1 ± 1.1	33.0 ± 10.6	47.7 ± 17.2	26.7 ± 11.9	26.2 ± 10.6	2
β-CN f40–52	17.2 ± 5.0	51.4 ± 42.7	82.7 ± 52.1	33.2 ± 31.4	44.6 ± 22.4	2
β-CN f41–52	58.0 ± 8.3	68.4 ± 27.6	127.6 ± 52.5	90.1 ± 72.8	96.9 ± 56.2	2
β-CN f43–52	45.9 ± 28.8	26.6 ± 5.9	43.3 ± 17.6	17.0 ± 7.5	18.0 ± 1.4	1
β-CN f44–52	20.4 ± 3.0	30.3 ± 3.9	8.9 ± 3.6	9.4 ± 1.3	9.9 ± 1.2	2
β-CN f45–52	3.8 ± 2.7	15.6 ± 3.2	13.2 ± 9.6	7.7 ± 2.1	13.2 ± 6.5	2
β-CN f46–52	0.6 ± 0.4	5.5 ± 1.0	81.0 ± 17.8	26.0 ± 32.2	76.3 ± 5.8	3
β-CN f47–52	0.3 ± 0.2	3.5 ± 1.2	55.1 ± 9.3	22.4 ± 27.0	39.4 ± 4.2	3
β-CN f57–72	3.8 ± 3.1	31.9 ± 12.1	35.7 ± 7.3	16.4 ± 3.5	15.3 ± 1.1	3
β-CN f58–68 (A^2, A^3)	2.8 ± 2.5	31.6 ± 9.6	49.2 ± 13.1	22.8 ± 2.2	16.6 ± 3.8	3
β-CN f58–72 (A^1, B)	3.4 ± 2.8	36.5 ± 12.4	28.2 ± 7.9	16.0 ± 3.5	13.1 ± 1.8	2
β-CN f59–72 (A^1, B)	3.8 ± 2.8	41.0 ± 17.7	60.3 ± 9.4	21.3 ± 6.5	15.6 ± 8.5	2
β-CN f59–68 (A^2, A^3)	4.3 ± 3.7	52.0 ± 14.2	60.5 ± 11.8	29.2 ± 3.2	23.0 ± 5.5	2
β-CN f60–68 (A^2, A^3)	16.5 ± 10.4	45.2 ± 15.1	42.9 ± 9.4	12.1 ± 10.1	8.0 ± 3.4	2
β-CN f60–69 (A^1, B)	17.4 ± 7.5	27.7 ± 6.0	16.4 ± 6.1	9.4 ± 3.5	10.1 ± 2.6	2
β-CN f60–72 (A^1, B)	63.4 ± 35.2	133.8 ± 44.7	108.3 ± 32.5	42.0 ± 11.2	60.8 ± 25.6	2
β-CN f60–72 (A^2, A^3)	11.1 ± 4.6	23.0 ± 3.9	18.8 ± 12.2	7.9 ± 8.1	4.7 ± 6.9	1
β-CN f62–72 (A^1, B)	11.2 ± 2.8	24.5 ± 9.2	21.6 ± 8.7	8.0 ± 1.7	11.7 ± 2.0	2
β-CN f64–72 (A^1, B)	30.8 ± 2.4	175.4 ± 36.0	131.9 ± 35.3	50.2 ± 3.8	56.8 ± 9.0	2
β-CN f69–93	49.7 ± 9.8	55.3 ± 8.7	97.4 ± 30.0	68.3 ± 11.9	65.6 ± 10.4	3
β-CN f72–93	1.0 ± 1.0	30.6 ± 8.9	47.7 ± 29.4	12.3 ± 3.9	15.6 ± 15.0	2
β-CN f73–91	28.6 ± 5.8	67.8 ± 8.3	60.3 ± 21.7	37.6 ± 14.1	25.2 ± 10.7	2
β-CN f73–93	103.0 ± 32.5	145.3 ± 23.9	191.1 ± 28.4	124.9 ± 23.5	109.3 ± 31.8	2

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Table 3 (Continued). List of the most abundant peptides (μmol/kg of cheese) derived from β-CN, α_{S1}-CN, and α_{S2}-CN detected in water-soluble extract of Old Saare by liquid chromatography-MS/MS[1]

Fragment	Ripening time					Groups
	1 d	1 mo	3 mo	5 mo	8 mo	
β-CN f73–96	56.0 ± 16.5	41.5 ± 17.0	45.0 ± 21.9	28.8 ± 19.3	19.6 ± 14.9	1
β-CN f74–91	5.2 ± 1.4	61.0 ± 7.4	39.7 ± 16.1	9.4 ± 5.9	6.3 ± 3.3	2
β-CN f74–93	7.9 ± 1.4	132.7 ± 39.6	82.9 ± 29.3	26.0 ± 12.9	19.7 ± 9.7	2
β-CN f74–96	2.0 ± 1.3	16.8 ± 7.7	19.6 ± 15.7	6.6 ± 4.6	4.8 ± 4.0	2
β-CN f77–93	4.3 ± 2.4	37.8 ± 9.3	40.8 ± 9.1	18.1 ± 3.8	18.3 ± 2.8	2
β-CN f78–88	5.6 ± 2.5	55.2 ± 5.2	44.9 ± 4.5	30.4 ± 9.9	18.1 ± 6.5	2
β-CN f78–91	3.8 ± 1.3	39.9 ± 3.5	15.0 ± 12.6	18.8 ± 7.9	11.5 ± 8.6	2
β-CN f78–93	12.2 ± 4.4	74.2 ± 6.6	80.1 ± 19.8	47.8 ± 11.3	45.0 ± 18.3	2
β-CN f79–88	4.6 ± 0.6	42.9 ± 6.3	59.5 ± 9.3	29.9 ± 9.1	19.0 ± 9.5	2
β-CN f79–93	8.8 ± 2.2	42.9 ± 17.1	52.5 ± 22.7	32.8 ± 25.4	39.7 ± 26.2	3
β-CN f80–88	12.2 ± 5.3	34.4 ± 12.0	50.5 ± 23.1	14.2 ± 3.5	13.7 ± 6.3	2
β-CN f80–93	18.1 ± 2.4	38.0 ± 5.9	32.9 ± 9.3	9.6 ± 2.3	6.4 ± 2.8	2
β-CN f82–88	2.1 ± 1.4	64.2 ± 13.2	108.8 ± 33.5	26.9 ± 15.0	21.8 ± 8.7	2
β-CN f82–90	0.3 ± 0.2	18.3 ± 2.7	36.0 ± 6.1	9.5 ± 3.3	5.8 ± 2.3	2
β-CN f82–93	7.7 ± 5.2	25.7 ± 3.8	29.8 ± 8.2	12.1 ± 3.9	10.9 ± 4.3	2
β-CN f83–88	2.3 ± 0.2	44.1 ± 7.3	46.1 ± 12.5	11.8 ± 3.9	12.1 ± 5.0	2
β-CN f83–90	0.4 ± 0.2	28.1 ± 1.8	27.6 ± 9.0	7.2 ± 2.0	6.3 ± 3.3	2
β-CN f84–93	8.5 ± 1.7	33.7 ± 4.5	27.6 ± 7.0	10.0 ± 1.1	12.8 ± 3.1	2
β-CN f94–101	68.7 ± 36.0	34.2 ± 6.3	18.0 ± 10.5	8.2 ± 5.9	6.6 ± 3.6	1
β-CN f94–105	59.6 ± 50.9	18.7 ± 10.9	5.6 ± 2.7	3.2 ± 1.3	7.4 ± 4.6	1
β-CN f97–105	36.2 ± 21.7	10.5 ± 8.6	14.1 ± 5.0	3.3 ± 1.0	5.1 ± 3.1	1
β-CN f106–119 (A^2)	14.0 ± 5.0	87.1 ± 29.2	35.9 ± 10.6	27.8 ± 11.3	46.9 ± 3.6	4
β-CN f108–119	31.3 ± 16.1	131.3 ± 38.2	77.5 ± 16.6	62.1 ± 25.7	112.2 ± **24**.8	4
β-CN f109–119	75.5 ± 25.9	82.2 ± 21.5	43.4 ± 8.5	16.3 ± 9.1	43.9 ± 19.3	4
β-CN f111–119	110.9 ± 12.3	116.7 ± 24.1	43.8 ± 13.0	33.5 ± 3.9	77.2 ± 11.5	4
β-CN f120–128 (A^1, A^2, A^3)	25.2 ± 2.3	6.8 ± 1.5	3.4 ± 1.4	2.6 ± 1.7	6.7 ± 2.9	1
β-CN f129–136	15.5 ± 7.4	1.8 ± 0.3	0.5 ± 0.5	1.5 ± 0.9	1.8 ± 0.9	1
β-CN f129–141	24.0 ± 9.2	16.0 ± 6.0	18.8 ± 3.5	15.7 ± 4.4	29.8 ± 4.2	4
β-CN f170–175	4.6 ± 5.7	32.1 ± 12.0	28.7 ± 11.9	19.4 ± 5.6	68.2 ± 24.1	4
β-CN f177–189	7.6 ± 0.9	6.8 ± 0.9	10.9 ± 8.1	19.3 ± 7.0	43.5 ± 14.1	4
β-CN f178–189	14.2 ± 5.5	9.5 ± 0.7	17.7 ± 4.3	17.0 ± 8.2	48.4 ± 10.5	4
β-CN f183–190	7.1 ± 2.3	12.8 ± 4.7	13.3 ± 10.3	10.0 ± 8.7	56.1 ± 15.8	4
β-CN f191–209	7.1 ± 0.9	10.7 ± 4.5	17.2 ± 4.4	19.7 ± 1.9	30.6 ± 6.0	3
β-CN f193–206	3.6 ± 1.1	27.9 ± 9.0	32.4 ± 10.1	20.8 ± 2.8	68.4 ± 6.0	4
β-CN f193–209	43.2 ± 5.2	90.5 ± 41.8	88.4 ± 68.6	87.5 ± 53.0	101.3 ± 92.8	3
β-CN f194–206	9.3 ± 2.0	25.3 ± 7.8	14.3 ± 4.5	10.2 ± 2.0	36.7 ± 3.0	4
β-CN f194–209	71.7 ± 11.4	121.8 ± 27.8	149.6 ± 27.5	84.0 ± 17.5	223.0 ± 35.9	4
β-CN f195–209	41.5 ± 12.9	35.3 ± 6.6	32.3 ± 4.4	12.7 ± 2.4	25.7 ± 5.0	4
α_{S1}-CN f1–7	27.5 ± 26.3	6.7 ± 2.1	3.4 ± 5.7	0.0 ± 0.0	0.3 ± 0.6	1
α_{S1}-CN f1–13	16.8 ± 4.2	5.9 ± 4.0	0.8 ± 1.6	0.0 ± 0.0	0.8 ± 1.5	1
α_{S1}-CN f7–13	4.7 ± 1.6	33.3 ± 23.1	34.9 ± 14.5	11.3 ± 2.3	8.8 ± 3.4	2
α_{S1}-CN f7–14	5.5 ± 2.3	56.3 ± 6.1	40.2 ± 19.0	15.9 ± 1.8	13.6 ± 4.3	2
α_{S1}-CN f8–14	50.9 ± 14.2	76.8 ± 10.2	56.1 ± 43.0	22.5 ± 13.9	55.7 ± 15.5	2
α_{S1}-CN f8–16	0.5 ± 0.3	45.0 ± 28.8	39.8 ± 34.4	14.6 ± 7.0	46.7 ± 18.1	4
α_{S1}-CN f8–22	8.4 ± 4.6	36.0 ± 17.6	32.2 ± 10.2	21.5 ± 10.0	36.7 ± 14.3	2
α_{S1}-CN f8–23	16.9 ± 1.0	37.6 ± 20.6	70.7 ± 14.1	69.0 ± 7.3	56.0 ± 48.1	3
α_{S1}-CN f9–23	15.6 ± 8.9	23.1 ± 10.9	19.4 ± 5.5	16.4 ± 6.8	26.2 ± 10.7	4
α_{S1}-CN f10–23	39.4 ± 23.1	39.3 ± 17.1	61.5 ± 31.7	55.3 ± 28.7	100.6 ± 66.2	4
α_{S1}-CN f10–34	8.1 ± 3.6	8.1 ± 5.3	18.3 ± 16.7	31.2 ± 19.5	44.7 ± 47.9	3
α_{S1}-CN f14–22	44.2 ± 19.7	45.9 ± 15.3	35.3 ± 15.8	21.2 ± 10.6	42.6 ± 33.4	2
α_{S1}-CN f14–23	61.0 ± 17.1	95.8 ± 20.9	129.2 ± 20.8	84.8 ± 7.1	107.0 ± 19.1	4
α_{S1}-CN f15–22	30.4 ± 10.5	66.3 ± 11.8	57.3 ± 21.1	26.2 ± 10.6	43.2 ± 28.0	2
α_{S1}-CN f15–23	48.4 ± 7.2	91.3 ± 15.4	118.3 ± 19.2	200.7 ± 255.5	107.8 ± 12.0	3
α_{S1}-CN f16–22	30.4 ± 3.5	50.1 ± 5.3	15.9 ± 10.0	5.0 ± 3.8	9.2 ± 9.7	2
α_{S1}-CN f16–23	15.5 ± 10.6	39.7 ± 4.2	48.2 ± 21.9	11.3 ± 4.5	22.8 ± 5.7	2
α_{S1}-CN f17–22	62.0 ± 6.4	58.4 ± 22.6	32.8 ± 12.4	12.0 ± 0.9	19.3 ± 13.8	1
α_{S1}-CN f17–23	60.0 ± 15.6	111.4 ± 28.2	110.2 ± 100.1	40.3 ± 22.3	63.7 ± 3.8	2
α_{S1}-CN f17–24	15.2 ± 5.2	15.8 ± 4.7	14.6 ± 1.4	6.2 ± 0.5	12.4 ± 2.2	1
α_{S1}-CN f18–23	17.2 ± 2.6	14.9 ± 5.5	20.5 ± 21.3	4.8 ± 2.4	9.9 ± 3.9	1
α_{S1}-CN f24–30	19.7 ± 10.0	71.4 ± 23.8	72.1 ± 39.7	54.1 ± 16.4	59.3 ± 19.7	2
α_{S1}-CN f24–34	47.3 ± 6.3	43.0 ± 31.0	87.1 ± 18.9	97.2 ± 33.9	100.2 ± 35.0	3
α_{S1}-CN f24–36	16.5 ± 7.2	12.5 ± 3.9	33.3 ± 5.9	37.1 ± 10.2	49.0 ± 2.5	3
α_{S1}-CN f25–30	23.0 ± 5.2	62.0 ± 15.3	60.6 ± 12.0	49.8 ± 33.4	53.5 ± 22.6	2

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Table 3 (Continued). List of the most abundant peptides (μmol/kg of cheese) derived from β-CN, α_{S1}-CN, and α_{S2}-CN detected in water-soluble extract of Old Saare by liquid chromatography-MS/MS[1]

Fragment	Ripening time					Groups
	1 d	1 mo	3 mo	5 mo	8 mo	
α_{S1}-CN f25–32	10.3 ± 3.5	19.9 ± 5.4	32.1 ± 6.1	13.4 ± 4.1	25.4 ± 8.5	2
α_{S1}-CN f25–34	60.7 ± 11.0	31.9 ± 5.3	95.7 ± 29.0	66.9 ± 12.1	99.2 ± 8.5	4
α_{S1}-CN f26–34	15.7 ± 5.1	2.8 ± 0.4	5.5 ± 1.8	1.5 ± 0.7	4.5 ± 1.5	1
α_{S1}-CN f33–40	21.4 ± 9.6	52.7 ± 21.1	32.9 ± 6.4	13.1 ± 6.6	15.2 ± 2.5	2
α_{S1}-CN f34–40	0.4 ± 0.7	30.1 ± 6.8	31.9 ± 21.2	10.4 ± 1.5	12.7 ± 3.7	2
α_{S1}-CN f41–60	1.6 ± 1.0	25.4 ± 12.8	43.0 ± 25.7	31.2 ± 1.7	38.5 ± 14.4	3
α_{S1}-CN f42–54	0.3 ± 0.2	5.9 ± 5.0	49.9 ± 29.5	32.7 ± 15.8	64.4 ± 30.6	3
α_{S1}-CN f42–60	0.4 ± 0.1	11.4 ± 11.0	62.4 ± 33.8	30.7 ± 8.7	31.0 ± 12.6	3
α_{S1}-CN f44–58	0.1 ± 0.1	1.3 ± 0.7	11.5 ± 4.2	16.4 ± 5.1	31.4 ± 6.7	4
α_{S1}-CN f45–58	0.3 ± 0.3	10.3 ± 2.3	47.4 ± 34.9	33.7 ± 5.2	56.1 ± 37.6	4
α_{S1}-CN f46–58	0.1 ± 0.1	9.5 ± 0.4	58.7 ± 16.3	51.1 ± 9.0	58.4 ± 18.2	3
α_{S1}-CN f46–60	0.0 ± 0.0	12.1 ± 4.9	31.1 ± 32.8	27.4 ± 23.3	22.5 ± 21.9	4
α_{S1}-CN f65–74	0.0 ± 0.0	0.5 ± 0.4	16.4 ± 2.4	68.1 ± 33.6	103.9 ± 21.1	4
α_{S1}-CN f65–79	0.0 ± 0.0	1.1 ± 0.4	14.5 ± 9.7	17.5 ± 4.6	29.0 ± 13.3	4
α_{S1}-CN f67–79	0.0 ± 0.1	5.8 ± 1.8	26.7 ± 28.0	68.9 ± 19.6	152.8 ± 32.0	4
α_{S1}-CN f75–82	0.5 ± 0.3	19.5 ± 7.0	39.8 ± 2.6	15.8 ± 5.0	23.8 ± 12.1	3
α_{S1}-CN f80–98	0.7 ± 0.2	2.5 ± 0.5	8.2 ± 6.2	24.0 ± 8.2	52.8 ± 21.7	4
α_{S1}-CN f85–93	12.1 ± 1.7	62.3 ± 41.5	25.1 ± 30.0	24.8 ± 22.7	23.9 ± 18.7	2
α_{S1}-CN f105–124	1.3 ± 0.4	6.7 ± 1.4	6.4 ± 3.4	13.3 ± 4.8	64.7 ± 18.0	4
α_{S1}-CN f115–121	9.6 ± 8.1	30.3 ± 23.0	67.2 ± 40.1	6.3 ± 6.5	3.0 ± 0.6	2
α_{S1}-CN f115–122	6.3 ± 7.2	26.8 ± 13.5	79.0 ± 5.8	47.7 ± 8.6	55.6 ± 62.7	3
α_{S1}-CN f131–142	7.2 ± 4.0	33.9 ± 6.9	11.0 ± 3.4	3.1 ± 0.6	3.1 ± 1.5	2
α_{S1}-CN f180–199 (B)	25.3 ± 7.7	3.6 ± 0.8	3.2 ± 2.0	5.4 ± 2.3	10.6 ± 7.8	1
α_{S2}-CN f100–114	11.0 ± 1.9	18.3 ± 3.4	25.0 ± 2.3	26.8 ± 8.6	46.4 ± 6.4	4
α_{S2}-CN f100–115	10.8 ± 6.8	25.8 ± 10.4	26.1 ± 8.4	16.7 ± 4.2	27.0 ± 4.2	4
α_{S2}-CN f101–114	31.7 ± 3.6	23.7 ± 4.0	19.6 ± 9.8	21.5 ± 3.1	51.5 ± 7.1	4
α_{S2}-CN f101–115	19.8 ± 2.7	16.5 ± 6.1	18.5 ± 5.6	8.5 ± 0.8	13.2 ± 1.1	4
α_{S2}-CN f125–140 (A)	1.4 ± 1.4	1.7 ± 0.8	17.4 ± 11.1	8.3 ± 3.7	17.4 ± 7.6	3
α_{S2}-CN f128–137 (A)	2.0 ± 2.4	5.8 ± 0.7	40.8 ± 7.5	47.6 ± 18.1	83.4 ± 18.7	3
α_{S2}-CN f128–138 (A)	5.3 ± 6.1	4.8 ± 2.9	19.0 ± 15.5	47.5 ± 3.4	69.0 ± 8.4	3
α_{S2}-CN f128–140 (A)	3.0 ± 3.3	8.0 ± 3.4	53.3 ± 23.3	54.8 ± 30.9	74.1 ± 22.7	3
α_{S2}-CN f128–142 (A)	2.9 ± 2.6	6.4 ± 2.5	28.9 ± 8.4	36.0 ± 3.3	36.2 ± 20.9	3
α_{S2}-CN f129–142 (A)	17.2 ± 8.6	27.2 ± 10.1	100.6 ± 35.8	66.0 ± 11.6	47.6 ± 29.4	3
α_{S2}-CN f150–156	17.6 ± 9.0	3.7 ± 1.3	15.1 ± 13.6	7.9 ± 4.7	17.9 ± 5.5	3
α_{S2}-CN f151–162	19.5 ± 4.5	8.4 ± 3.9	13.1 ± 1.6	15.3 ± 3.2	25.0 ± 6.1	4
α_{S2}-CN f152–162	41.7 ± 21.4	23.3 ± 7.5	19.4 ± 5.5	44.3 ± 38.3	33.1 ± 13.0	1
α_{S2}-CN f153–161	29.6 ± 13.8	8.5 ± 3.4	11.4 ± 6.5	4.3 ± 3.4	12.7 ± 13.3	1
α_{S2}-CN f153–162	94.1 ± 35.0	44.8 ± 15.8	85.8 ± 60.6	34.8 ± 21.3	80.3 ± 43.6	1
α_{S2}-CN f175–182	13.1 ± 2.8	35.2 ± 6.2	104.1 ± 38.6	56.0 ± 14.8	73.3 ± 26.3	3
α_{S2}-CN f176–182	21.3 ± 4.5	12.2 ± 2.4	31.9 ± 11.1	21.0 ± 8.0	36.0 ± 10.7	3
α_{S2}-CN f187–197	29.1 ± 14.5	31.0 ± 21.7	21.1 ± 18.8	18.5 ± 4.7	40.6 ± 21.4	4
α_{S2}-CN f189–197	17.2 ± 6.0	24.9 ± 6.7	16.6 ± 10.4	10.2 ± 5.2	24.7 ± 21.1	4
α_{S2}-CN f191–197	35.4 ± 6.9	14.1 ± 8.0	6.3 ± 3.6	3.0 ± 2.1	18.4 ± 14.9	1

[1]Values are the means of estimated concentrations of duplicated samples from each of 2 cheesemaking trials (mean ± SD, n = 4). Potential phosphopeptides determined by MaxQuant (version 1.5.2.8; Cox and Mann, 2008) search based on MS/MS spectra are marked in bold. The MaxQuant search was performed against casein sequences of the following genetic variants: A^1, A^2, A^3, and B for β-CN; A, B, and C for α_{S1}-CN; and A and C for α_{S2}-CN. The peptides marked in italics could be derived from the genetic variant A of α_{S1}-CN with the different index numbers of AA, as AA 14 to 26 are deleted from the sequence. Numbers in the last column refer to the groups showing similar evolutionary trends of peptides according to the clustering analysis.

ing peptides determined in considerable amounts: β-CN f106/108/111–119 and f193/194–206/209 (Table 3).

A very low concentration of peptides was released from the region β-CN f120–170 (Figure 4c). This was in agreement with the observation by Gagnaire et al. (2001), who have indicated the absence of peptides among the most abundant ones in even a wider region of β-CN sequence (f108–192) in the case of Emmental cheese. Gagnaire et al. (2001) have explained the lack of hydrolysis of the preferred in vitro cleavage sites by the difference of the physicochemical conditions in cheese changing the specificity of proteolytic enzymes and the different accessibility of cleavage sites of caseins in cheese protein matrix in comparison with in vitro conditions, as proposed by Exterkate et al. (1997). Nevertheless, the full analysis of MS/MS profiles in our study showed that peptides were released from all the regions of the sequence of β-CN including the region

f120–170 (Supplemental Figure S1c, https://doi.org/10.3168/jds.2017-12944). The latter region did not contain plasmin hydrolysis sites, but has been shown to include some in vitro cleavage sites of bacterial proteases [e.g., Thr_{128}-Asp_{129} and Gln_{141}-Ser_{142} (Kunji et al., 1996; Sadat-Mekmene et al., 2011; Miclo et al., 2012)], which were found cleaved in Old Saare cheese (Figure 5c). We even determined a few peptides (i.e., β-CN f120–128 and f129–141), formed in high concentrations presumably in the result of the cleavage of aforementioned sites (Table 3).

Several peptides of β-CN shown to have bitter taste in Gouda cheese (Toelstede and Hofmann, 2008) were present also in Old Saare cheese in substantial amounts (e.g., peptides f59–68, f60–68/69, and f193–209). The latter peptide was detected in the highest concentration in comparison with the other bitter peptides and accumulated in the end of 8 mo ripening. In contrast, the content of the former peptides was the highest at the beginning of ripening, and they were degraded afterward.

α_{S1}-CN-Derived Small Peptides. In case of α_{S1}-CN, the majority of peptides arose from the N-terminal part of the protein, f1–124 (Figure 4a). Fewer peptides in much lower amounts originated from the C-terminal part of the sequence, f125–199 (Supplemental Figure S1a, https://doi.org/10.3168/jds.2017-12944).

The peptides from the region f1–23 comprised approximately 49% of all peptides formed from α_{S1}-CN in the 1-d-old cheese. Further, their relative content decreased gradually up to 23% in the 4-mo-old cheese, remaining rather constant until the end of the ripening. It means that the α_{S1}-CN f1–23 region was preferably hydrolyzed in Old Saare cheese compared with the rest of the protein sequence at the beginning of ripening, similarly to the ripening of Emmental (Gagnaire et al., 2001; Sadat-Mekmene et al., 2013). The high content of peptides from region f1–23 (Figure 4a) could be considered as an indication of high activity of chymosin in proteolysis of α_{S1}-CN, cleaving the preferable bond Phe_{23}-Phe_{24}, and subsequent hydrolysis by the microbial enzymes of the resulting peptides (Fox and McSweeney, 1996). However, taking into account the presumably high thermal inactivation of chymosin during the cooking of cheese grains, the initial formation of α_{S1}-CN f1–23 can be assigned also to the action of cathepsin D (Larsen et al., 1996). Indeed, a more detailed analysis of the cleavage patterns supported the assumption that the indigenous milk proteinases (cathepsin D and plasmin) may participate in the sequential hydrolysis of the N-terminus of α_{S1}-CN (Figure 5a). Thus, we assigned the production of particular abundant peptides (e.g., f1–7 and f8–22), and the cutting in the N-terminus of the peptides f25–30/32/34 (Table 3), to an evident hydrolysis of the possible sites of plasmin: Lys_3-His_4, Lys_7-His_8, and Arg_{22}-Phe_{23} (McSweeney et al., 1993b), as well as of another preferable bond of cathepsin D: Phe_{24}-Val_{25} (Larsen et al., 1996).

We observed a high total concentration of released peptides from the N-terminal region of α_{S1}-CN, f1-40, at the beginning of the ripening, followed by a subsequent gradual increase of their contents (Figure 4a). However, only few peptides from this region among the numerous abundant ones could be characterized by a clear accumulating trend by the end of the ripening (i.e., f10-23/34 and f24-34/36; Table 3). The majority of the peptides revealed a fluctuating behavior indicating their simultaneous and unbalanced production and digestion to smaller degradation products during ripening. The oligopeptides f1-22/23/24, originating from primary hydrolysis of α_{S1}-CN by plasmin, cathepsin D, and residual chymosin, could have been degraded to smaller fragments by the cleavage of the bonds Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, Gln_{14}-Val_{15}, and Leu_{16}-Asn_{17} (Figure 5a) by CEP of *Lc. lactis* (Exterkate and Alting, 1993), resulting in the liberation of peptides f1-13, f10-23, f14/15-22/23, and 17-22/23/24 in high amounts already in the 1-d-old cheese (Table 3). The latter peptides were likely degraded further in Old Saare cheese by the proteolytic enzymes of starter bacteria containing also thermophilic in addition to mesophilic strains, differently from cheeses produced only with mesophilic starters (e.g., Cheddar and Västerbottenost), where the accumulation of peptides released by CEP of lactococci has been reported (Broadbent et al., 2002; Rehn et al., 2010). The CEP of *Lb. helveticus* have shown broader proteolytic activity on the N-terminus of α_{S1}-CN compared with CEP of *Lc. lactis*, acting on bonds Ile_6-Lys_7, Lys_7-His_8, His_8-Gln_9 in addition to all reported lactococcal preferable cleavage sites (Sadat-Mekmene et al., 2011). The proteolytic system of *St. thermophilus* could also have been involved in the hydrolysis of these bonds (Miclo et al., 2012). Furthermore, the proteolytic enzymes of *Lb. helveticus* and *St. thermophilus* have been shown to hydrolyze also the positions Glu_{30}-Val_{31}, Phe_{32}-Gly_{33}, and Leu_{40}-Ser_{41} (Sadat-Mekmene et al., 2011; Miclo et al., 2012), competing with chymosin for the latter 2 bonds (Exterkate et al., 1997). Accordingly, we associated the extensive cleavage of the aforementioned sites (Figure 5a) and the formation of corresponding small peptides f7-13/14 and the C-terminus of f24/25-30, f25-32, f33/34-40 to a high proteolytic activity of a thermophilic starter cultures found in higher relative abundance in Old Saare cheese (L. Blank, TUT, Tallinn, Estonia, personal communication). Additionally, we may relate the location of C-termini of the abundant

peptides f10/24/25/26-34 and f24-36 (Table 3) to the action of plasmin on the bonds Lys_{34}-Glu_{35} and Lys_{36}-Val_{37} (McSweeney et al., 1993b).

The next parts of the sequence, α_{S1}-CN f41–63 and f65–79 (Figure 4a), containing phosphorylated sites at Ser_{41}, Ser_{46}, Ser_{48}, Thr_{49} and Ser_{66}, Ser_{67}, Ser_{68}, Ser_{75}, showed the release of the numerous phosphopeptides accumulating mostly by the end of cheese ripening (e.g., f41/42/46–60, f44/45/46–58, f65–74/79, f67–79; Table 3). We observed an extensive cleavage in these regions of α_{S1}-CN as expected (Figure 5a). However, we could assign only a few hydrolysis sites to the activity of the particular proteinases: Lys_{42}-Asp_{43}, Lys_{58}-Gln_{59}, and Lys_{79}-His_{80} have been reported as possible cleavage sites of plasmin (McSweeney et al., 1993b), and the bond Asn_{74}-Ser_{75} has been shown to be cut by CEP of *Lc. lactis* (Kunji et al., 1996). The successive cutting patterns in the regions f41–49 and f64–72, and f58–62 demonstrated the presence of mainly either the N-terminal or only the C-terminal AA of peptides in the consecutive positions, respectively (Figure 5a). Therefore, we suggested that amino- and carboxypeptidases of LAB could have been likely involved in the formation of peptides from the respective regions. Interestingly, the phosphopeptides only from the narrower region of α_{S1}-CN, f61–79, have been identified in Grana Padano cheese (Ferranti et al., 1997a).

The central part of α_{S1}-CN, f80–124, was extensively hydrolyzed by different proteolytic enzymes in Old Saare cheese (Figure 5a). Plasmin could have cleaved this region at 7 sites: Lys_{79}-His_{80}, Arg_{90}-Tyr_{91}, Arg_{100}-Leu_{101}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Arg_{119}-Leu_{120}, and Lys_{124}-Glu_{125} (McSweeney et al., 1993b); chymosin at Leu_{98}-Leu_{99} and Leu_{101}-Lys_{102} (Exterkate et al., 1997); cathepsin D at Leu_{98}-Leu_{99} (Larsen et al., 1996); CEP of lactococci at Glu_{84}-Asp_{85}, Ser_{88}-Glu_{89}, Leu_{98}-Leu_{99}, and His_{121}-Ser_{122} (Kunji et al., 1996); CEP of *Lb. helveticus* at 17 sites (Sadat-Mekmene et al., 2011); and the enzymes of *St. thermophilus* at 13 sites (Miclo et al., 2012). Besides, the consecutive splitting pattern in the regions f97–101 and f119–124 may indicate a possible involvement of bacterial carboxypeptidases in the proteolysis of the central part of α_{S1}-CN (Figure 5a). Thus, the intensive cutting in this region resulted in the formation of abundant peptides α_{S1}-CN f85–93, f105–124, and f115–121/122 (Table 3).

We observed a moderate hydrolysis in the regions α_{S1}-CN f131–142 and f180–199 (Figure 4a, Supplemental Figure S1a, https://doi.org/10.3168/jds.2017-12944) that could be associated with the action of enzymes of mesophilic and thermophilic LAB at the ends of these regions (Kunji et al., 1996; Sadat-Mekmene et al., 2011; Miclo et al., 2012), as well as of chymosin on the bonds Leu_{142}-Ala_{143} and Phe_{179}-Ser_{180} (McSweeney et al., 1993a). We detected a very low hydrolysis in the region α_{S1}-CN f145–175 in Old Saare cheese (Figure 4a, Figure 5a, Supplemental Figure S1a, https://doi.org/10.3168/jds.2017-12944), although chymosin has been reported to have potential cleavage sites at Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160}, and Trp_{164}-Tyr_{165} (McSweeney et al., 1993a) as well as proteolytic enzymes of LAB could have also cut this region based on in vitro studies (Monnet et al., 1992; Sadat-Mekmene et al., 2011; Miclo et al., 2012). Our results were in agreement with the results obtained by Exterkate et al. (1997), who reported that chymosin has failed to cleave bonds in the C-terminal part of α_{S1}-CN f129–199 in Gouda cheese due to the specific structural arrangement of the α_{S1}-CN molecules in cheese, which could also be a reason for the worse susceptibility of the C-terminus of α_{S1}-CN to chymosin and proteolytic enzymes of LAB in Old Saare cheese.

It is worth mentioning that the observed occurrence of several hydrolysis sites of plasmin (Figure 5a), some specific and some overlapping with other proteinases, was an indication of its possible important role in the degradation of α_{S1}-CN in Old Saare cheese. This conclusion is supported by the fact that the high cooking temperature of curd grains (52°C) activated the enzyme, and increasing pH during the ripening of Old Saare cheese (Table 1) became more favorable for plasmin action.

α_{S2}-CN-Derived Small Peptides. Lower overall content of water-soluble peptides derived from α_{S2}-CN was in agreement with a lower abundance of this casein in cheese (Figure 4). Furthermore, a slower release of α_{S2}-CN-derived peptides compared with those from other caseins has been presumably associated with its lower accessibility to proteolytic enzymes within casein matrix in cheese (Farrell et al., 2009).

The peptides originating from the N-terminal region f1–24 and released in the result of primary action of plasmin on the preferable bonds Lys_{21}-Gln_{22} and Lys_{24}-Asn_{25} (Le Bars and Gripon, 1989), comprised only approximately 1% of all α_{S2}-CN derived peptides in the 1-d-old cheese and reached the maximum relative content of 10% in the 4-mo-old cheese. Peptides mainly from the phosphorylated region f6–21, with phosphate groups at Ser_8, Ser_9, Ser_{10}, Ser_{13}, Ser_{16}, and Thr_{19}, accumulated to some relatively low amounts in the cheese (Figure 4b). The peptide bond Glu_5-His_6 has been shown to be hydrolyzed by CEP of *Lb. helveticus* (Sadat-Mekmene et al., 2011) and proteolytic system of *St. thermophilus* (Miclo et al., 2012). Phosphopeptides in the N-terminal α_{S2}-CN region (i.e., f7–18/19/21) have been also found in Grana Padano cheese in the study of Ferranti et al. (1997a), where the authors have speculated that carboxypeptidases are involved in the

production of these peptides. According to our results, the cleavage pattern of the N-terminal region of α_{S2}-CN may also suggest a possible action of aminopeptidases as well as carboxypeptidases on the respective regions f5–14 and f15–21 (Figure 5b), where notable cleavage occurred at every bond either in the N- or C-terminal ends of peptides.

We found a very low hydrolysis and a low total amount of peptides released from the region α_{S2}-CN f25–80 (Figure 4b and Figure 5b). Although the MS/MS analysis determined some phosphopeptides with phosphorylation at Ser_{31}, Ser_{52}, Ser_{56}, Ser_{57}, Ser_{58}, Ser_{61}, and Thr_{66}, we did not notice any accumulation of phosphopeptides from this region, unlike in the case with other highly phosphorylated sequences in β-CN and α_{S1}-CN. This revealed that in this region chymosin and plasmin could not release oligopeptides during primary hydrolysis of α_{S2}-CN. The lack of cleavage in this part of the molecule agreed with 3-D model of α_{S2}-CN developed by Farrell et al. (2009) locating the cleavage sites of proteolytic enzymes mainly in the most surface-accessible portions of the molecule, the relatively hydrophobic and highly positively charged C-terminal half of the protein.

So, the peptides derived from the α_{S2}-CN determined in our study originated mainly from the C-terminal part of the sequence (Figure 4b). Accordingly, the most prevalent peptides from α_{S2}-CN derived from the following regions: f100–115, f125–142, f150–162, f175–182, and f187–197 (Table 3, Figure 4b). Four phosphopeptides, f128–137/138/140/142, phosphorylated at positions Ser_{129}, Thr_{130}, Ser_{131}, Ser_{135}, and Thr_{138}, accumulated by the end of the ripening and showed high concentrations among other α_{S2}-CN derived peptides (Table 3). The α_{S2}-CN has been considered to be a good substrate for plasmin (Fox and McSweeney, 1996). Many of the major cleavage sites in the α_{S2}-CN sequence: Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{150}-Thr_{151}, Lys_{188}-Asp_{189}, Lys_{197}-Thr_{198} (Figure 5b), involved in the extensive formation of peptides in the C-terminus of the α_{S2}-CN, may be related to plasmin action (Le Bars and Gripon, 1989). However, bacterial proteases could also largely contribute to the splitting of these sites; for example, CEP of *Lc. lactis* strains may hydrolyze α_{S2}-CN bonds Lys_{150}-Thr_{151} and Lys_{197}-Thr_{198} (Monnet et al., 1992), CEP of *Lb. helveticus* may hydrolyze the bond Arg_{114}-Asn_{115} (Sadat-Mekmene et al., 2011), and enzymes of *St. thermophilus* may act on all aforementioned plasmin-preferable sites, including Lys_{24}-Asn_{25} at the N-terminus, according to in vitro studies (Miclo et al., 2012). In general, α_{S2}-CN is relatively resistant to chymosin hydrolysis (Fox and McSweeney, 1996) with cleavage sites restricted to the hydrophobic regions of the molecule (McSweeney et al., 1994). According to our data, none of the peptides among most abundant ones (Table 3) could be ascribed to the potential chymosin action except α_{S2}-CN f175–182, released in the result of cleavage of the bond Phe_{174}-Ala_{175} (McSweeney et al., 1994). However, both CEP of lactococci (Monnet et al., 1992) and proteolytic enzymes of streptococci (Miclo et al., 2012) may also compete for this cleavage. It should be noted that the set of major α_{S2}-CN-derived peptides determined in Old Saare cheese differed essentially from the peptides identified in Grana Padano cheese, which have been assumed to originate from chymosin hydrolysis (Ferranti et al., 1997b). Explicitly, the only peptide found in common in these 2 cheeses was α_{S2}-CN f175–182. The potential cleavage sites of cathepsin D on α_{S2}-CN (Larsen et al., 1996) differ from those of chymosin, but coincide with in vitro hydrolysis positions of *St. thermophilus* proteases (Miclo et al., 2012). Among these sites, we should highlight the cleavage of the bonds Leu_{99}-Tyr_{100} and Thr_{182}-Val_{183} (may be also cleaved by CEP of lactococci; Monnet et al., 1992), accompanied by the release of corresponding peptides (f100–114/115 and f175/176–182) in noticeable amounts (Table 3, Figure 5b). The involvement of bacterial proteases in the hydrolysis of α_{S2}-CN was evident during the ripening of Old Saare cheese: the bond Asn_{115}-Ala_{116} has been shown potentially cut by *Lc. lactic*, *Lb. helveticus*, as well as *St. thermophilus* (Monnet et al., 1992; Sadat-Mekmene et al., 2011; Miclo et al., 2012); the extensive cleavage at Lys_{137}-Thr_{138} has been shown to be specific to CEP of lactococci (Monnet et al., 1992); the cleavage at Leu_{161}-Asn_{162} and Asn_{162}-Phe_{163} has been assigned to the action of *St. thermophilus* (Miclo et al., 2012). We also found a pronounced cleavage at other in vitro indicated potential hydrolysis sites of *Lc. lactis* and *St. thermophilus*: His_{186}-Gln_{187} and Tyr_{203}-Val_{204} (Monnet et al., 1992; Miclo et al., 2012; Figure 5b). The analysis of cleavage sites suggested the action of aminopeptidases on the consecutive bonds in the regions f100–106, f123–128, f151–155, and carboxypeptidases in the region f109–114 in the C-terminus of α_{S2}-CN (Figure 5b). In addition, we detected remarkable cleavage of the bonds Gln_{127}-Leu_{128}, Leu_{128}-Ser_{129}, Asp_{140}-Met_{141}, and Glu_{142}-Ser_{143} (Figure 5b), but we did not find the proteolytic agents potentially responsible for these sites in the literature.

κ-CN-Derived Small Peptides. The κ-CN-derived water-soluble peptides were present in Old Saare cheese in relatively low concentrations compared to those derived from other caseins (Figure 4d). Thus, none of the peptides from κ-CN were observed in cheese in the amounts comparable to the most abundant ones originating from the other caseins (Figure 4d, Table 3). Nevertheless, the analysis of cleavage sites allowed us to distinguish some cuttings indicated in the literature. It

has been noticed that plasmin has very low activity towards κ-CN (Fox and McSweeney, 1996). On the other hand, we found all 3 known hydrolysis sites of cathepsin D (Larsen et al., 1996) hydrolyzed in Old Saare cheese: Leu_{32}-Ser_{33}, Leu_{79}-Ser_{80}, as well as Phe_{105}-Met_{106} coinciding with the primary cleavage site of chymosin causing milk clotting (Figure 5d).

Although most of the GMP is suggested to be lost in the whey (Fox and McSweeney, 1996), our results showed that the water-soluble peptides originating from the GMP, especially κ-CN f114–169, actually dominated in the cheese among other κ-CN-derived small peptides at the beginning of ripening. The water-soluble peptides from the C-terminal part of the GMP, κ-CN f145–169, were produced intensively in fresh cheese, and then subsequently degraded during the first month of ripening (Figure 4d, Supplemental Figure S1d, https://doi.org/10.3168/jds.2017-12944). The action of CEP of lactococci, splitting the bonds Glu_{151}-Val_{152}, Asn_{160}-Thr_{161}, Val_{162}-Gln_{163}, Gln_{163}-Val_{164}, Thr_{165}-Ser_{166}, and Thr_{167}-Ala_{168} (Kunji et al., 1996), followed by the action of aminopeptidases on κ-CN f145–154, could explain the digestion of C-terminal peptides of GMP to FAA during the second half of Old Saare ripening (Supplemental Figure S1d, S2d, https://doi.org/10.3168/jds.2017-12944).

The peptides from the negatively charged N-terminus of *para*-κ-CN, f1–10, accumulated from the beginning of the ripening and prevailed in more ripened cheese (Figure 4d). The concentration of peptides derived from the *para*-κ-CN regions f33–75 and f96–106 appeared to be fluctuating throughout the ripening. During the whole period of ripening, the small peptides derived from the *para*-κ-CN part of the protein appeared to be produced by the action of CEP of lactococci on the bonds Asp_{20}-Lys_{21}, Leu_{32}-Ser_{33}, Asn_{41}-Tyr_{42}, Tyr_{60}-Tyr_{61}, Ala_{65}-Ala_{66}, Ala_{71}-Gln_{72}, Gln_{75}-Trp_{76}, Leu_{79}-Ser_{80}, Ala_{85}-Lys_{86}, Met_{95}-Ala_{96}, Pro_{99}-His_{100}, and His_{102}-Leu_{103} (Kunji et al., 1996), supported by the action of some bacterial endopeptidases with subsequent digestion by exopeptidases (Figure 5d).

We could not assign some extensive cleavages, for example, at the bond Arg_{10}-Cys_{11} and on fragment f48–60 (Figure 5d) of *para*-κ-CN, as well as at the bonds Asn_{123}-Thr_{124}, Glu_{137}-Ala_{138}, and Leu_{146}-Glu_{147}, on GMP (Supplemental Figure S2d, https://doi.org/10.3168/jds.2017-12944) to the activity of proteolytic enzymes found in the previously published literature.

As we saw from our results, the analysis of cleavage sites performed on all identified casein-derived peptides allowed to determine additional significant cleavages which could not be identified in case of performing the analysis only with the data on abundant peptides, even though our list of abundant peptides was broader (169 peptides) than usually taken in consideration in other studies (Gagnaire et al., 2001; Piraino et al., 2007; Sforza et al., 2012). The omission of less abundant peptides from the analysis would provide only limited information on the bonds in casein sequences that have been preferably cut by proteolytic enzymes, and some important cleavages would have been unnoticed.

Clustering Analysis. Because the amount of data obtained from the LC-MS/MS analysis was both massive and complex, we performed the PCA and clustering analysis based on the selection of the most abundant peptides for better understanding and overall assessment of the evolutionary trends in peptide profiles during ripening (Figure 6). The PCA revealed that the most prominent changes in the profiles of water-soluble peptides occurred within the first 5 mo of Old Saare cheese ripening; subsequently, 5- to 8-mo-old cheeses clustered together on the PCA plot (Figure 6a). The first PC explained 37.7% and the second PC explained 27.5% of the variance. To follow the differences in characteristic trends of the evolution of peptides, we clustered the abundant peptides included in PCA into 4 groups according to their changing patterns throughout maturation. An overall allocation of the peptide groups on PCA plot was in accordance with the different ripening stages of Old Saare cheese (Figure 6a). The first group of peptides was associated with unripened, up to the 2-wk-old cheeses, the second group related to the 1- to 3-mo-old cheeses, and the third related to the 3- to 5-mo-old cheeses. The remaining period of ripening (6–8 mo) was characterized mainly by the peptides from the fourth group. Similar clustering analysis of 57 most abundant peptides was performed using PCA in Parmigiano-Reggiano cheese during 24 mo of ripening (Sforza et al., 2012); each group of peptides was characterized by respective aging time and different composition.

So, in the case of Old Saare, the first group included the peptides exhibiting the highest content in the 1-d-old cheese; then, their content mostly dropped abruptly during 2 wk, followed by a slower decrease during the next mo of ripening (Figure 6b). Twelve peptides from β-CN, 7 peptides from α_{S1}-CN, and 4 peptides from α_{S2}-CN belonged to the group (Table 3). For instance, this group included some longer water-soluble peptides (i.e., β-CN f29–52 and f73–96), easily released at the beginning of the ripening and giving further rise to numerous smaller peptides at the later stages of the ripening (assigned mostly to groups 3 and 2, respectively).

The peptides clustered in the second group (33 from β-CN, 17 from α_{S1}-CN, and none from α_{S2}-CN) were characterized by rapid increase during the 1 to 3 mo of the ripening, followed by a decrease to the 5 mo of ripening, then leveling off (Figure 6b). This group

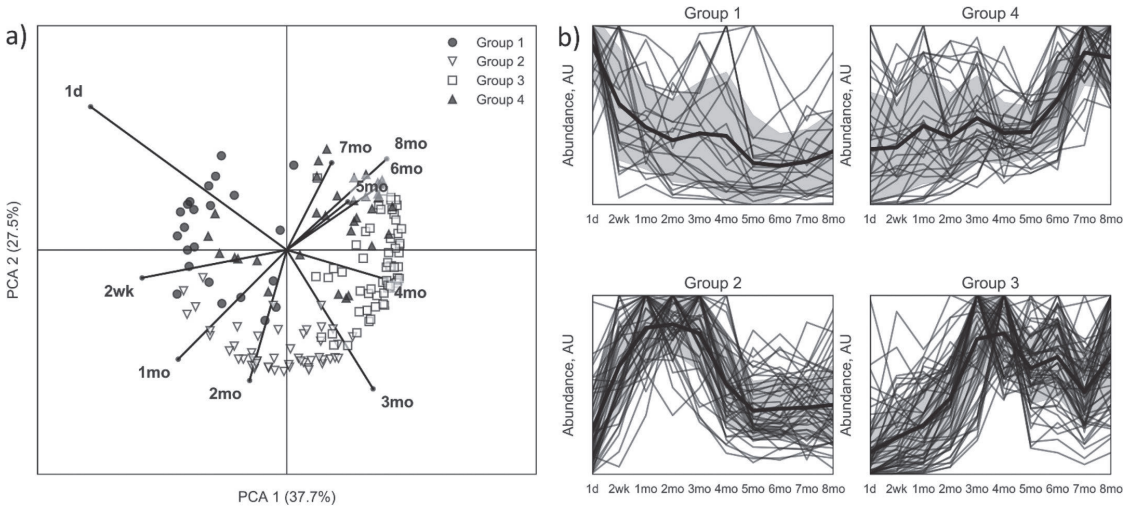

Figure 6. Principal component analysis (PCA; a) and clustering analysis (b) of the most abundant water-soluble peptides determined during Old Saare cheese ripening. Calculations were made using mean values of duplicated samples from each of 2 cheesemaking trials (n = 4). AU = arbitrary units.

consisted of mostly nonphosphorylated peptides; almost all peptides were derived from β-CN region f39–93 and peptides randomly distributed mainly along the N-terminus of α_{S1}-CN f7–40 (Table 3).

The peptides in the groups 3 and 4 can be largely characterized by accumulating behavior during the ripening. The third group contained the highest number of β-CN peptides, 42; among those are almost all phosphopeptides from region f1–29, two-thirds of the peptides from region f30–52, including most phosphopeptides, and few peptides from the rest of the sequence (Table 3). The α_{S1}-CN and α_{S2}-CN contributed to the group with 11 and 9 peptides, respectively, again, the majority being potentially phosphorylated. The contents of these peptides increased rapidly at the beginning of the ripening to their maximum value at 3 to 4 mo, then slightly decreased by 7 mo, followed by another increase in the end of ripening (Figure 6b). Similar to Old Saare, 3 β-CN-derived phosphopeptides, β-CN f11/12/15–28, were identified in Parmigiano-Reggiano cheese showing the increasing trend and reaching maximum around 5 mo of aging (Sforza et al., 2012). Two accumulating peptides from α_{S1}-CN attributed to the third group in Old Saare cheese, α_{S1}-CN f24–34/36, were also found in Parmigiano-Reggiano, but differently from Old Saare were shown to have maximum values in cheese curd with a constant decrease in amounts during further ripening (Sforza et al., 2012). In Old Saare cheese, the fourth group was formed by 14 peptides from β-CN, almost all located in the C-terminal part f106–209; 13 from α_{S1}-CN, a half of them being phosphopeptides from region f44–79; and 7 from α_{S2}-CN, inconsistently distributed along the C-terminal half of the protein (Table 3). A common feature of these peptides was a slight increase in concentration during 5 mo and subsequent notable increase to the maximum value by the end of ripening (Figure 6b). As confirmed by CA, the phosphopeptides, being less available for further digestion by proteolytic agents, accumulated greatly since the middle of cheese ripening. Two peptides from the fourth group, β-CN f194/195–209, were also found in Parmigiano-Reggiano but with different behavior, reaching the maximum values around 1 to 2 mo of ripening and constantly decreasing thereafter (Sforza et al., 2012). According to our results, the PCA and clustering analysis can be easily applied to full data from MS/MS analysis, providing an interesting general characterization of the complex dynamics of peptides during cheese maturation.

Accumulation of FAA

The content of total FAA increased from 1.2 ± 0.1 g/kg in the 1-d-old cheese to 59.9 ± 1.1 g/kg in the 8-mo-old cheese (Table 2). Accumulation was the fastest during the first 4 mo of ripening and slowed down afterward (Figure 1, Table 2).

Figure 7 shows the changes in the relative content of all FAA in the cheese simultaneously at all ripening

time points and also allows to compare them with AA composition of intact caseins present initially before any hydrolysis in cheese had started (initial intact casein). Thus, the relative content of FAA being higher or lower than in intact caseins allows to determine tentatively either the more preferable cutting regions or the parts of the sequences that have been hydrolyzed to a lesser extent, respectively. The relative content of individual FAA deviated greatly from AA composition of initial intact caseins in the 1-d-old Old Saare cheese (Figure 7). Most but not all FAA stabilized at levels close to their relative molar content in caseins by around 3 to 4 mo (Figure 7). The correlation coefficient between the relative contents of FAA in Old Saare cheese and in initial intact caseins changed from 0.57 in the 1-d-old cheese to 0.76 in the 8-mo-old cheese. The relative concentration of free Lys was more than 2 times higher in the 1-d-old cheese compared with its content in initial intact casein fraction of Old Saare, and its higher amounts were also maintained during the later stages of the ripening. We explain the intensive production of Lys by the fact that Lys-Lys and Lys-X are the preferable bonds hydrolyzed by plasmin (Fox and McSweeney, 1996), exposing Lys to the action of bacterial exopeptidases. Our results showed that in Old Saare cheese, in addition to β-CN and α_{S2}-CN, the plasmin was active also in hydrolysis of α_{S1}-CN (Figure 5a), even though it is not the primary target of the enzyme. High production of Lys can be also attributed to the fact that the intracellular enzymes released into the cheese environment during the lysis of bacterial cells can produce much more AA than active bacteria are further able to consume (Ardö et al., 2002). *Lactobacillus helveticus* was shown to be a most abundant metabolically active species among the other starter bacteria in 1-d-old Old Saare cheese, which abundance decreased during further ripening (L. Blank, TUT, Tallinn, Estonia, personal communication) indicating its potential lysis. Thus, *Lb. helveticus* could have been actively involved in the release of free Lys. Similarly, Ardö et al. (2002) have found a substantial increase in Lys content in a semi-hard cheese produced with an adjunct culture of *Lb. helveticus*. The relative content of free Glu and Arg, which are located at relatively less preferable cleavage sites of plasmin (McSweeney et al., 1993b), was also higher at the beginning of the ripening than in initial intact caseins. The intense conversion of Glu to γ-aminobutyric acid (**GABA**) took place during the further ripening by the GABA-producing Glu decarboxylase activity found in *St. thermophilus* strains (Ardö et al., 2002). We assigned the pronounced decrease in Arg content during the later stages of the ripening (Table 4, Figure 7) to its conversion to the breakdown product Orn and to the production of ATP and carbamyl phosphate, which may be used by cheese microflora for the formation of flavor compounds (Ardö, 2006). Conversely, the relative

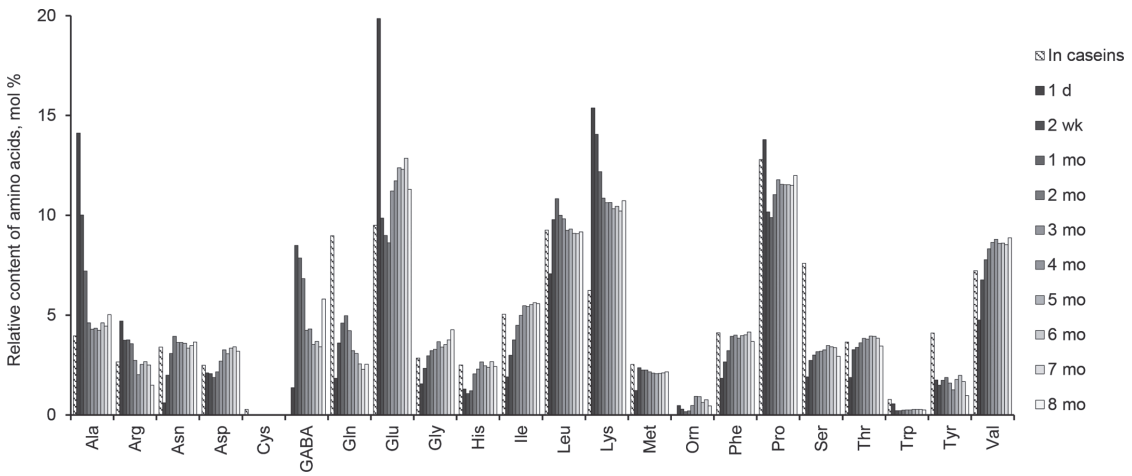

Figure 7. Changes in relative abundance (mol %) of free AA in Old Saare cheese during the ripening compared to the relative molar AA composition of initial intact caseins before any hydrolysis had started in the cheese (striped bars). The ratios of different caseins for the calculation of the AA composition of initial intact casein were obtained from the data from capillary electrophoresis (CE) of 1-d-old cheese, recalculating the content of the hydrolysis products of β-CN, α_{S1}-CN, and κ-CN (γ-CN, α_{S1}-I-CN, and para-κ-CN, respectively) into the content of the initial unhydrolyzed caseins, which was added to the intact casein fractions (β-CN, α_{S1}-CN, and α_{S2}-CN) directly determined by CE by separate peaks. Results represent the mean values of duplicated samples from each of 2 cheesemaking trials (n = 4).

Table 4. Formation of free AA (mmol/kg of cheese) during the ripening of Old Saare cheese[1]

	Ripening time									
AA	1 d	2 wk	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo
Ala	1.25 ± 0.05	2.41 ± 0.37	4.67 ± 0.18	7.25 ± 0.66	11.18 ± 1.92	17.22 ± 3.92	18.17 ± 0.75	22.15 ± 3.68	22.64 ± 2.72	23.47 ± 2.28
Arg	0.42 ± 0.03	0.90 ± 0.23	2.43 ± 0.37	5.60 ± 0.62	7.14 ± 0.60	7.99 ± 0.79	10.84 ± 0.64	12.83 ± 1.47	12.76 ± 0.96	7.01 ± 1.11
Asn	0.05 ± 0.02	0.48 ± 0.12	2.00 ± 0.40	6.19 ± 0.45	9.44 ± 1.25	14.36 ± 4.23	15.35 ± 1.37	16.09 ± 0.70	17.70 ± 1.82	17.10 ± 2.01
Asp	0.19 ± 0.03	0.50 ± 0.14	1.21 ± 0.11	3.40 ± 0.34	7.02 ± 1.16	12.92 ± 3.08	13.14 ± 0.57	16.08 ± 1.48	17.41 ± 1.93	14.92 ± 0.60
Cys	0.03 ± 0.01	0.08 ± 0.00	0.15 ± 0.01	0.16 ± 0.02	0.15 ± 0.06	0.21 ± 0.04	0.24 ± 0.03	0.24 ± 0.08	0.24 ± 0.06	0.23 ± 0.03
GABA	0.12 ± 0.03	2.05 ± 0.79	5.10 ± 1.70	10.73 ± 0.99	11.06 ± 0.47	17.03 ± 2.43	15.13 ± 2.63	17.68 ± 0.07	17.42 ± 2.14	27.12 ± 5.79
Gln	0.16 ± 0.03	0.87 ± 0.23	2.98 ± 0.37	7.81 ± 0.84	10.99 ± 1.24	12.77 ± 1.31	13.20 ± 0.60	12.25 ± 0.93	11.63 ± 0.83	11.91 ± 0.81
Glu	1.76 ± 0.14	2.38 ± 0.23	5.82 ± 1.04	13.55 ± 1.00	29.19 ± 6.95	46.40 ± 11.69	52.97 ± 4.36	59.05 ± 4.34	65.51 ± 4.62	52.84 ± 4.40
Gly	0.14 ± 0.05	0.56 ± 0.20	1.92 ± 0.16	5.06 ± 0.47	8.54 ± 1.13	14.54 ± 4.11	14.56 ± 0.38	16.95 ± 1.10	19.19 ± 1.24	19.95 ± 1.09
His	0.12 ± 0.02	0.26 ± 0.05	0.78 ± 0.14	3.22 ± 0.35	6.00 ± 0.85	10.50 ± 3.62	10.53 ± 1.41	11.49 ± 0.65	13.58 ± 2.09	11.34 ± 2.15
Ile	0.17 ± 0.02	0.72 ± 0.15	2.43 ± 0.18	7.06 ± 0.64	12.99 ± 2.07	21.64 ± 4.98	23.26 ± 1.59	26.50 ± 2.26	28.69 ± 2.49	26.03 ± 0.66
Leu	0.63 ± 0.04	2.36 ± 0.42	7.02 ± 0.49	15.72 ± 1.60	25.56 ± 4.41	36.59 ± 7.34	39.83 ± 1.97	43.64 ± 2.50	46.30 ± 3.92	42.86 ± 0.37
Lys	1.36 ± 0.09	3.39 ± 0.50	7.89 ± 0.61	17.08 ± 1.97	27.67 ± 4.85	42.13 ± 9.78	44.19 ± 2.15	50.13 ± 3.43	52.07 ± 6.01	50.13 ± 0.88
Met	0.11 ± 0.03	0.57 ± 0.10	1.45 ± 0.06	3.54 ± 0.45	5.62 ± 0.94	8.31 ± 1.70	8.88 ± 0.52	10.01 ± 0.80	10.77 ± 0.78	10.11 ± 0.49
Orn	0.04 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.31 ± 0.09	1.24 ± 0.47	3.68 ± 3.03	3.91 ± 0.23	2.98 ± 0.41	3.89 ± 1.84	2.12 ± 0.10
Phe	0.16 ± 0.02	0.64 ± 0.14	2.09 ± 0.16	6.18 ± 0.65	10.41 ± 1.83	15.21 ± 3.37	16.99 ± 1.21	19.28 ± 1.13	21.18 ± 0.77	17.23 ± 0.47
Pro	1.22 ± 0.08	2.45 ± 0.53	6.40 ± 0.77	17.35 ± 1.45	30.66 ± 5.41	45.69 ± 9.39	49.33 ± 2.29	55.34 ± 3.72	58.63 ± 4.86	56.08 ± 0.60
Ser	0.17 ± 0.05	0.66 ± 0.18	1.94 ± 0.23	4.98 ± 0.51	8.32 ± 1.06	12.91 ± 2.69	14.87 ± 1.32	16.40 ± 0.83	17.20 ± 1.15	13.75 ± 1.14
Thr	0.17 ± 0.02	0.79 ± 0.13	2.19 ± 0.11	5.69 ± 0.66	10.00 ± 1.90	15.02 ± 2.88	16.90 ± 1.00	18.9 ± 1.80	19.60 ± 0.99	16.16 ± 0.60
Trp	0.05 ± 0.03	0.05 ± 0.01	0.14 ± 0.01	0.38 ± 0.05	0.67 ± 0.17	1.00 ± 0.25	1.21 ± 0.11	1.33 ± 0.15	1.44 ± 0.13	1.19 ± 0.06
Tyr	0.15 ± 0.03	0.36 ± 0.08	1.13 ± 0.06	2.95 ± 0.36	4.16 ± 0.80	4.98 ± 0.89	7.59 ± 0.68	9.50 ± 0.51	8.51 ± 0.62	4.52 ± 0.83
Val	0.42 ± 0.05	1.63 ± 0.37	5.04 ± 0.40	13.08 ± 1.18	22.51 ± 3.64	34.82 ± 7.70	36.74 ± 1.36	41.29 ± 2.43	43.46 ± 3.84	41.50 ± 0.53

[1]Values are the means of duplicated samples from each of 2 cheesemaking trials (mean ± SD, n = 4). GABA = γ-aminobutyric acid.

concentration of free Ser was about 2 times lower than could be expected from the AA composition of initial intact caseins. We explained this difference by extensive phosphorylation of Ser in casein sequences indicated by the MS/MS results and significant accumulation of the phosphopeptides, which are not degraded during the cheese ripening. The level of free Gln was lower than expected from the AA composition of initial intact caseins due to its rapid conversion to Glu and GABA.

Proline, Glu, Lys, Leu, and Val (in descending order) were the quantitatively dominating FAA by the end of the ripening in Old Saare cheese (Table 4). We found dissimilarities in the occurrence of dominating FAA and in the order of their relative abundance in the 8-mo ripened Old Saare cheese compared with other cheeses. The content of Pro, present in the highest amounts in Old Saare cheese (Table 4), has been among the less abundant AA in Cheddar, Edam, and Gouda cheeses (Fox and Wallace, 1997) manufactured with mesophilic starter. Additionally, Rehn et al. (2010) found Pro in fifth place among the dominating AA in Västerbottenost cheese, which has also been produced with mesophilic starter using high cooking temperatures. We suggest that the thermophilic starter, used in addition to DL-starter in Old Saare cheese production, may be important in the hydrolysis of Pro-containing peptides by proline-specific peptidases. We should mention that nonstarter lactic acid bacteria, present in cheese as adventitious microflora, may have also been responsible for the degradation of water-soluble peptides and catabolizing of AA to flavor-forming compounds. We did not consider the proteolytic action of nonstarter lactic acid bacteria in a more detail in this study.

CONCLUSIONS

This work presents the results of the application of different experimental methods and in silico tools that allowed us to carry out the first detailed characterization of the dynamics of different proteinaceous fractions, starting with the degradation of intact caseins followed by the release of peptides of different length and the AA throughout ripening of cheese. The results obtained showed that the majority of the events of casein hydrolysis took place during the first 4 to 5 mo of ripening of Old Saare cheese, suggesting that the cheese may be regarded as practically fully ripened upon expiration of that time. On the other hand, as seen from the further changes in the patterns of cleavage sites, in the dynamics of peptide groups obtained in the clustering analysis, and in the relative abundance of free AA, the ripening events continued until the very end of Old Saare cheese maturation. Application of the high-resolution and high-accuracy LC-MS/MS method allowed determination of a large number (3,266) of small water-soluble peptides. Analysis of cleavage sites based on the detailed LC-MS/MS data proved the necessity of also including the less abundant peptides in the analyses. Important cleavages were assigned to the known specificities of proteolytic agents and new phosphopeptides were detected in the result of the analysis of MS/MS data. More efforts should be undertaken in the future to complete an overall quantitative scheme of proteolysis taking into account also the fractions not fully covered in the present study [i.e., long water-soluble peptides (longer than 25 AA in the sequence) and very short ones (di-, tripeptides)]. The mathematical modeling techniques could and should be applied to the detailed proteolysis data for building models of cheese ripening and exploring the kinetics of production and degradation of peptides.

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Appendix 2

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Composition and renneting properties of raw bulk milk in Estonia

Tiina Kriščiunaite [a,b,*], Irina Stulova [a,b], Anastassia Taivosalo [a,b], Tiiu-Maie Laht [a,b], Raivo Vilu [a,b]

[a] Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia
[b] Competence Centre of Food and Fermentation Technologies (CCFFT), Akadeemia tee 15b, 12618 Tallinn, Estonia

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ABSTRACT

Seasonal variations in milk composition and renneting properties were determined in more than 1000 raw bulk milk samples from individual farms in Estonia. Pooled milk from tank trucks and dairy silos originating from the same milkings was also analysed. Changes in whey protein to casein ratio, due to partial degradation of κ- and β-casein fractions, were observed in pooled milk from tank trucks and dairy silos. Likewise, rennet flocculation time (RFT) was 1 min longer compared with that of milk from individual farms. Milk pH correlated significantly with curd firmness and RFT, whereas curd firmness correlated also with protein content and with κ-casein fraction in particular ($r = 0.45$). The important correlations between milk composition and renneting characteristics observed earlier in the case of milk from individual cows were essentially the same in the case of bulk milk delivered for processing.

1. Introduction

Milk as a raw material for cheesemaking has to meet certain requirements to allow the production of high quality cheese without modifying the routine technological processes. Milk has to be of sufficient microbiological quality and has to have stable chemical composition and renneting properties. The evaluation of the microbiological and hygienic quality and safety of milk is the main priority for the local authorities. The microbiological composition of raw milk produced in Estonian dairy farms has been reported recently and the high hygienic quality of Estonian milk was documented (Stulova, Adamberg, Kriščiunaite, Blank, & Laht, 2010). Milk fat and protein content are of major interest for dairy farmers, as these parameters determine the price paid for milk, and are measured routinely for individual cows and farm herds by the Milk Analysis Laboratory of Estonian Animal Recording Centre. Technological characteristics of milk are of the major concern for the dairy industry and are not routinely determined by the authorities.

According to data from the Estonian Animal Recording Centre (2010), annual yield of milk per cow in Estonia doubled from 3666 kg in 1995 to 7447 kg in 2009. The increase in milk production is considered a result of breeding strategies and increasing prevalence of Estonian Holstein-Friesian (EHF) breed over Estonian Red (ER). The percentage of ER breed in the Estonian herd dropped from 38.0% in 1995 to 23.3% in 2009; accordingly, the share of EHF breed rose from 61.5% to 76.1% during the same time (Estonian Animal Recording Centre, 2010). The increasing predominance of the Holstein-Friesian breed in the dairy herds has become a popular trend in Estonian farms, in line with the situation in other European countries. Changes of the general composition of milk – protein, fat and lactose content, have been also observed during the past decades (Estonian Animal Recording Centre, 2010). Thus, the alternations in Estonian milk renneting properties can also be expected to have taken place. In spite of the higher milk productivity of Holstein-Friesian breed, Malacarne et al. (2006) have shown that milk from Italian Friesian cows had lower protein content, lower curd firming rate, weaker coagulum and lower cheese yield than milk from Italian Brown breed. On the contrary, Holstein-Friesian cows from Finnish herds showed better milk coagulating ability than Finnish Ayrshire cows (Tyrisevä, Vahlsten, Ruottinen, & Ojala, 2004).

Several studies have been carried out to evaluate composition of cows' milk in a number of countries with respect to seasonal, geographical or annual variations. Lindmark-Månsson, Fondén, and Pettersson (2003) investigated the detailed composition of Swedish dairies' milk with respect to seasonal and geographical variations. Heck, van Valenberg, Dijkstra, and van Hooijdonk (2009) reported the seasonal variations in the composition of average dairy milk of the Netherlands, based on the single pooled milk sample collected and analysed weekly within the year. However, all the studies mentioned were performed without turning a special attention on the rennetability of milk. Changes in milk composition and technological properties during the spring flush period were

* Corresponding author. Tel.: +372 6 202 831; fax: +372 6 202 828.
E-mail address: tiina@tftak.eu (T. Kriščiunaite).

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determined in bulk milk from a small number of herds (Grimley, Grandison, & Lewis, 2009). The relationship between chemical composition and clotting properties of milk from several farmhouses and the properties and yield of the ripened cheese were also studied (Martin, Chamba, Coulon, & Perreard, 1997). Surveys carried out on individual cow's milk samples, collected from a small number of animals in experimental herds have provided insight on the changes in milk composition throughout a lactation (Jõudu, Henno, Kaart, Püssa, & Kärt, 2008; Tsioulpas, Lewis, & Grandison, 2007a,b) and on the correlation between different milk characteristics (Jõudu et al., 2008; Tsioulpas et al., 2007b; Wedholm, Larsen, Lindmark-Månsson, Karlsson, & Andrén, 2006). Unfortunately, studies performed on individual cow's milk or bulk milk originating from a small number of herds do not characterise the overall quality of commercial pooled milk delivered to dairy plants in practice. To the best of our knowledge, there are no published data on the composition and rennetability of raw milk throughout the country, characterising the processing quality of pooled milk utilised by dairies.

The aim of the present study was to document the quality of raw bulk milk collected from individual farms in Estonia in relation to its cheesemaking properties, to find out seasonal and geographical variations, to compare bulk milk from individual farms and pooled milk from dairy silos, and to establish correlations between milk composition and renneting properties of commercial milk delivered to a dairy plant throughout different seasons during 3.5 years.

2. Materials and methods

2.1. Milk samples and sampling procedure

Raw bulk milk samples (>1000) from individual dairy farms (>170) of geographically different locations in Estonia, delivering milk to two large dairies, were collected during the period February 2004–June 2007. Milk samples were collected from 7 of the 15 counties of Estonia, comprising more than half (53%) of the total land area, and was regarded as representing overall Estonian milk quality. Samples were collected in 34 batches, and each time approximately 30 milk samples were analysed. The majority of samples were collected during the first two years of the project and were distributed randomly throughout all seasons. Later the samples were collected more rarely but still taking into account the need to have data on seasonality. During the 3.5 year period, 247 bulk milk samples from farms were collected in winter, 530 in spring, 135 in summer, and 93 in autumn. On most sampling days, samples were also taken from tank trucks and dairy silos where the milk from examined individual farms was pooled. Milk general composition and rennet flocculation time (RFT) were determined in case of all collected raw bulk milk samples. Measurement of curd firmness with penetration test was performed in milk samples from 11 batches (439 samples) during two years. Na^+, K^+ and Cl^- were determined in five batches (176 milk samples) during one year, and protein composition in samples from one batch (47 milk samples) as additional analyses.

Two sterile plastic tubes of 50 mL were filled with the same milk sample by licenced milk hauliers during the sampling procedure. Samples from individual farms were collected when pumping milk from farm tank to refrigerated tank truck. Samples from trucks were collected when pumping milk from truck to dairy silo, and dairy silos were sampled after all trucks had delivered milk to the dairy. After taking the samples from farms, tank trucks and dairy silos sampling tubes were kept in cool bags at the same conditions until all samples were collected and sent to two laboratories for simultaneous analyses. Milk samples from tank trucks represented pooled milk from several farms also sampled on the same occasion, and milk in dairy silos consisted practically entirely of milk originating from trucks sampled on that day. The general characteristics of milk samples (fat, protein, lactose, freezing point, urea, somatic cell count and total bacteria count) were determined by Milk Analysis Laboratory of Estonian Animal Recording Centre (Tartu, Estonia); other analyses were performed in the laboratory of Competence Centre of Food and Fermentation Technologies (CCFFT) as described below.

2.2. Milk general characteristics

The pH measurements were conducted using Seven Easy S20 pH-meter equipped with InLab 413 electrode (Mettler-Toledo GmbH, Greifensee, Switzerland).

Analysis of fat, protein, lactose, freezing point and urea of milk of individual dairy farms was performed with infrared spectrometry, according to IDF standard 141C:2000 (International Dairy Federation, 2000), using a MilcoScan (Foss Electric, Hillerød, Denmark), somatic cell count (SCC) was determined using a Fossomatic (Foss Electric), and total bacteria count (TBC) was determined using a BactoScan FC (Foss Electric).

2.3. Analysis of soluble cations and anions in rennet whey

Rennet whey was separated from the coagulated milk samples, prepared as described in Section 2.6, after the measurements of curd firmness. Rennet whey samples were filtered and mixed 1:1 with isopropanol for sedimentation of proteins, centrifuged at 14,000 × *g* for 5 min and diluted with the proper eluent before analysis. A HPLC system (Waters Corporation, Milford, MA, USA) equipped with Waters 1515 isocratic pump and Waters 432 conductivity detector was used for determination of ions. Cations were separated on Waters IC-Pac C column (3.9 × 150 mm) at 25 °C; eluent 3.0 mM nitric acid, flow rate 1.0 mL min^{-1}. Anions were separated using Waters IC-Pac A column (4.6 × 50 mm) at 25 °C; eluent lithium borate/gluconate containing n-butanol and acetonitrile, flow rate 1.2 mL min^{-1}.

2.4. Capillary electrophoresis of milk proteins

Protein fractions of milk were analysed by capillary electrophoresis (CE) according to the method described for milk by Ardö and Polychroniadou (1999). Samples were prepared by mixing 300 μL of skimmed milk (3000 × *g* at 10 °C for 10 min) with 700 μL of sample buffer, incubated for 1 h at room temperature, filtered through a 0.45-μm pore size polyethersulfone (PES) filter (Whatman, Maidstone, UK) and loaded into the 200 μL vials for CE analysis. The sample buffer (pH 8.6) consisted of 10 mM urea, 0.83 g L^{-1} methylhydroxyethylcellulose (MHEC), 167 mM hydroxymethylaminomethane (Tris), 67 mM ethylenedinitrilo tetraacetic acid disodium salt dihydrate (disodium EDTA), 42 mM 3-morpholino-propanesulphonic acid (MOPS) and 17 mM DL-dithiothreitol (DTT). CE analyses were carried out with a Beckman P/ACE™ MDQ instrument controlled by 32 Karat™ version 8.0 software (Beckman Coulter, Inc., Brea, CA, USA). The separations were performed using a neutral PVA coated capillary column (Agilent Technologies Finland Oy, Espoo, Finland) of 40 cm effective length and 50 μm i.d., with a slit opening of 100 × 800 μm, at a temperature of 45 °C with a linear voltage gradient from 0 to 20 kV in 3 min, followed by constant voltage of 20 kV. The separation buffer (pH 3.0) contained 20 mM trisodium citrate dihydrate, 190 mM citric acid, 0.5 g L^{-1} MHEC and 6 M urea. All buffers were filtered through a 0.45 μm pore size filter (Whatman). Before each separation, the capillary was flushed in the reverse direction with water and electrophoresis buffer, each for 3 min. The samples were

Table 1
The general characteristics of raw bulk milk samples collected from individual farms and pooled milk samples collected from trucks and dairy silos. The *p*-value indicates the difference between the mean values of bulk milk from individual farms and pooled milk from trucks and silos.

Component	Raw bulk milk collected from individual farms					Pooled milk from trucks and dairy silos			*p*-value
	n	Mean	SD	Range		*n*	Mean	SD	
				Minimum value	Maximum value				
Protein (g 100 g^{-1})	859	3.29	0.16	2.45	3.84	108	3.30	0.09	NS
Fat (g 100 g^{-1})	859	4.14	0.27	3.08	5.42	108	4.07	0.46	NS
Lactose (g 100 g^{-1})	859	4.75	0.09	4.28	4.99	108	4.74	0.07	NS
Urea (mg L^{-1})	859	253	64	53	500	114	249	59	NS
Freezing point (°C)	859	−0.528	0.005	−0.503	−0.541	102	−0.526	0.004	<0.01
TBC (10^3 cfu mL^{-1})	859	32	80	1	1265	112	45	87	NS
SCC (10^3 cells mL^{-1})	859	277	103	52	1147	108	284	67	NS
Na^+ (mM)	165	16.42	3.18	9.40	28.25	11	17.96	2.01	<0.05
K^+ (mM)	165	31.13	5.68	14.76	47.84	11	36.66	2.84	<0.001
Cl^- (mM)	165	28.82	4.02	21.53	44.25	11	32.94	1.41	<0.001

injected by pressure injection at 3.4 kPa for 20 s. Protein fractions were detected by UV absorbance at 214 nm.

All milk samples were analysed in duplicate. Identification of proteins was done by injection of milk protein standards (α-LA, β-LG, κ-, β- and α_s-CN; Sigma, St. Louis, MO, USA) and by comparison of the electropherograms obtained with those presented in literature (Heck et al., 2008; Miralles, Ramos, & Amigo, 2003; Otte, Zakora, Kristiansen, & Kvist, 1997). Integration of the peaks was carried out after setting the baseline from valley to valley (Miralles et al., 2003). To obtain the relative concentration of each protein fraction the following equation was used (Heck et al., 2008):

$$C_x = \frac{\frac{A_x}{t_x}}{\sum_{i-1}^{n}\frac{A_i}{t_i}} \times 100\%$$

where C_x is the relative concentration of fraction x (%), A_x – the area in the electropherogram of fraction x, t_x – the migration time of fraction x and n – the total number of identified peaks that comprise 100% of the area.

2.5. Rennet flocculation time

The rennet flocculation time (RFT) was measured visually in milk samples using a modified Berridge method (Berridge, 1952). Twenty mL of raw milk samples were equilibrated in glass tubes at 35 °C for at least 30 min using a circulating water bath, followed by addition of 200 μL of a 10 g L^{-1} aqueous solution of chymosin (Sigma). After the addition of rennet, samples were mixed by inversion of test tubes three times and then gently stirred immersed in the water bath until RFT was determined. RFT was defined as the time from rennet addition until the formation of the first visible flocks of coagulated protein on the walls of the glass tube.

2.6. Curd firmness

Rennet gels were prepared by addition of 100 μL of a 10 g L^{-1} chymosin solution to 10 mL of skimmed milk (3000 × *g* at 10 °C for 10 min) previously equilibrated at 35 °C for 30 min. Skimmed milk was used to eliminate the effect of fat content on coagulum strength. Gels were set in plastic tubes with inner diameter of 22.5 mm at 30 °C for 50 min and equilibrated at 22 °C for 10 min before measurement. Penetration test with 10-mm displacement at a crosshead speed of 1 mm s^{-1} was performed using a TA.XT2i Texture Analyzer (Stable Micro Systems Ltd, Godalming, UK), equipped with a 5 kg load cell and a P0.5 cylinder ebonite probe and operated using Texture Expert Exceed software. The firmness of pre-set gels was determined as the force (g) at structure breaking, defined as the first significant discontinuity produced in a force-time curve. Measurements were replicated on three samples.

2.7. Statistical analyses

The means with the standard deviation (SD) were calculated based on the overall data collected throughout the survey. For the dairy farms that were sampled at least three times during the observed period, the mean milk composition and renneting characteristics were calculated. Single-factor analysis of variance was used to evaluate the significant differences in the properties of milk collected in different seasons. Student's *t*-test was used to compare the properties of milk produced during the indoor and outdoor periods, and to verify whether the mean characteristics of milk from individual farms and pooled milk from tank trucks and dairy silos were different. The differences were considered significant if *p* values were less than 0.05. Principal component analysis (PCA) was applied to previously autoscaled and mean centred data to visualize the differentiation of raw bulk milk samples collected from Estonian farms according to season or geographical location. Pearson's product moment correlation coefficient was calculated to determine correlations between milk composition and renneting characteristics. Significance of correlations was determined, taking into account the number of samples and the strength of relationships.

3. Results and discussion

3.1. Milk composition

3.1.1. General characteristics

The mean general composition of Estonian raw bulk milk samples collected from individual farms in 2004–2007 is presented in Table 1. Fat was the most variable from the main milk components (relative standard deviation, RSD, 6.5%), lactose (RSD 2.0%) was the least variable constituent in Estonian milk, with protein being in between (RSD 4.8%); this is in accordance with the findings reported by Heck et al. (2009). According to the data obtained in the present investigation, the mean values of protein (3.29 ± 0.16 g 100 g^{-1}), fat (4.14 ± 0.27 g 100 g^{-1}) and freezing point (−0.528 ± 0.005 °C) were lower, but mean lactose (4.75 ± 0.09 g 100 g^{-1}), urea (253 ± 64 mg L^{-1}) and SCC (277,000 ± 103,000 cells mL^{-1}) were higher in Estonian milk, than values reported for Dutch milk in 2005 (Heck et al., 2009). Conversely, Estonian milk had markedly higher total protein and

Table 2
The relative protein composition of raw bulk milk samples collected from individual farms and pooled milk samples from trucks and dairy silos in one batch. The *p*-value indicates the difference between the mean values of bulk milk from individual farms and pooled milk of the same milking collected from trucks and silos.

Component	Raw bulk milk collected from individual farms					Pooled milk from trucks and dairy silos			*p*-value
	n	Mean	SD	Range		*n*	Mean	SD	
				Minimum value	Maximum value				
α-LA (%)	36	2.8	0.4	1.7	3.7	11	2.9	0.6	NS
β-LG (%)	36	8.8	1.2	6.9	12.0	11	10.1	1.3	<0.05
α_{s1}-CN (%)	36	26.5	1.4	23.7	29.9	11	26.7	1.4	NS
α_{s2}-CN[a] (%)	36	5.3	0.7	4.0	7.1	11	6.2	0.4	<0.001
κ-CN[b] (%)	36	10.0	1.0	7.9	12.1	11	9.5	0.6	<0.05
β-CN (%)	36	37.1	1.4	30.9	38.9	11	35.3	2.1	<0.05
WP (%)	36	11.7	1.4	9.3	15.5	11	13.0	1.8	<0.05
CN (%)	36	78.9	1.4	75.1	81.3	11	77.6	1.8	<0.05
WP:CN	36	0.148	0.021	0.115	0.206	11	0.168	0.028	<0.05

[a] Recalculated assuming that major fraction comprises 50% of the total α_{s2}-CN area.
[b] Recalculated assuming that major fraction comprises 50% of the total κ-CN area.

fat content compared with the results reported for Navarra region in Spain, i.e., 3.19 and 3.81 g 100 g^{-1}, respectively (Sola-Larrañaga & Navarro-Blasco, 2009). According to Estonian Animal Recording Centre (2010), the mean protein content of milk from individual cows in Estonia has increased gradually as a result of breeding strategies during the past 15 years, from 3.17 g 100 g^{-1} in 1995 to almost 3.37 g 100 g^{-1} in 2009; milk fat content increased steadily until the year 2000, and then remained rather stable during the past decade or even has become lower in the period 2005–2009.

Based on the data collected during the survey, the mean values of the milk general composition were calculated for each dairy farm sampled at least three times during the observed period (134 farms). Mean fat content varied from 3.71 ± 0.27 to 4.98 ± 0.29 g 100 g^{-1} between dairy farms, and protein from 2.93 ± 0.15 to 3.59 ± 0.15 g 100 g^{-1}; the mean value of urea varied remarkably from 178 ± 57 to 369 ± 74 mg L^{-1}.

3.1.2. Ions in rennet whey

The mean concentrations of Na^+ (16.42 ± 3.18 mM), K^+ (31.13 ± 5.68 mM) and Cl^- (28.82 ± 4.02 mM) in rennet whey separated from Estonian milk were close to the values reported in other studies (Grimley et al., 2009; Lindmark-Månsson et al., 2003; Sola-Larrañaga & Navarro-Blasco, 2009). The variation in the content of ions between the milk samples was very prominent, particularly concerning Na^+ and K^+ values (RSD 19.4% and 18.3%, respectively). Markedly increased Na^+ and Cl^- values can be an indicator for mastitic milk (Gaucheron, 2005); in Estonian bulk milk samples from individual farms no evidence of mastitis could be found.

3.1.3. Milk protein composition

The six major protein fractions (α-lactalbumin, α-LA; β-lactoglobulin, β-LG; α_{s1}-casein, α_{s1}-CN; α_{s2}-casein, α_{s2}-CN; β-casein, β-CN; κ-casein, κ-CN) detected and quantified in milk in the present study comprise near 90.6% of total protein content (Walstra & Jenness, 1984). The κ-CN exists in milk in one major carbohydrate-free form (Farrel et al., 2004), which is separated well on electropherograms and comprises ~50% of the total κ-CN peak area (Otte et al., 1997), and at least six minor fractions (Farrel et al., 2004), which migrate at the same time as other caseins and are not separated by CE in milk. The α_{s2}-CN has multiple phosphorylation states (Farrel et al., 2004), and the major fraction with 11 phosphates (α_{s2}-CN-11P) comprises also near 50% of the total α_{s2}-CN area (Heck et al., 2008). Only the major fractions of κ- and α_{s2}-CN were quantified in the present study, therefore, to obtain the correct relative protein composition of raw milk, the integrated areas of κ- and α_{s2}-CN identified major peaks were multiplied by two.

The mean protein composition of raw bulk milk from 36 individual farms (Table 2) was very close to that reported for milk of individual cows from the research farm in Estonia (Jõudu et al., 2008), and also in line with the protein composition determined in milk of Italian (Bonizzi, Buffoni, & Feligini, 2009) or Swedish and Danish cows (Wedholm et al., 2006). Estonian milk showed higher β-CN than α_{s1}–CN content (37.1 ± 1.4% and 26.5 ± 1.4%, respectively), in contrast to α_{s1}–CN prevailing over β-CN in Dutch milk (Heck et al., 2008, 2009).

3.2. Milk renneting properties

The mean values of pH, RFT and firmness of the rennet coagulum of Estonian raw bulk milk samples collected from individual farms in the period 2004–2007 are presented in Table 3. Comparing the mean pH value obtained in the present investigation (6.76 ± 0.04) with the mean pH value in 1984–1991 (6.67 ± 0.03; unpublished data), measured in Estonian milk intended for cheesemaking and collected from the set of 11–15 dairy herds distributed throughout the country, the shift in milk pH upwards by 0.1 units was observed taking place during the past two decades. The mean pH of raw bulk milk in Estonia in 2004–2007 was higher than was reported for individual cow's milk samples in UK (Tsioulpas et al., 2007a) or bulk milk samples analysed in France, Sweden and Italy (Lindmark-

Table 3
The renneting properties of raw bulk milk samples collected from individual farms and pooled milk samples collected from trucks and dairy silos. The *p*-value indicates the difference between the mean values of bulk milk from individual farms and pooled milk from trucks and silos.

Parameter	Raw bulk milk collected from individual farms					Pooled milk from trucks and dairy silos			*p*-value
	n	Mean	SD	Range		*n*	Mean	SD	
				Minimum value	Maximum value				
pH	1002	6.76	0.04	6.56	6.86	223	6.76	0.05	NS
RFT (min)	961	5.45	1.06	2.07	10.35	119	6.48	1.11	<0.001
Curd firmness (g)	366	21.54	4.46	9.69	36.44	73	23.47	5.08	<0.01

Table 4
Mean values of the general characteristics and renneting properties of raw bulk milk samples collected from individual farms by season. The *p*-value indicates the difference between the mean values obtained in milk samples collected within different seasons.

Parameter	Season when raw bulk milk samples were collected												*p*-value
	Winter			Spring			Summer			Autumn			
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	
General characteristics													
Protein (g 100 g^{-1})	226	3.31	0.13	421	3.27	0.16	123	3.25	0.13	89	3.41	0.17	<0.001
Fat (g 100 g^{-1})	226	4.17	0.25	421	4.14	0.27	123	3.98	0.20	89	4.30	0.27	<0.001
Lactose (g 100 g^{-1})	226	4.73	0.10	421	4.78	0.08	122	4.77	0.07	89	4.67	0.10	<0.001
Urea (mg L^{-1})	226	254	58	421	268	65	123	220	53	89	231	68	<0.001
Freezing point (°C)	226	−0.528	0.005	421	−0.528	0.005	123	−0.526	0.004	89	−0.527	0.005	NS
TBC (10^3 cfu mL^{-1})	226	33	96	421	33	79	123	34	81	89	19	15	NS
SCC (10^3 cfu mL^{-1})	226	266	89	421	273	105	123	300	93	89	287	134	<0.05
Renneting properties													
pH	246	6.77	0.04	529	6.77	0.04	134	6.74	0.03	93	6.72	0.05	<0.001
RFT (min)	214	5.65	0.99	525	5.37	1.08	133	5.35	0.93	89	5.54	1.23	<0.01
Curd firmness (g)	123	20.03	4.06	47	20.58	3.23	127	22.03	4.30	69	24.00	4.94	<0.001

Månsson et al., 2003; Malacarne et al., 2006; Martin et al., 1997). Higher pH values of milk have been associated with the increased SCC, if the high SCC levels of over 800,000 cells mL^{-1} were observed (Moslehishad, Ezzatpanah, & Aminafshar, 2010). In bulk milk collected from Estonian farms in 2004–2007, only one sample of 859 exceeded that boundary and the mean SCC (277,000 cells mL^{-1}; Table 1) was three times lower than the critical level. It was assumed that the increase of milk pH throughout the country may be associated with the changes in feeding strategies of cows (increased share of concentrates) as a result of industrialisation of dairy farming. Taking into account the strong negative correlation between pH and curd firmness determined in present study, $r = -0.49$ (see Section 3.7), higher losses of curd fines in whey during vat process and lower cheese yield could be expected with the increase in milk pH.

Milk renneting characteristics of the individual milk samples varied in the wide range: values of RFT varied five-fold, roughly from 2 to 10 min, and curd firmness from less than 10 into more than 36 g (Table 3). Although curd firmness of individual milk samples from farms differed to a great extent, pooling of milk decreased the variability a lot, i.e., firmness of the curd obtained from the milk collected from dairy silos varied from 19 to 27 g depending on the sampling occasion. Only one sample that did not result in any measurable coagulum was discovered during the observed period; and even in this case milk sampled from the same farm on the other days clotted well, and the coagulum firmness was satisfactory. Unfortunately, published data on the rennetability of bulk milk throughout the country in other dairying world are not available.

The greatest variation of the mean values of renneting characteristics determined for milk from individual farms was observed in the case of curd firmness: farm values differed two-fold from 14.0 ± 3.2 to 28.6 ± 5.3 g. The mean RFT estimated for milk from different farms varied from 4.5 ± 1.0 to 6.8 ± 1.0 min, and pH varied from 6.68 ± 0.04 to 6.81 ± 0.05.

3.3. Seasonal variations in milk general composition and renneting properties

The seasonal variations in milk composition and renneting properties in Estonia are presented in Table 4. The mean protein and fat contents in Estonian milk were the lowest in summer (3.25 ± 0.13 and 3.98 ± 0.20 g 100 g^{-1}, respectively) and the highest in autumn (3.41 ± 0.17 and 4.30 ± 0.27 g 100 g^{-1}, respectively). In the Netherlands, summer season was also characterised by low protein and fat content, but differently from our results, the highest values were observed in winter (Heck et al., 2009). Lindmark-Månsson et al. (2003) reported minimum values for protein content in spring and maximum values in autumn, but fat did not show any significant seasonal variation in Swedish milk. Nevertheless, despite the clear variation in the total protein content during the year, the protein composition of Swedish and Dutch milk was rather constant (Heck et al., 2009; Lindmark-Månsson et al., 2003). Although the climate, calving patterns and feeding strategies are relatively similar in Estonia compared with the Netherlands and Sweden, Estonian milk showed somewhat different seasonal changes in the two main components, protein and fat. In Estonian milk, lactose was the lowest in autumn, 4.67 ± 0.10 g 100 g^{-1}, and the highest in spring, 4.78 ± 0.08 g 100 g^{-1}, which is in line with other studies (Heck et al., 2009; Lindmark-Månsson et al., 2003). Regarding the entire data set presented in Table 4, milk with the best cheesemaking properties (low pH, low TBC, high protein and fat, firmer coagulum) was produced in autumn. However, the shortest mean RFT in Estonian milk was recorded in summer, 5.35 ± 0.93 min. The lowest mean urea, 220 ± 53 mg L^{-1}, and the highest mean SCC, 300,000 ± 93,000 cells mL^{-1}, were also observed during summer (Table 4). The highest mean urea was observed in spring, 268 ± 65 mg L^{-1}, which is in line with the results of Grimley et al. (2009) showing elevated urea concentrations during turn-out from dry feed to spring pasture. To the best of our knowledge, our study has shown for the first time that the renneting properties of milk also display the seasonal pattern, as it was previously widely shown for milk chemical composition (Heck et al., 2009; Lindmark-Månsson et al., 2003).

A PCA applied to the data obtained for raw bulk milk collected from individual farms showed differentiation of samples according to the season. Principal component one (PC1; 21.4% of the total variation) distinguished autumn milk (higher scores) from summer milk (lower scores). High PC1 values were related mainly to high firmness of the rennet curd and higher protein and fat content of the milk. The best discrimination of the samples between the seasons was obtained on the basis of the principal component two (PC2, 13.4% of the total variation) versus principal component three (PC3, 12.7% of the total variation) plot (Fig. 1). Autumn samples scored positively on PC2 and PC3 axes; spring and winter milk samples were shifted to the negative PC2 values; and summer milk samples were shifted to the positive PC2 and negative PC3 values. Analysis of the eigenvector loadings revealed that high PC2 values were determined mainly by low pH, and high PC3 by high fat, protein and RFT.

The seasonal changes in milk composition may be caused by the changes in diet or the calving pattern of a dairy herd. In Estonia, the

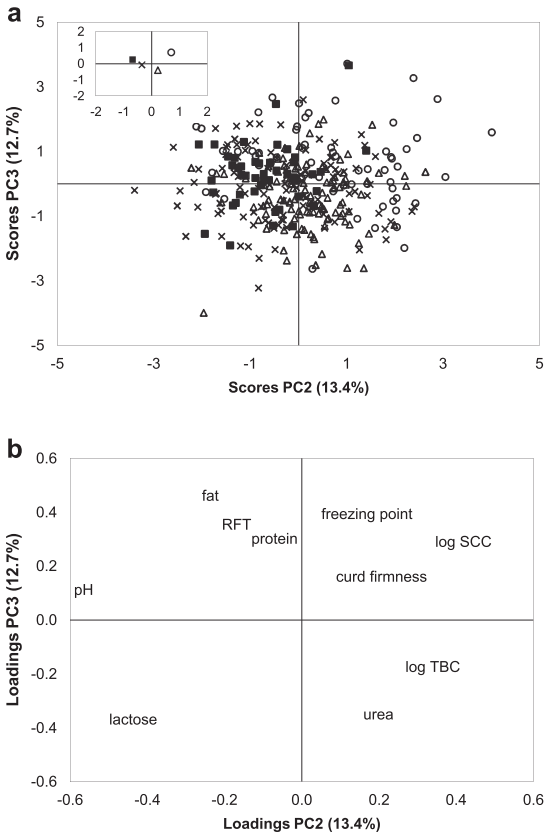

Fig. 1. Scores (a) and loadings (b) of the second (PC2) and third (PC3) principal components obtained by PCA of milk general composition and renneting properties of raw bulk milk samples collected from individual farms: milk samples collected in spring (■), summer (△), autumn (○) and winter (×). Averages ("centres of gravities") of the PCA scores of milk collected in different seasons are presented in the insert to panel a.

calving of cows is distributed evenly throughout the year (Estonian Animal Recording Centre, 2010). The majority of milk (55.2%) is produced by the herds of a size ranging from 100 to 600 cows (Estonian Animal Recording Centre, 2010), which are predominantly held in the barn throughout the year. Still, the majority of cows receive fresh grass as a part of their diet in summer independently of whether the herd is freely grazing or held indoors. The transition from forage feeding to free pasturing (if any occurs) takes place in late spring, and the grazing period lasts normally until the end of September. The data obtained in the present survey were also conventionally divided into the indoor housing (from October to April) and the outdoor grazing (from May to September) period (data not shown). Raw bulk milk samples collected from individual farms during the grazing period showed lower mean fat and urea values ($p < 0.001$), higher freezing point ($p < 0.001$) and higher SCC ($p < 0.01$). On the other hand, during the grazing period, dairy farms produced milk with lower mean pH that resulted in firmer coagulum ($p < 0.001$). The differences in protein, lactose, TBC and RFT of milk between the indoor and the outdoor housing periods were insignificant ($p > 0.05$). Concerning the soluble ions in rennet whey, milk produced during the grazing period showed lower mean Na^+ and Cl^- ($p < 0.001$), whereas the seasonal changes of K^+ content were insignificant ($p > 0.05$).

3.4. Geographical variations in milk composition and renneting properties

A PCA applied to the data on milk general composition and rennetability showed some discrimination of raw bulk milk samples according to the location of the farms (data not shown). Milk produced in the southwest part of Estonia (Pärnumaa) was characterised by higher protein and fat contents, and resulted in a firmer coagulum after renneting. On the contrary, milk collected from the farms located in the northern and central part of the country showed lower protein and fat. Geographical differences in milk composition and properties observed should not be accounted to climatic or geomorphological differences, as these are fairly similar throughout the territory of Estonia. These can be most likely explained by the regional differences in feeding practice.

3.5. Stability of the quality of milk produced in Estonia

Farms engaged in our investigation and sampled at least three times in the period 2004–2007 were evaluated for stability of the composition and renneting characteristics of the milk they produced. The quality of milk was considered variable if the SD calculated for the values recorded for a farm were higher than the SD calculated for all analysed milk samples throughout the whole period. More than half of the farms produced milk with the uniform quality concerning cheesemaking, showing stable protein, fat, pH value and curd firmness during the observation period. The quality of milk was variable in 42.5% of the farms concerning RFT, and in 20–25% of the farms concerning fat, urea and curd firmness; 13% of the farms were variable in milk protein and 7% in lactose content. Large variations in the composition of milk produced by the same farm indicate most probably the lack of the proper management of cows feeding. Cheese producers can take into account seasonal changes in milk quality, but random fluctuations in milk composition, not related to season, cannot be forecasted.

3.6. Comparison of raw bulk milk samples from individual farms with pooled milk from tank trucks and dairy silos

The mean relative concentrations of whey proteins and caseins, as well as their ratios were different in milk from tank trucks and dairy silos in comparison with the samples from individual farms ($p < 0.05$) (Table 2). Notably higher mean TBC in pooled milk (45,000 cfu mL^{-1}) than in milk obtained from separate farms (32,000 cfu mL^{-1}) was also observed (Table 1), however the difference was not statistically significant ($p > 0.05$). Psychrotrophic bacteria have been shown to become the predominant species in Estonian milk (Stulova et al., 2010). They also possess proteolytic activity on milk proteins (Ercolini, Russo, Ferrocino, & Villani, 2009), and they can activate plasmin activators under cheesemaking conditions (Frohbieter, Ismail, Nielsen, & Hayes, 2005) enhancing further degradation of β-casein by plasmin. A protease isolated from *Pseudomonas fluorescence*, the most common species of psychrotrophic bacteria found in milk, was shown to hydrolyse preferentially κ- and β-CN when incubated with artificial micelles (Koka & Weimer, 2000). Lower relative total casein content in pooled milk from tank trucks and dairy silos, essentially lower κ- and β-CN fractions (see Table 2), refers to a certain degree of proteolysis that could have taken place in pooled milk during transportation to the dairy. The appearance of para-κ-CN, which has a migration time close to that of β-LG, has been discovered in raw bulk milk after few days of refrigerated storage,

Table 5
Pearson's correlation matrix of milk renneting properties, milk general and relative protein composition, and ions in rennet whey.[a]

Parameter	pH	RFT	Curd firmness
Renneting properties			
pH	1.00		
RFT	0.17***	1.00	
Curd firmness	−0.49***	−0.07	1.00
Milk general characteristics			
Protein	−0.13***	0.01	0.41***
Fat	0.07*	−0.01	0.24***
Lactose	0.08*	0.00	0.19***
Urea	0.02	−0.09**	0.03
Freezing point	0.02	0.06	−0.15**
TBC	−0.06	−0.03	0.02
SCC	0.01	0.00	0.01
Ions in rennet whey			
Na^+	−0.32***	0.10	0.11
K^+	−0.52***	−0.06	0.19*
Cl^-	−0.20*	0.12	−0.03
Relative protein composition			
α-LA	0.06	−0.19	0.31
β-LG	0.02	0.05	−0.17
α_{s1}-CN	0.24	−0.07	−0.11
α_{s2}-CN[b]	−0.23	−0.07	0.04
κ-CN[c]	−0.10	−0.23	0.45**
β-CN	−0.09	0.29	−0.18
WP:CN	0.04	−0.02	−0.05

[a] Asterisks indicate level of correlation: * significant at the 0.05 level; ** significant at 0.01 level; *** significant at 0.001 level.
[b] Recalculated assuming that major fraction comprises 50% of the total α_{s2}-CN area.
[c] Recalculated assuming that major fraction comprises 50% of the total κ-CN area.

causing an overestimation of the β-LG fraction in milk if proteolysis has taken place (Miralles et al., 2003). Similarly, our results showed significant increase in estimated β-LG fraction in pooled milk from tank trucks and dairy silos compared to bulk milk collected at the farms ($p < 0.05$).

Pooled milk from tank trucks and dairy silos showed 1 min longer mean RFT ($p < 0.001$), which is almost 20% longer than mean RFT value measured for bulk milk from individual farms (see Table 3). We propose that the action of proteases originating from psychrotrophic bacteria on κ-CN located on the surface of the casein micelles could have affected the affinity of the chymosin binding sites for rennet action. In contrast, mean curd firmness turned to be even slightly higher ($p < 0.01$) if prepared from pooled milk. However, considering high overall variability of curd firmness among individual farm milk samples and among samples from tank trucks (differences between boundary values 27 g and 23 g, respectively), and seasonal changes among milk from dairy silos (difference of 7.5 g), the difference of under 2 g between mean curd firmness of farm milk and pooled milk from tank trucks and silos seemed to be rather inessential. It should be noted, however, that as the amounts of milk collected from every farm, truck or silo were not taken into account in the calculation of means in the present study (the mean values were not weighted), the conclusions drawn could not be considered completely impeccable.

3.7. Correlation between milk chemical composition and renneting properties

The Pearson's coefficients between the renneting properties, milk composition and ions in rennet whey are shown in Table 5. The pH of milk correlated negatively with curd firmness and protein content and positively with RFT ($p < 0.001$). Low correlations of milk pH with fat and lactose content seem incidental and may be caused by close relationship between protein, and fat and lactose content (data not shown). The pH of milk also correlated significantly with ions determined in rennet whey: negative correlations with Na^+, K^+ and Cl^- were obtained. The RFT did not correlate significantly with any of the determined parameters except pH, and only a small negative correlation with urea was also observed. A high positive correlation was observed between curd firmness and protein content ($p < 0.001$). Correlations between curd firmness, and fat and lactose content were also noticed (Table 5). However, this was probably due to the close relationship of fat, lactose and protein contents (data not shown). The positive correlation between curd firmness and K^+ content in rennet whey was also established ($p < 0.05$). Protein and fat content of milk and curd firmness have been shown to affect cheese yield (Martin et al., 1997). The relative κ-CN composition correlated with curd firmness even more than total protein content (Table 5), $r = 0.45$ ($p < 0.001$). Our results, obtained from individual dairy farms representing the commercial bulk milk quality utilised for processing, revealed similar findings reported for milk from individual cows (Wedholm et al., 2006) that showed the association of low concentration of κ-CN with poor clotting properties of milk, resulting in weak curd and lower cheese yield.

4. Conclusions

The composition together with the renneting properties of raw bulk milk collected from a large number of individual farms of a country were determined for the first time. Our study has shown that pH of the Estonian bulk milk became 0.1 units higher during the past decades, and also was higher than that of milk produced in other countries in Europe. No published data concerning the changes in milk pH over the last decades in traditional dairying countries are available. Renneting characteristics of the individual milk samples, as well calculated mean values for separate farms varied in the wide range; besides, clear seasonal changes in milk rennetability and firmness of coagulum were determined. Taking into account the similarity of milk composition in Estonia and traditional dairying Europe, the seasonal trends in renneting characteristics could also be similar.

Technological properties of bulk milk from individual farms and pooled milk from dairy silos were shown to be different. This provides us with awareness that, in addition to the original milk composition and microbiological quality, the weak exogenous influencing factors encountered after milking at the farm and before processing at dairy plant (refrigerated transportation of milk from farms to dairy silos, milk vigorous pumping or any other mechanical treatment, occurrence of disinfectant residues, etc.) should be considered and need further investigation.

Our study of bulk milk originating from large number of farms showed that correlations between milk composition and renneting properties exist not only in milk from individual cows from experimental herds but also in pooled milk utilised for processing. The data obtained could help dairies to establish raw milk quality parameters essential from the technological point of view and may be a motivation for careful selection of farms according to the suitability of their milk for cheesemaking.

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Appendix 3

Publication III

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Ripening of Hard Cheese Produced from Milk Concentrated by Reverse Osmosis

Anastassia Taivosalo [1,2,*], Tiina Kriščiunaite [1], Irina Stulova [1], Natalja Part [1], Julia Rosend [1,2], Aavo Sõrmus [1] and Raivo Vilu [1,2]

[1] Center of Food and Fermentation Technologies, Akadeemia tee 15A, 12618 Tallinn, Estonia; tiina@tftak.eu (T.K.); irina.stulova@tftak.eu (I.S.); natalja@tftak.eu (N.P.); julia@tftak.eu (J.R.); aavo@tftak.eu (A.S.); raivo@tftak.eu (R.V.)

[2] Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

* Correspondence: anastassia@tftak.eu

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Abstract: The application of reverse osmosis (RO) for preconcentration of milk (RO-milk) on farms can decrease the overall transportation costs of milk, increase the capacity of cheese production, and may be highly attractive from the cheese manufacturer's viewpoint. In this study, an attempt was made to produce a hard cheese from RO-milk with a concentration factor of 1.9 (RO-cheese). Proteolysis, volatile profiles, and sensory properties were evaluated throughout six months of RO-cheese ripening. Moderate primary proteolysis took place during RO-cheese ripening: about 70% of α_{s1}-casein and 45% of β-casein were hydrolyzed by the end of cheese maturation. The total content of free amino acids (FAA) increased from 4.3 to 149.9 mmol kg^{-1}, with Lys, Pro, Glu, Leu, and γ-aminobutyric acid dominating in ripened cheese. In total, 42 volatile compounds were identified at different stages of maturation of RO-cheese; these compounds have previously been found in traditional Gouda-type and hard-type cheeses of prolonged maturation. Fresh RO-cheese was characterized by a milky and buttery flavor, whereas sweetness, saltiness, and umami flavor increased during ripening. Current results prove the feasibility of RO-milk for the production of hard cheese with acceptable sensory characteristics and may encourage further research and implementation of RO technology in cheese manufacture.

Keywords: reverse osmosis; concentrated milk; hard cheese; cheese ripening; volatile compounds

1. Introduction

The application of a membrane separation technology has gained increasing attention in the dairy industry. The substantial continuing increase in the global milk and cheese production [1] calls for innovations in cheese milk pretreatment to improve the efficiency of cheese manufacturing plants retaining high quality of cheese. Preconcentration of large volumes of raw milk prior to transportation to a cheese plant reduces the delivery costs of milk, and potentially increases the plant capacity and cheese yields.

Ultra- (UF) and microfiltration (MF) for the concentration of cheese milk are the most widely and successfully applied techniques in cheesemaking [2] and have been of enormous research interest. UF retentates have been used to standardize milk protein content by entrapping the whey proteins into the cheese curd matrix to improve the cheese yield and composition of Mozzarella, Cheddar, Camembert, and Brie cheeses [3,4], and semihard cheese made from a mixture of ewes' and cows' milk [5]. Fewer studies have been performed on UF-Feta cheese, where the cheese rheological [6] and microstructural changes in fat globules during ripening [7] as well as accelerating the cheese ripening by the addition

of lipase have been evaluated [8]. MF retentates with increased casein (CN) content can be added to UF retentates to optimize the cheesemaking process by improving rennet coagulability, which results in a firmer gel and increased cheese yield [2,9]. CN enrichment by MF has been applied to produce Mozzarella [10], Cheddar [11,12], and semihard [13] cheeses with a good sensory quality. The effect of both MF and UF on Edam cheese yield and ripening has been evaluated by Heino et al. [14].

Reverse osmosis (RO) is a filtration method that separates the solutes with a molecular weight of 150 Da and less [2]. Thus, water passes through the membrane, while fats, proteins, lactose, and minerals are retained [2]. The process of RO in the dairy industry has been developed during the last 25 years [15,16] and has received considerable attention, especially that of whey protein concentration [17,18]. Moreover, this dewatering technology has been used for the concentration of skim milk before drying in the production of milk powders [19], and prior to yoghurt manufacture [20]. However, only a limited number of studies have demonstrated the application of RO in cheese technology. Agbevavi et al. [21] made the first attempt to produce Cheddar cheese from whole milk concentrated by RO (RO-milk) on a pilot plant scale. The texture of cheese was assumed to be not uniform because of the high lactose content of the retentate. Moreover, the unacceptable bacterial contamination of the final cheese was observed due to the poor sanitary conditions of the RO system [21]. Barbano and Bynum [22] evaluated the effect of different water reduction levels of whole milk obtained by RO on Cheddar cheese produced in a commercial cheese plant. The authors succeeded in the manufacture of the cheese with increased yield by using conventional cheesemaking equipment. The CN breakdown in Cheddar cheese produced from RO-milk has been shown to be similar to that of the control cheese at earlier stages of ripening, while the slower proteolysis has been noted in aged cheese [23]. Additionally, a significantly higher lactose content in Cheddar cheese produced from RO-milk compared to the cheese from unconcentrated milk was determined, which could cause increased lactic acid fermentation, effecting the sensory properties of cheese. Hydamaka et al. [24] used the whole milk RO retentate to produce the direct acidified cheese with a higher cheese yield and good sensory characteristics. At the moment, there have been no published data on the production and ripening of hard-type cheeses from RO-milk.

In this research, a concentrated milk was manufactured using RO filtration technology. The aim of the study was to produce a hard cheese from the RO-milk (RO-cheese) and to study the impact of milk concentration on cheese ripening. Both mesophilic and thermophilic starter cultures and high curd scalding temperature (48 °C) were used in RO-cheese production. The breakdown of caseins, release of amino acids and volatile compounds, as well as sensory properties of RO-cheese were evaluated during six months of ripening.

2. Materials and Methods

2.1. RO-Cheese Manufacture and Sampling

A Gouda-type cheese technology with certain modifications into the manufacturing protocol (i.e., addition of thermophilic starter cultures and high scalding temperature) was applied to obtain a cheese with hard texture and low moisture content. A hard cheese was produced in the pilot plant at the School of Service and Rural Economics (Olustvere, Estonia) from 100 L of whole bovine RO-milk (concentration factor of 1.9, dry matter 21.7% w/w, pH 6.44) pasteurized at 74 °C for 15 s. After heat treatment, the RO-milk was cooled to 32 °C and inoculated with mixed starter culture (10 U 100 L^{-1}) 30 min prior to addition of microbial rennet (8 g 100 L^{-1}; 1300 IMCU g^{-1}, Chymax, Chr. Hansen Ltd., Hørsholm, Denmark). The starter consisted of multiple strains of mesophilic and thermophilic lactic acid bacteria (LAB)—FD-DVS CHN-11, FD-DVS LH-B02, and FD-DVS ST-B01 (Chr. Hansen Ltd., Hørsholm, Denmark) mixed in the proportion 10:5:1, respectively—*Lactococcus lactis ssp. lactis*, *Lc. lactis ssp. cremoris*, *Lc. lactis ssp. diacetylactis*, *Leuconostoc sp.*, *Streptococcus thermophilus*, and *Lactobacillus helveticus*. After coagulation, the curd was cut into 0.7 cm × 0.7 cm × 0.7 cm cubes and then slowly stirred for 20 min followed by removing 20 L of whey. Warm water was added to the vat and cheese

grains were continuously mixed and heated at 48 °C for 30 min. After pre-pressing at 0.5 bar for 10 min and 1 bar for 10 min in the vat under the whey, the whey was drained off and pre-pressed grain cubes were transferred into low cylinder cheese molds. The cheeses were pressed three times for 20 min at 1, 1.8, and 2.1 bar, and brine-salted (20% NaCl, *w/v*) for 16 h at room temperature. Approximately 1 kg wheel-shaped cheeses were coated with wax (6.6 g kg^{-1} of cheese; Ceska®-coat WL01; CSK Food Enrichment, Netherlands) and ripened at 13 °C for six months. Samples were taken from the inner part of the cheese at 0 (fresh cheese before salting), 0.5, 1, 2, 4, and 6 months of ripening and stored at −20 °C until analysis.

2.2. Compositional and Microbiological Analyses of Cheeses

Moisture content and pH of the RO-milk and cheeses were measured using a Mettler-Toledo HR83 moisture analyzer (Mettler-Toledo AG, Greifensee, Switzerland) [25] and a pH meter (Mettler-Toledo Ltd., Leicester, UK), respectively. The pH of the cheese was measured by inserting a glass electrode into the compressed grated cheese samples. Total fat content of the cheese was determined by the method of the Association of Official Analytical Chemists AOAC 933.05 [26] in the grated lyophilized cheese samples.

2.3. Analysis of Caseins

Fractions of CN and their primary degradation products in cheeses were analyzed by Agilent Capillary Electrophoresis (CE, Agilent Technologies, Waldbronn, Germany) according to the method of Ardö and Polychroniadou [27] as described by Taivosalo et al. [28]. CN fractions were identified and labeled based on the results of Otte et al. [29], Miralles et al. [30], Albillos et al. [31], and Heck et al. [32]. 'Valley-to-valley' integration of the peaks was used [30]. The ratio of peak area to the total peak area adjusted to the migration time was used to calculate the relative concentration of caseins in the cheese samples [32].

2.4. Analysis of Free Amino Acids

Composition of the free amino acids (FAA) of RO-cheeses was obtained by UPLC (Acquity UPLC, Waters Corp., Milford, MA, USA) controlled by Waters Empower 2.0 software (Waters Corp., Milford, MA, USA) as described by Taivosalo et al. [28]. The absolute concentrations of AA were calculated using standard curves and expressed as mmol kg^{-1} of cheese for total FAA and relative content as mol% for individual FAA.

2.5. Analysis of Volatiles by SPME-GC-TOF-MS

The extraction of volatile compounds from RO-cheese samples was carried out using the headspace solid-phase microextraction (HS-SPME) method based on Bezerra et al. [33]. Grated cheese was measured (0.1 g) into a 20 mL glass autosampler vial capped with a PTFE/silicone septum. Vials were preincubated at 40 °C for 5 min. A SPME fiber (30/50 µm DVB/Car/PDMS Stableflex, length 2 cm; Supelco, Bellefonte, PA, USA) was used to extract the volatile compounds from the headspace for 20 min under stirring at 40 °C.

Identification of cheese volatiles was performed using a Micromass GCT Premier gas chromatograph system (Waters, Milford, MA, USA) coupled with a CombiPAL autosampler (CTC Analytics AG, Lake Elmo, MN, USA). After the SPME procedure, the volatile compounds were desorbed in splitless mode into a GC injection port equipped with a 0.75 mm internal diameter liner at 250 °C for 10 min. A DB5-MS column (30 m length × 0.25 mm i.d. × 1.0 µm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a flow rate of 1.0 mL min^{-1}. GC conditions were based on the method employed by Lee et al. [34]. The initial oven temperature was 35 °C with a holding time of 3 min. Then, the oven was programmed to ramp-up from 35 °C at a rate of 5 °C min^{-1} to 110 °C, and then from 110 °C at a rate of 10 °C min^{-1} to a final temperature of 240 °C with an additional holding time of 4 min (35 min of total run time). Mass spectra were obtained at

ionization energy of 70 eV and a scan speed of 10 scans s^{-1}, with a mass-to-charge ratio scan range of 35 to 350. Three analytical replicates were used for each cheese.

Nontargeted identification of volatile compounds was carried out using the ChromaLynx application (version 4.1; MassLynx software; Waters, Milford, MA, USA) and theoretical calculation of Kovats retention indices (RI). Theoretical RI were calculated using the retention times (RT) of the eluting compounds normalized to the RT of adjacent n-alkanes. Accurate identification of the compounds was verified by comparing theoretical RI to the NIST database (US Department of Commerce, Gaithersburg, MD, USA). The quantities of identified compounds were expressed in peak area units (AU) and as a percentage of a peak area against total ion chromatogram (%TIC).

2.6. Sensory Analysis

Descriptive sensory analysis was performed by a local sensory panel of eight trained assessors. The testing rooms were in compliance with ISO standard [35]. In total, 35 attributes (11 for odor, five for appearance, 14 for flavor, and five for texture) were assessed on a scale of 0−15. Commercial 6-month-old Old Saare (Saaremaa Dairy Factory, Kuressaare, Estonia), made with both mesophilic and thermophilic starters, and 8-month-old Gouda (Valio Eesti AS, Võru, Estonia) cheeses were chosen as references. A complete list of sensory attributes, attribute definitions, and anchor-values of the reference materials on the scale is presented in Table 1.

For sensory analysis, cheese samples were cut into 6 cm × 1 cm × 1 cm pieces. Three pieces of each sample were served to panel members on a white plate. As an exception, appearance attributes were assessed separately from a cross-section of the cheese wheel. Randomized blind-tasting with sequential monadic order of presentation was used. Two replicate assessments were done for each cheese sample. Water was provided as a palate cleanser between the samples.

Table 1. A complete list of sensory attributes, attribute definitions, reference materials, and their anchor-values on the scale.

Sensory Attributes	Attribute Definition	Reference	
		Commercial 6-Month-Old Old Saare Cheese	Commercial 8-Month-old Gouda Cheese
Appearance			
Color	Indicates overall color hue of the sample. The highest score on the scale implies deep orange hue of the sample; the lowest score – off-white hue of the sample	8	15
Hole size	Indicates the average size of holes. The attribute is assessed from the cross-section of a cheese wheel The highest score on the scale implies the presence of large holes in the cross-section; the lowest score—no holes are present in the cross-section of a cheese wheel	0	5
Hole shape	Indicates uniformity and roundness of holes. The attribute is assessed from the cross-section of a cheese wheel. The highest score on the scale implies that the holes (if present) are all round and even; the lowest score—the holes (if present) and misshapen and uneven	0	15
Hole distribution	Indicates the degree of evenness of hole distribution. The attribute is assessed throughout the cross-section of a cheese wheel. The highest score on the scale implies that there is an even distribution of the holes (if present); the lowest score – uneven distribution of the holes (if present)	0	15
Hole merging	Indicates the degree of hole merging (webbing). The attribute is assessed throughout the cross-section of a cheese wheel. The highest score on the scale implies that there is a sever merging of holes; the lowest score—the absence of visible merging	0	0
Odor *			
Intensity	Indicates the strength of the overall perceived odor	8	12
Milk	Indicates overall strength of odor characteristic to milk-based products	8	5
Sour	Indicates overall strength of all sour odors	6	3
Sweet	Indicates overall strength of all sweet odors	5	10
Buttery	Indicates the strength of odor sensation characteristic to butter	5	2
Animalic	Indicates the strength of odor sensation characteristic to musky civet and castoreum [1]	1	0
Sulfur	Indicates the strength of odor sensation characteristic to rotten eggs [1]	0	0
Animal feed	Indicates the strength of odor sensation characteristic to cattle feed yards [1]	0	0
Rancid	Indicates the strength of odor sensation characteristic to products of oxidation [1]	0	0
Yeasty	Indicates the strength of odor sensation characteristic to active yeast [1]	0	0
Metallic	Indicates the strength of odor sensation characteristic to metal or steel [1]	0	0
Flavor *			
Intensity	Indicates the overall strength of perceived flavor (basic taste + retronasal olfaction)	10	13
Sweet	Indicates the strength of overall sweet sensation (basic taste + retronasal olfaction)	12	8
Caramel	Indicates the strength of retronasal olfaction sensation characteristic to caramel, which is formed as a result of cheese maturation	0	5
Sour	Indicates the strength of overall sour sensation (basic taste + retronasal olfaction) characteristic to acids formed as a result of fermentation	4	6

Table 1. *Cont.*

Sensory Attributes	Attribute Definition	Reference	
		Commercial 6-Month-Old Old Saare Cheese	Commercial 8-Month-old Gouda Cheese
Bitter	Indicates the strength of bitter taste characteristic to small peptides in cheese (basic taste)	2	3
Salty	Indicates the strength of salty taste characteristic to table salt (basic taste)	5	8
Umami	Indicates the strength of savory taste characteristic to monosodium glutamate (basic taste)	8	6
Milk	Indicates the strength of retronasal olfaction characteristic to milk-based products	8	4
Animalic	Indicates the strength of retronasal olfaction sensation characteristic to musky civet and castoreum [2]	2	0
Sulfur	Indicates the strength of retronasal olfaction sensation characteristic to rotten eggs [2]	0	0
Animal feed	Indicates the strength of retronasal olfaction sensation characteristic to cattle feed yards [2]	0	0
Rancid	Indicates the strength of retronasal olfaction sensation characteristic to products of oxidation [2]	0	0
Yeasty	Indicates the strength of retronasal olfaction sensation characteristic to active yeast [2]	0	0
Metallic	Indicates the strength of sensation in the mouth characteristic to metal or steel [2]	0	0
Texture			
Crumbliness	Indicates the number of particles released when breaking the sample in half. The highest score on the scale implies that no particles were released when breaking the sample in half (the sample does not crumble); the lowest score implies a significant release of particles (the sample crumbles)	3	1
Hardness	Indicates the force required to bite through the sample. The highest score on the scale implies that a lot of force is required to make an initial bite through the sample (the samples is hard); the lowest—barely any force is required to bite through the sample (the samples is soft)	5	10
Rubbery	Indicates the rubbery texture characteristic to squeaky cheeses. The highest score on the scale implies that the sample texture is the least similar to that of squeaky cheeses (the samples is not rubbery); the lowest score on the scale implies that the sample texture is similar to that of squeaky cheeses (the samples is rubbery)	3	7
Adhesiveness	Indicates the amount of sample particles that remain on the teeth after chewing the sample for 5 times. For the adhesiveness assessment, a bite of approx. 1 cm × 1 cm piece should be taken. The highest score on the scale implies that the sample leaves behind a significant residue on the teeth after chewing and swallowing (the samples is adhesive); the lowest—no residue is left behind on the teeth after chewing and swallowing (the sample is not adhesive)	2	8

* The highest score on the scale implies very intense sensation; the lowest score – no sensation.[1] Possible off-odor; [2] possible off-flavor.

3. Results and Discussion

A cheese, produced in this study from RO-milk concentrated 1.9-fold on a dry matter basis, was characterized as hard cheese, with the calculated moisture in nonfat substance (MNFS) content of 45.5% (w/w) and fat content of 37.1% (w/w) as determined in 0.5-month-old cheese and pH ranging from 5.10 to 5.23 during ripening for six months.

3.1. Proteolysis During RO-Cheese Ripening

Figure 1 shows the electropherograms of the intact CN fractions (α_{s1}-CN (8P and 9P), α_{s2}-CN (11P, 12P, and nP), β-CN (genetic variants A^1, A^2, and B), and para-κ-CN), and their primary hydrolysis products (α_{s1}-I-CN (8P and 9P), γ_1-CN (A^1 and A^2), and γ_2-CN and γ_3-CN) identified in RO-cheese during ripening.

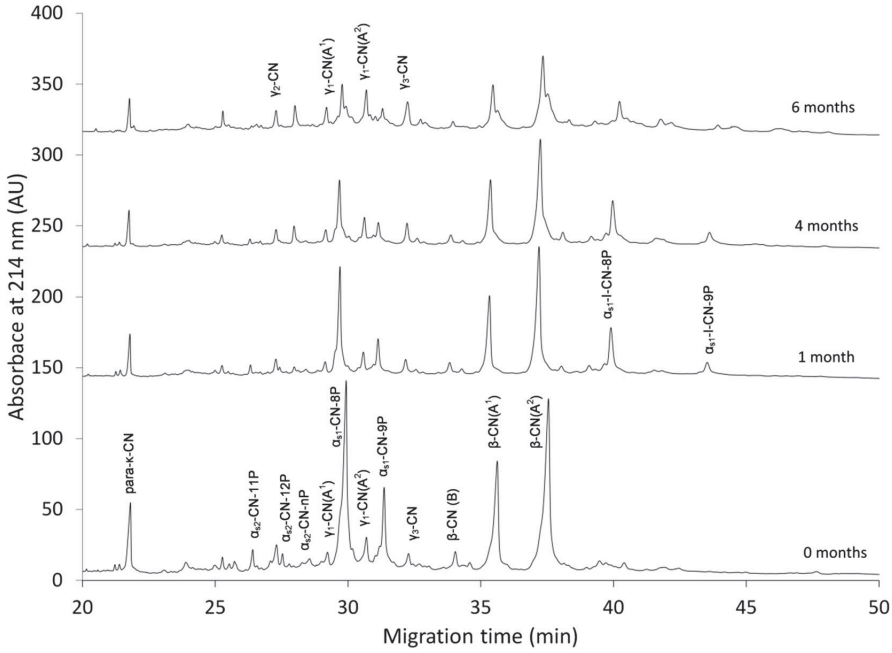

Figure 1. The electropherograms of RO-cheese obtained by CE at 0, 1, 4, and 6 months of ripening. CN: casein; para-κ-CN: κ-CN f1−105; γ_1-CN: β-CN f29−109; γ_2-CN: β-CN f106−209; γ_3-CN: β-CN f107−209; α_{s1}-I-CN-8P: α_{s1}-CN f24−199; α_{s1}-I-CN-9P: α_{s1}-CN f24−199 9P; A^1, A^2, and B: genetic variants of β-CN; 11P, 12P, 9P, 8P, and nP: phosphorylation states of caseins.

Intact α_{s1}-CN was subjected to more rapid degradation than β-CN: approximately 60% of α_{s1}-CN breakdown was observed within the first month and 70% was hydrolyzed by the end of the sixth month of ageing (Figures 1 and 2a). The peaks corresponding to β-CN also showed obvious changes throughout ripening, but still ~55% of the initial β-CN remained intact by the end of the ripening period. The rate of the hydrolysis of the initial CN fraction in RO-cheese was the highest during the first months of ripening (Figure 2).

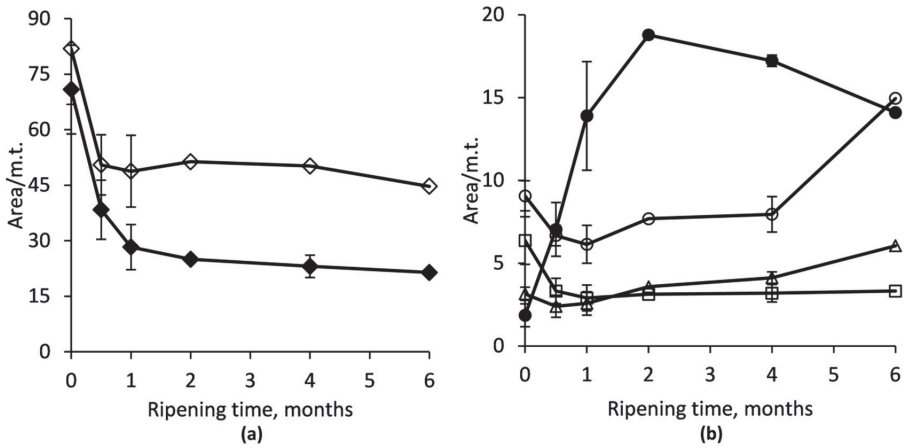

Figure 2. Change of the content (normalized peak area) of main intact CN (**a**) and their primary degradation products (**b**) during RO-cheese ripening. α_{s1}-CN (⧫): sum of α_{s1}-CN-8P and α_{s1}-CN-9P; β-CN (◊): sum of β-CN(A^1), β-CN(A^2), and β-CN(B); α_{s1}-I-CN (●): sum of α_{s1}-I-CN-8P and α_{s1}-I-CN-9P; γ_1-CN (○): sum of γ_1-CN(A^1) and γ_1-CN(A^2); (□): γ_2-CN; (Δ): γ_3-CN; m.t., migration time.

Due to the lack of published data on RO-cheeses and the large diversity of the manufacturing parameters of traditional cheeses, a direct comparison of the ripening characteristics of our RO-cheese to those of other cheeses was fairly complicated. Nevertheless, it was instantly evident that the course of primary proteolysis in RO-cheese was comparable with those of traditional cheeses. In traditional cheese varieties with similar manufacturing technology, primary proteolysis is characterized by a rapid breakdown of intact caseins to high molecular weight peptides by an activity of both the rennet (chymosin) retained in the curd and the milk native proteinase plasmin. The addition of rennet based on the initial amount of milk used for RO-milk production resulted in an adequate degree of primary degradation of α_{s1}-CN in our RO-cheese (Figures 1 and 2a), which is consistent with results on proteolysis in RO-Cheddar cheese [23]. Quite similar results on the breakdown of α_{s1}-CN have been reported in a traditional hard cheese Old Saare, with manufacturing and ripening conditions very similar to our RO-cheese (a high curd scalding temperature and both mesophilic and thermophilic LAB as starters), where the same share—~70% of α_{s1}-CN—was hydrolyzed during six months of ripening [28]. In 26-week-ripened traditional extra-hard Västerbottenost cheese, manufactured with high scalding temperature and mesophilic starter, the initial peaks of both α_{s1}- and β-CN had almost completely disappeared according to the CE profiles [36]. Proteolysis in a typical Gouda-type cheese is determined by an ~70−80% decrease in α_{s1}-CN content, mainly by the action of chymosin, already within the first month of ripening [37–39], which is consistent with our results for α_{s1}-CN degradation; during the production of RO-cheese, the curd scalding temperature was higher (48 °C) compared to Gouda-type cheeses, which could have caused a partial inactivation of chymosin that affected the degradation of α_{s1}-CN [40]. Indeed, a rather high percentage of α_{s1}-CN breakdown determined in our cheese can be associated with a rather low pH of the cheese environment (in the range of 5.10 to 5.23 during the ripening period) being more favorable for the activity of chymosin as well as also the reversible and incomplete thermal inactivation of chymosin after cooking the cheese curds at 48 °C [40,41].

The substantial contribution of plasmin to the primary proteolysis in cheeses with a high curd cooking temperature is evident [42]. Nevertheless, conversely to α_{s1}-CN degradation, significantly less β-CN was hydrolyzed in RO-cheese (45% of intact β-CN hydrolyzed by the end of ripening) compared to the levels reported in Old Saare and even in Gouda-type cheeses (78% and 60−50% of

β-CN hydrolyzed by six months of ripening, respectively) [28,37–39]. The greater activity of plasmin on β-CN could be expected in RO-cheese, as high cooking temperature inactivates inhibitors of plasminogen activators, leading to the conversion of plasminogen to the active plasmin [43]. Both the higher curd scalding temperature (52 °C) and higher pH during ripening (5.3–5.5) of Old Saare [28], as well as in Västerbottenost cheeses (5.3–5.6) [36], than that of RO-cheese could have exerted an impact on more intensive primary hydrolysis of β-CN in those cheeses [43]. Considerable lower pH values in RO-cheese were evidently decisive for the reduced activity of the alkaline proteinase, plasmin [43], which resulted in impaired hydrolysis of β-CN in RO-cheese.

Large peptides obtained from the primary hydrolysis serve as substrates for subsequent generation of shorter peptides and FAA by complex action of different proteolytic enzymes from LAB [39]. Figures 1 and 2b show that the production rate of chymosin-derived peptide α_{s1}-I-CN was the highest during the first two months, while during the next four months of ageing, the degradation rate of that peptide became higher than the production. At the beginning of the ripening, the content of plasmin-derived peptides γ-CNs decreased moderately during the first month, suggesting that they could have been more rapidly degraded further to shorter peptides by the LAB enzymes. The γ-CNs accumulated during further months of aging, showing an increase in the production rate in the period between four and six months (Figures 1 and 2b).

The final step of proteolysis is the release of FAA, those enzymatic and chemical conversions to volatile compounds lead to the development of the characteristic cheese flavor [39,44]. The total and relative (mol%) content of FAA released during six months of RO-cheese ripening is shown in Figures 3 and 4, respectively.

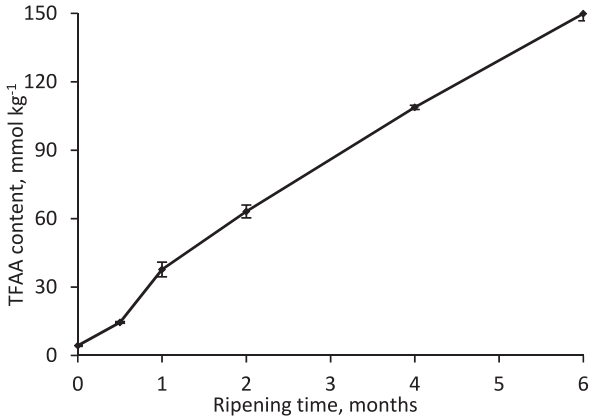

Figure 3. The change in total free amino acids (TFAA) content during RO-cheese ripening.

The total content of the FAA increased from 4.3 ± 0.3 to 149.9 ± 3.2 mmol kg^{-1} of cheese during maturation. The use of a thermophilic starter in cheese production could have considerably increased the total FAA content [45,46] in RO-cheese. Nevertheless, the level of total FAA in 6-month-old RO-cheese was three-fold lower than that in hard Old Saare cheese (450 mmol kg^{-1} of cheese) [28], it is also made with both mesophilic and thermophilic starters but shows intensive degradation of β-CN, unlike the RO-cheese. Moreover, the total FAA content in RO-cheese was somewhat lower than that in 6-month-old extra-hard Västerbottenost (150–260 mmol kg^{-1}) and semihard Herrgård (190 mmol kg^{-1}) cheeses manufactured only with mesophilic starters [36,46]. This clearly reveals that the lower total FAA content in RO-cheese can be attributed to the lower degree of hydrolysis of one of the main casein—β-CN—on the first stage of proteolysis mediated by plasmin activity.

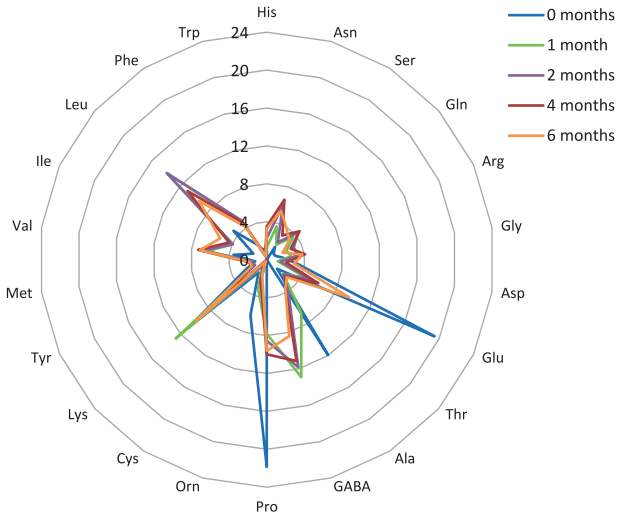

Figure 4. Radar diagram showing the relative content of individual free amino acids (FAA) during six months of RO-cheese ripening (mol%). Results presented are the means of two measurements. Amino acids: GABA, γ-aminobutyric acid; Ala, alanine; Thr, threonine; Glu, glutamate; Asp, aspartate; Gly, glycine; Arg, arginine; Gln, glutamine; Ser, serine; Asn, asparagine; His, histidine; Trp, tryptophan; Phe, phenylalanine; Leu, leucine; Ile, isoleucine; Val, valine; Met, methionine; Tyr, tyrosine; Lys, lysine; Cys, cysteine; Orn, ornithine; and Pro, proline.

The quantitatively dominating amino acids in the ripened 6-month-old RO-cheese cheese were Lys, Pro, Glu, Leu, and γ-aminobutyric acid (GABA) (Figure 4). The first four were found at very similar concentrations (14.2–14.8 mmol kg^{-1} of cheese). It is noteworthy that the same four AA have been shown to be among the quantitatively dominating ones in matured Old Saare cheese [28]. A large amount of Pro (14.4 mmol kg^{-1} in ripened RO-cheese) obviously produced by peptidases of *Lb. helveticus*, may introduce the sweet note in the cheese flavor profile [47]. Amino acid Val, reported to be within the quantitatively dominating amino acids in ripened Old Saare cheese [28], was determined within the dominating ones in the middle of the RO-cheese ripening process (2–4 months), and may contribute to the bitter and sweet flavors of cheese [47]. High levels of Lys and Leu could have contributed to the bitter note [47] in the taste of ripened RO-cheese. GABA was determined in large amounts from the first month of ripening. GABA is not originally present in caseins, but several LABs, including *St. thermophilus* and *Lb. helveticus*, have been shown to exert a GABA-producing ability through the decarboxylation of Glu [47,48].

3.2. Volatile Compounds

A total of 42 volatile compounds were identified in RO-cheese: nine alcohols, six ketones, seven acids, seven esters, six aldehydes, four aromatic compounds, and three of other groups. Table 2 shows the volatile compounds grouped by classes and the changes in content throughout the ripening process. Volatile acids and alcohols were the main volatiles identified in RO-cheese (1.5–46.3 and 0.0–10.4%TIC, respectively). Esters and ketones comprised the smaller share (0.0–6.7 and 0.0–2.9%TIC, respectively, except for the ketones at the initial point of ripening), while aldehydes and aromatic compounds were among the minor ones (0.0–0.3 and 0.0–0.4%TIC, respectively). The identified volatile compounds showed a dynamic behavior during cheese maturation; however, the total amounts within most detected chemical groups (except for the ketones and aromatic compounds) increased by the end of ripening (Figure 5).

Table 2. Volatile compounds identified in RO-cheese during ripening (AU × 10^4). Results are the means of three GC-MS measurements. AU, arbitrary units (peak area).

Compound	RT	RI, Exp	RI, Theor [1]	Odor Description [2]	Ripening Time, Months					
					0	0.5	1	2	4	6
Alcohols										
Isopropyl Alcohol	2.15	510	515	Woody, musty	–	t	–	–	t	0.10
2-Butanol, 3-methyl	7.92	715	700	Musty, vegetable, cheesy	1.34	–	–	–	–	–
3-Buten-1-ol, 3-methyl-	8.33	725	720	Fruity	–	0.04	–	–	–	–
1-Butanol, 2-methyl-	8.34	726	740	Fruity, whiskey	–	–	–	–	–	0.04
1-Butanol, 3-methyl-	8.80	738	750	Fruity, banana, cognac	–	–	–	–	0.09	0.18
2,3-Butanediol	10.34	778	779	Creamy, buttery	0.15	1.72	–	1.73	t	0.86
1,3-Butanediol	10.96	794	789	Odorless	–	–	–	0.32	2.57	2.47
1-Hexanol, 2-ethyl	18.93	1009	1025	Fruity, floral, fatty	0.02	0.03	–	–	–	0.03
1-Undecanol	26.03	1358	1370	Soapy, citrus	–	0.04	–	–	–	–
Total					1.51	1.85	0.00	2.05	2.66	3.69
Aldehydes										
Butanal, 3-methyl-	5.49	640	652	Fruity, cocoa, nutty	–	–	–	–	–	0.03
Butanal, 2-methyl-	5.78	650	664	Musty, nutty, fermented	–	–	–	–	–	0.06
Octanal	18.49	995	1000	Citrus, orange peel, waxy	–	0.06	–	–	–	–
Nonanal	20.97	1085	1099	Citrus, green, cucumber	0.01	0.03	0.01	–	t	–
Decanal	23.08	1183	1188	Citrus, orange peel, waxy	0.02	–	–	–	–	–
Undecanal	25.07	1295	1310	Soapy, citrus	–	0.02	–	–	–	–
Total					0.03	0.12	0.01	0.00	0.00	0.09
Ketones										
Acetone	2.12	509	509	Solvent, apple, pear	0.24	0.30	–	–	–	–
2,3-Butanedione	3.68	577	574	Buttery, creamy, milky	0.17	–	–	0.17	–	0.94
2-Pentanone	7.26	697	697	Fruity, banana, fermented	7.79	–	–	–	–	–
2-Butanone, 3-hydroxy	7.42	711	706	Creamy, dairy, butter	–	–	t	–	0.13	0
2-Heptanone	14.00	873	887	Cheesy, fruity, banana	0.04	–	–	–	0.01	0.06
2-Nonanone	20.62	1072	1090	Fruity, dairy, soapy	t	–	–	–	–	–
Total					8.23	0.30	0.00	0.17	0.15	1.01
Acids										
Acetic acid	5.25	633	640	Pungent	0.71	t	0.47	4.05	14.02	12.62
Propanoic acid	7.11	692	700	Pungent, dairy	–	–	–	–	0.00	–
Propanoic acid, 2-methyl-	9.15	747	758	Acidic, cheesy, dairy	–	–	–	–	0.05	0.04
Butanoic acid	10.14	773	790	Sharp, cheesy	0.01	0.00	003	0.50	1.17	t
Butanoic acid, 3-methyl	12.35	830	848	Cheesy, dairy, fermented, berry	–	–	–	0.00	0.38	0.21
Butanoic acid, 2-methyl	12.74	840	846	Fruity, dairy, cheesy	–	–	–	–	0.05	0.09
Hexanoic acid	17.34	963	990	Fatty, cheesy	–	t	–	0.05	0.11	–
Total					0.72	t	0.50	4.60	15.80	12.95

Table 2. *Cont.*

Compound	RT	RI, Exp	RI, Theor [1]	Odor Description [2]	Ripening Time, Months					
					0	0.5	1	2	4	6
Esters										
Ethyl Acetate	4.44	607	610	Ethereal, fruity, grape, cherry	–	t	0.01	–	t	t
Butanoic acid, ethyl ester	10.91	793	798	Fruity, sweet, apple	–	0.07	0.04	t	0.55	2.03
Butanoic acid, butyl ester	18.09	984	994	Fruity, banana, pineapple	–	–	–	0.08	–	0.02
Hexanoic acid, ethyl ester	18.23	988	996	Fruity, banana, pineapple, green	–	–	0.06	0.13	0.06	0.23
Octanoic acid, ethyl ester	23.00	1179	1190	Fruity, pineapple, musty	–	–	–	–	t	0.07
Decanoic acid, ethyl ester	26.27	1373	1381	Fruity, apple	–	–	–	–	t	0.02
Dodecanoic acid, ethyl ester	28.97	1572	1581	Floral, creamy, dairy, soapy	–		–	–	–	0.01
Total					–	0.07	0.11	0.20	0.61	2.37
Aromatics										
Toluene	9.43	770	774	Sweet, pungent, caramel	t	0.02	0.01	–	0.01	–
Benzaldehyde	16.87	950	955	Almond, cherry	0.06	0.06	0.02	–	0.00	–
Acetophenone	20.17	1055	1062	Almond, cherry, fruity, floral	t	0.02	0.04	0.02	0.02	–
Indole	24.84	1282	1292	Animal, fecal	t	–	–	–	–	–
Total					0.06		0.10	0.02	0.03	–
Others										
n-Butyl ether	14.05	883	888	Ethereal	–	t	–	0.07	0.01	0.05
Dimethyl sulfone	14.94	897	918	Sulfur	t	0.06	–	–	0.03	0.03
Dimethyl sulfide	2.45	523	530	Sulfur, onion, cabbage	t	0.02	t	–	–	–
Total					t	0.08	t	0.07	0.05	0.09

[1] NIST database (US Department of Commerce, Gaithersburg, MD, USA). [2] The Good Scents Company Information System (Oak Creek, WI, USA). t (traces), in nonquantifiable amounts; –, not detected.

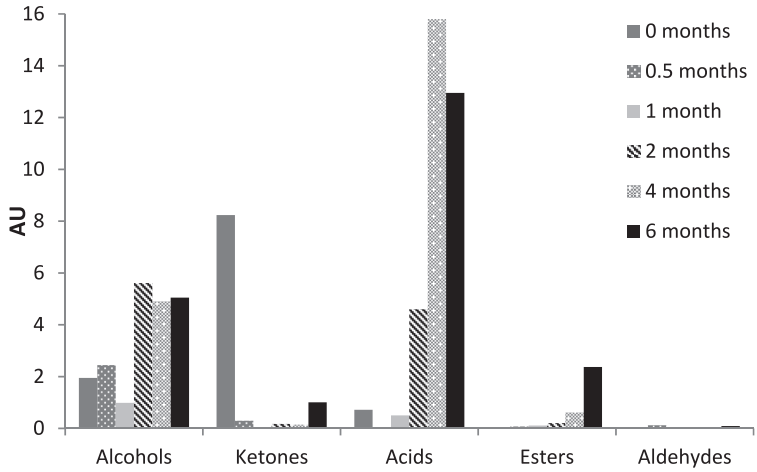

Figure 5. Changes in the content of the main chemical groups of the volatile compounds (AU × 10^4) identified during RO-cheese ripening. AU: arbitrary units (peak area).

3.2.1. Carboxylic Acids

The total amount of volatile carboxylic acids increased during the ripening of RO-cheese for six months, with a slightly higher content in 4-month-old than in 6-month-old cheese (Figure 5). The relative content of carboxylic acids was the highest in the 4-month-old cheese (46.3%TIC), but also retained high levels toward the end of the ripening period. Acetic acid was the most abundant compound with a very high relative content (1.3–41.0%TIC) during all stages of cheese maturation. The content of acetic acid increased greatly after one month of ripening (Table 2). Acetic and propanoic acids may have a microbial origin and could have been formed as a product of lactose metabolism [44]. Hexanoic acid was present in relatively low levels, exhibiting an ascending tendency solely in the middle of RO-cheese ripening until disappearing after the fourth month of ripening. Butanoic acid was present in comparable amount at the beginning of ripening and showed a substantial increase in amounts up to the fourth month of maturation and disappeared thereafter. Short-chain fatty acids and butanoic and hexanoic acids could be produced as a result of the enzymatic hydrolysis of triglycerides, which is also one of the biochemical pathways essential for cheese flavor development [35]. It is likely that the linear-chain fatty acids could have been transformed into esters [49] since their content was higher at the end of RO-cheese ripening (Figure 5). The main branched-chain fatty acids in RO-cheese—2- and 3-methylbutanoic acid—were present in substantial amounts after four months of ripening (Table 2) and could provide sweaty, sour, fruity, and buttery flavor notes [47,49]. Methylated acids as well as methylated aldehydes and methylated alcohols have a proteolytic origin and, obviously, are produced by the catabolism of branched-chain amino acids (Leu, Ile, and Val) by aminotransferases [47,49]. The prompt decreased in the relative content of Leu in RO-cheese from the fourth month of ripening could be associated with the higher content of 3-methyl-butanoic acid, 3-methyl-1-butanol, and 3-methyl-butanal in ripened 4- and 6-month-old RO-cheeses. In addition, the relative content of Val did not increase much after the second month of ripening, which correlated to the appearance of 2-methylpropanoic acid. Volatiles 2-methylbutanoic acid, 2-methyl-1-butanol, and 2-methyl-butanal originate from Ile, but we could not relate the occurrence of these components in the ripened RO-cheeses to the content of Ile, as it still steadily increased during the RO-cheese maturation. All volatile carboxylic acids identified in RO-cheese (except for propanoic acid) have been previously reported as aroma-active in a wide range of young, medium, and aged traditional Gouda-type cheeses by SPME-GC-Olfactometry [50]. In addition, the same acids (except for acetic and propanoic acid) have been detected in 6-week-old Gouda-type cheeses, while aged cheeses have not shown them at all [51].

Acetic, butanoic, and hexanoic acids, as determined by SPME-GC-Olfactometry, have been found to contribute greatly to the characteristic cheesy sharp and mild to strong savory aroma of typical hard Parmesan and Grana Padano cheeses [52]. Along with the mentioned carboxylic acids, octanoic and decanoic acids were among the abundant aroma compounds in Parmigiano-Reggiano of different ages reported by Bellesia et al. [53].

3.2.2. Alcohols

Alcohols were the second major class of volatile compounds identified in 0-month-old cheese (6.5%TIC) and in cheeses after the second month (up to 10.4%TIC) of ripening (Table 2). The total alcohol content showed an increasing trend throughout cheese maturation up to the second month of ripening with a more rapid change of content after the first month. The total content of alcohols was more or less similar during further RO-cheese ripening (Figure 5). Among the alcohols, 3-methyl-2-butanol was abundant in fresh cheese, whereas in the middle and the later stages of ripening dominated 2,3- and 1,3-butanediol, respectively (Table 2). 2,3-Butanediol could be formed during the citrate or Asp metabolism from 2,3-butanedione (diacetyl) [44], and has been previously reported to be among the important flavor compounds in 0.5- and 4-month-old Gouda cheese [51]. Branched-chain alcohols—2-methyl-1-butanol and 3-methyl-1-butanol—and their corresponding methylated aldehydes—2- and 3-methylbutanal—identified in RO-cheese have been previously detected among the aroma-active compounds in extra-hard Västerbottenost [36], as well as in young and aged Gouda-type cheeses [50,51]. The levels of these compounds were reported to be higher in the matured cheeses, which is consistent with our results obtained for RO-cheese, where these alcohols were detected after four months of ripening. 3-Methyl-2-butanol, 3-methyl-1-butanol, and 2,3- and 1,3-butanediol were detected at trace amounts or among the less abundant alcohols in Parmigiano-Reggiano [53].

3.2.3. Esters

Esters were found in relatively small amounts in the volatile fraction of RO-cheese. This chemical group was presented at a low level from the second week until the second month of ripening and then increased at the later stages of cheese ripening (Figure 5). Butanoic acid ethyl ester and hexanoic acid ethyl ester were dominant among the esters during the entire period of ripening (Table 2). The former was present especially in high amounts in the 4- and 6-month-old cheese and composed 1.6 and 5.7%TIC, respectively. Octanoic acid, decanoic acid, and dodecanoic acid ethyl esters were found only in the 6-month-old cheese. Ethyl esters originate from the enzymatic or chemical esterification of the fatty acids and characterize a cheese by sweet and fruity notes [44]. Butanoic acid ethyl ester and hexanoic acid ethyl ester have been shown to have the highest contribution to the cheese flavor within the esters in young and matured Gouda [50] and hard Parmesan-type and Grana Padano [52,53]. Other ethyl esters identified in RO-cheese have also been quantified, but only in matured Gouda-type cheeses [51], which is in agreement with our results.

3.2.4. Ketones

Ketones were the most abundant class in fresh cheese (35.4%TIC), showing very high amounts compared to the other chemical groups of volatile compounds identified in RO-cheese, mainly because of 2-pentanone (Table 2 and Figure 5). The level of ketones decreased promptly by the second week of ripening. Ketones were found in low levels at the middle stages of maturation. A substantial amount of ketones was observed again at the end of the ripening process in the 6-month-old RO-cheese, mainly because of the increase in content of 2,3-butanedione, which was observed at all stages of ripening. 2,3-butanedione (diacetyl) is one of the products of citrate or Asp metabolism with a sweet buttery aroma [44] and has been demonstrated to be an important aroma-active compound in hard Parmesan cheese [52], considered to be characteristic to Gouda cheeses of different ages [50]. Methyl ketones (2-pentanone, 2-heptanone, and 2-nonanone) can be produced from fatty acids through

β-decarboxylation and may be transformed to secondary alcohols [44]. Methyl ketones have been identified as important constituents in blue-cheese aroma [52], although small amounts of those have also been observed in some Gouda [50,51] and Parmigiano-Reggiano of different ages [53]. Moreover, methyl ketones have been found in the fraction of volatile ketones in Västerbottenost cheese with the highest abundance of 2-pentanone [36].

3.2.5. Aldehydes

Aldehydes were present among the volatiles of RO-cheese in very small amounts with a fluctuating behavior during ripening (Table 2). The total content of aldehydes was the highest both in the 0.5- and 6-month-old cheese (0.12 and 0.09%TIC) (Figure 5). Only 2- and 3-methylbutanal were detected in matured 6-month-old cheese, whereas cheeses up to one month of ripening contained the linear-chain aldehydes octanal, nonanal, decanal, and undecanal. Aldehydes are minor volatile components present at low levels in cheese because they are rapidly converted to alcohols or corresponding acids [44]. Aldehydes 2-methyl-butanal and 3-methyl-butanal are the products of the catabolism of branched-chain amino acids Ile and Leu, and have been shown to give the malty, fruity, cocoa, and nutty flavors to cheese [47,49]. The decreasing relative content of Leu in RO-cheese after the fourth month of ripening (Figure 3) can be related to the appearance of low levels of these aldehydes due to their further rapid transformation into the corresponding alcohols 2- and 3-methyl-1-butanol or 2- and 3-methylbutanoic acids (Table 2). The above-mentioned aldehydes have been found among the strong aroma-active compounds at higher concentrations in matured 9- and 10-month-old Gouda than in younger cheeses [50]. In addition, 2-methyl-butanal and 3-methyl-butanal have been detected in Västerbottenost [36] and in some Parmigiano-Reggiano cheeses [53].

3.2.6. Aromatics and Other Compounds

Among the aromatic compounds benzaldehyde, toluene, and acetophenone were found in RO-cheeses up to the fourth month of ripening with a larger share of benzaldehyde in the two first ripening points. Benzaldehyde can be produced from the aromatic amino acid Phe via the α-oxidation of phenyl acetaldehyde and have been shown to give notes of bitter almond to aged Gouda [50,51], Parmigiano-Reggiano [53], and Västerbottenost cheeses [36]. Acetophenone has been found in aged Gouda [50]. Sulfur compounds derive from the amino acid Met and are essential components in many cheeses, giving the boiled cabbage and potatoes, garlic, and egg flavors [47].

3.3. Sensory Properties

Within sensory perception, an appearance modality revealed the most obvious changes during RO-cheese maturation. The 6-month-old cheese was considerably darker and richer in color than the young RO-cheeses (Figure 6). The size of holes within the RO-cheese matrix grew rapidly during the first month of ripening and then remained relatively the same throughout maturation. The distribution of holes within the cheese matrix became more uniform after the first month of ripening, and some partial merging of the holes was noticed at all ripening stages. The rubberiness decreased, and crumbliness increased dramatically during ripening. In matured 6-month-old RO-cheese, small, white crystals were observed and perceived in the interior of the cheese and on the surface of the holes. The crystals are commonly formed due to the crystallization of amino acids, e.g., Tyr, or calcium lactate, when lactobacilli-containing (including *Lactobacillus helveticus*) starters are used, and have been shown to occur after prolonged maturation of Gouda-type, Cheddar, and Parmesan cheeses [38,54].

Figure 7 shows the principal component analysis (PCA) carried out on the scores of RO-cheese odor and taste evaluation. The first two principal components (PC) explained 81.49% of the variability (PC1: 58.72%; PC2: 22.77%). The odor and taste attributes were related more to the younger, up to 2-month-old cheeses located on the negative axis of PC1, whereas on the positive axis, the attributes received high scores for the matured 4- and 6-month-old RO-cheeses. Young cheeses were characterized by high scores of milky odor and taste and buttery odor, which diminished with maturing time. With

maturation, the cheese became more intense in overall intensity (14 out of 15 for odor; 12 for taste), sweetness (8 for odor; 10 for taste), saltiness (seven), and umami taste (seven), and gained low scores for bitterness (three) and caramel taste (two). A slight yeasty odor and flavor (both scored 0.5) were noted in the ripened 6-month-old cheese. These results of the sensory evaluation of odor and taste of ripened 6-month RO-cheese were comparable with those reported for aged 9- and 12-month-old traditional Gouda, where cheeses have been characterized by sweet, salty, and umami tastes and low intensities of caramel and fruity notes [50]. However, an opposite trend in the development of the sweet attribute in cheese maturation, compared to the RO-cheese, has also been observed in Gouda cheese [51].

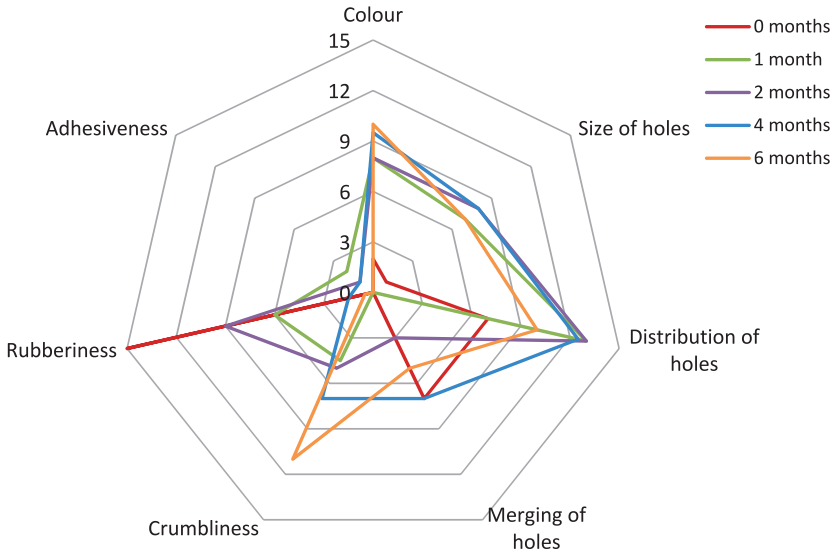

Figure 6. Radar diagram of the appearance and texture attributes of RO-cheese during ripening.

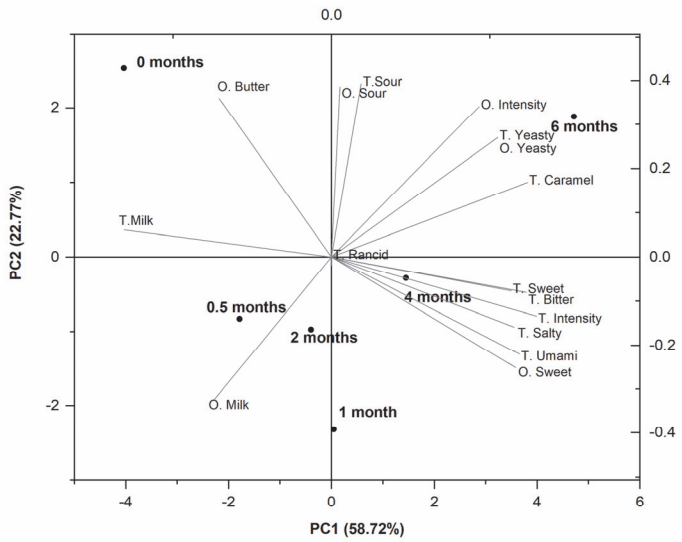

Figure 7. Principal component analysis (PCA) biplot of odor (O) and taste (T) attributes of RO-cheese during ripening. Cheeses are indicated by ripening time. PC: principal component.

No defects in descriptive texture and no off-flavor formation were detected during the descriptive sensory analysis of RO-cheese.

4. Conclusions

In the present study, the hard high cooked cheese was produced in a pilot plant from 1.9-fold concentrated RO-milk. The RO-cheese produced and evaluated in this study was considered to be of satisfactory quality based on sensory testing by a panel of trained assessors. The evaluation of the primary proteolysis, formation of FAA and volatile compounds proved that the patterns of biological processes essential for cheese maturation which took place in RO-cheese are essentially similar to those in traditional Gouda-type and other hard-type cheeses.

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Curriculum vitae

Personal data

Name:	Anastassia Taivosalo
Date of birth:	02.10.1985
Place of birth:	Tallinn, Estonia
Citizenship:	Estonian

Contact data

E-mail:	anastassia@tftak.eu

Education

2009–current	Tallinn University of Technology. School of Engineering. PhD studies
2007–2009	Tallinn University of Technology Faculty of Chemical and Materials Technology, Department of Food Processing. MSc
2004–2007	Tallinn University of Technology Faculty of Chemical and Materials Technology, Department of Food Processing. BSc
1991–2004	Loksa Russian Gymnasium

Language competence

Russian	Native
English	Good
Estonian	Good

Professional employment

2007–current	Center of Food and Fermentation Technologies, Tallinn, Estonia. Research & Development

Special courses

08.–13.07.2018	10th MaxQuant Summer School on Computational Mass Spectrometry-Based Proteomics, Barcelona, Spain
26.08.–02.09.2010	PhD course: Advanced Food Microbiology and 22nd International ICFMH Symposium, Food Micro 2010, Copenhagen, Denmark
23.–27.08.2010	PhD course: Ripening of Northern European Cheeses, Copenhagen, Denmark
13.–19.02.2010	Course: Modern Methods of Data Analysis. St. Petersburg State Technological University of Plant Polymers, Russian Federation
16.–18.09.2009	Course: Nordic Informatics User Training (Waters). Oscarsborg, Norway
24.–28.08.2009	Course: From Milk to Cheese. Norwegian University of Life Sciences, Norway

Supervising

2018	Maarika Tärk. MSc. Characteristics of Proteolysis in Gouda-type Cheese Manufactured with Different Starters
2017	Riin Vent. MSc. Optimization of Soy-Yogurt Technology and Valorisation of Okara
2012	Marin Pugi. MSc. Quantitative Cheese Ripening Proteomics: Mass Balance Equations of Amino acids

Elulookirjeldus

Isikuandmed

Nimi:	Anastassia Taivosalo
Sünniaeg:	02.10.1985
Sünnikoht:	Tallinn, Eesti
Kodakondsus:	Eesti

Kontaktandmed

E-post:	anastassia@tftak.eu

Hariduskäik

2009–…	Tallinna Tehnikaülikool, Inseneriteaduskond. PhD
2007–2009	Tallinna Tehnikaülikool, Keemia ja materjalitehnoloogia, Toidutehnika ja tootearendus. MSc
2004–2007	Tallinna Tehnikaülikool, Keemia ja materjalitehnoloogia, Toidutehnika ja tootearendus. BSc
1991–2004	Loksa Vene Gümnaasium

Keelteoskus

Vene keel	Emakeel
Inglise keel	Kõrgtase
Eesti keel	Kõrgtase

Teenistuskäik

2007–…	Toidu- ja fermentatsioonitehnoloogia arenduskeskus, Tallinn, Eesti. Teadur

Täiendusõpe

08.–13.07.2018	10th MaxQuant Summer School on Computational Mass Spectrometry-Based Proteomics. Praktiline kursus, Barcelona, Hispaania
26.08.–02.09.2010	Doktorikursus: Advanced Food Microbiology and 22nd International ICFMH Symposium, Food Micro 2010, Kopenhaageni, Taani
23.–27.08.2010	Doktorikursus: Ripening of Northern European Cheeses, Kopenhaageni, Taani
13.–19.02.2010	Kursus: Modern Methods of Data Analysis. St. Petersburg State Technological University of Plant Polymers, Venemaa
16.–18.09.2009	Kursus: Nordic Informatics User Training (Waters). Oscarsborg, Norra
24.–28.08.2009	Kursus: From Milk to Cheese. Norwegian University of Life Sciences, Norra

Juhendamine

2018	Maarika Tärk. MSc. Erinevate juuretistega valmistatud Gouda juustude proteolüüsi eripära
2017	Riin Vent. MSc. Sojajogurti valmistamise tehnoloogia optimeerimine ja okara väärindamine
2012	Marin Pugi. MSc. Kvantitatiivne juustu valmimisprotsesside proteoomika - aminohapete bilansivõrrandid