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A Study of Milk Coagulability

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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 any academic degree.

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CONTENTS

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation:

I. Kriščiunaite, T., Stulova, I., Taivosalo, A., Laht, T.-M., and Vilu, R. **Composition and renneting properties of raw bulk milk in Estonia.** *International Dairy Journal, 23(1)*, 45-52 (2012)

II. Kriščiunaite, T., Stulova, I., Kabanova, N., Laht, T.-M., and Vilu, R. **The effect of hydrogen peroxide on the growth of thermophilic lactic starter and acid gelation of UHT milk.** *International Dairy Journal, 21(4),* 239-246 (2011)

III. Kriščiunaite, T., Seiman, A., Laht, T.-M., and Vilu, R. **Front-face fluorescence spectroscopy studying rennet coagulation of milk.** Manuscript

IV. Stulova, I., Kabanova, N., Kriščiunaite, T., Taivosalo, A., Laht, T.-M., and Vilu, R. **Fermentation of reconstituted milk by *Streptococcus thermophilus*: effect of irradiation on skim milk powder.** *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

SUMMARY OF AUTHOR'S CONTRIBUTION

I. The author participated in planning the study and handling of the milk samples, was responsible for evaluation of milk coagulation properties, put together and analysed the data, and wrote the manuscript.

II. The author planned and performed the experiments, analysed the data, and wrote the manuscript.

III. The author planned and performed the experiments, calculated and interpreted the data, and wrote the manuscript.

IV. The author participated in planning the experiments, was responsible for rheological and fluorescence measurements and interpretation of the data, and participated in writing the manuscript.

ADDITIONAL PUBLICATIONS

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Stulova, I., Adamberg, S., Kriščiunaite, T., Kampura, M., Blank, L., and Laht T.-M. **Microbiological quality of raw milk produced in Estonia.** *Letters in Applied Microbiology, 51(6)*, 68-690 (2011)

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ABBREVIATIONS

$(dG'/dt)_{max}$	gelation rate
μ_{max}	maximum specific growth rate
CCP	colloidal calcium phosphate
CE	capillary electrophoresis
CN	casein
EHF	Estonian Holstein-Friesian
ER	Estonian Red
FA	Finnish Ayrshire
G'	elastic or storage modulus
G''	viscous or loss modulus
GDL	glucono-δ-lactone
GMP	glycomacropeptide
HF	Holstein-Friesian
irrLHSMP	irradiated LHSMP
irrRSM	RSM prepared from irrLHSMP
LAB	lactic acid bacteria
LHSMP	low-heat skimmed milk powder
LPO	lactoperoxidase
NIR	near infrared
NS	not significant
PC1/PC2/PC3	principal component one/two/three
PCA	principal component analysis
RFT	rennet flocculation time
RSM	reconstituted skimmed milk
SCC	somatic cell count
SD	standard deviation
SFS	spectral fluorescence signatures
t_{2min}	time to the minimum of the second order derivative
tan δ	loss tangent
TBC	total bacteria count
t_g	gelation time
$t_{G''}$	time to the initial viscosity increase
UHT	ultra-high temperature treated
WP	whey protein
α-LA	α-lactalbumin
β-LG	β-lactoblobulin
γ_0	shear strain
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
τ_0	shear stress

INTRODUCTION

Milk is defined as the secretion of the mammary glands of mammals and its primary natural function is nutrition of the young (Walstra & Jenness, 1984). This study focuses on bovine milk, which will be referred to as milk throughout this dissertation.

Milk is a raw material for production of wide variety of fermented dairy foods and cheese. Milk has to meet certain requirements to allow the manufacturing of high quality products without modifying the routine technological processes. Raw milk has to be of sufficient microbiological quality and has to have favourable and stable chemical composition.

In 2010 there were approximately 96 thousand dairy cows in Estonia (Estonian Animal Recording Centre, 2012) producing 700 thousand tons of milk. Only 9% of all raw milk produced was consumed as drinking milk. Major part of milk was utilised for manufacturing of dairy products: 35% for cheese, 23% for butter and 33% for other products (Estonian Dairy Association, 2012). The annual yield of milk per cow doubled from 3666 kg in 1995 to 7613 kg in 2010. The increase in milk production was 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 22.3% in 2010; accordingly, the share of EHF breed rose from 61.5% to 76.8% during the same time (Estonian Animal Recording Centre, 2012). 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, 2012). Hence, as a result of breeding strategies and changes in the composition of dairy herd, the alternations in the renneting properties of milk produced in Estonia can also be expected to have taken place.

Enzymatic coagulation of milk is an essential process in cheesemaking which peculiarities define in large extent the quality and yield of the final product (Fagan et al., 2007a; Johnson et al., 2001; Riddell-Lawrence & Hicks, 1989). Thus, the assessment of milk coagulation characteristics should be of high importance to the dairy industry due to the economic reasons. Numerous studies have been performed on the composition of cow's milk. The relation of milk coagulation ability to milk composition reported in literature has been based on the studies performed mainly on the samples collected from individual cows (Jõudu et al., 2008; Tsioulpas et al., 2007b; Tyrisevä et al., 2004; Wedholm et al., 2006), which allows to gain insight into the changes of milk composition throughout the lactation, differences among breeds or the correlations between different milk characteristics. This provides us with the understanding, which components determine milk coagulation ability, but does not allow to evaluate how the pooling of milk affects this technologically important factor in practice.

The recent large-scale studies of pooled milk from dairies in Sweden (Lindmark-Månsson et al., 2003) and the Netherlands (Heck et al., 2009) provide detailed information on the average milk composition in these countries, but one can only tentatively make conclusions about the appropriateness of that milk for cheesemaking. To the best of our knowledge there have been no publications on the composition and rennetability of raw milk, characterising the actual quality of commercial pooled milk utilized for processing with a country-wide coverage. Hence, a part of this dissertation focuses on the evaluation of the renneting properties of raw bulk milk produced at the farms throughout Estonia by direct determination of rennet flocculation time (RFT) by classical Berridge method (Berridge, 1952) and measurement of curd firmness. Further, we developed a novel method based on front-face fluorescence spectroscopy for instrumental determination of coagulation time and monitoring the process of milk rennet coagulation in detail.

The activity of starter cultures in milk is another aspect essential in the manufacturing of fermented dairy foods. Disinfectant residues getting incidentally into milk after milking or added on purpose by unfair producers are potential inhibitors of starter bacteria (Tamime & Robinson, 1999). Starter cultures containing lactic acid bacteria (LAB) produce lactic acid lowering the pH for controlling rate and extent of syneresis, retention and activity of proteolytic enzymes and further proteolysis in cheese mass. For example in case of yoghurt production, lowering the pH by LAB initiates acid-induced gelation of milk, which determines the body of the product. Furthermore, LAB may contribute to aroma, flavour, texture and mouthfeel of the end products (Leroy & de Vuyst, 2004). The growth and activity of starter bacteria in milk in case of occurrence of low concentrations of disinfectants or structural modifications of milk constituents caused by gamma-irradiation were monitored in the present study applying microcalorimetry and front-face fluorescence spectroscopy techniques.

1. LITERATURE REVIEW

1.1. Milk composition

The composition of milk determines its nutritional value and its properties as raw material for manufacturing food products.

The average content of milk principal constituents and approximate range of variation are presented in Table 1. The most abundant component of milk is water, which comprises about 87% of its content. Lactose comprises approximately 4.6%, fat 4.0% and protein 3.3% of milk composition. Milk also contains vitamins, enzymes, minerals (primarily K, Na, Ca, Mg, Cl and phosphate), organic acids and miscellaneous components (Walstra et al., 2006).

Table 1. Approximate composition and range of variation of milk (adapted from Walstra et al., 2006).

Component	Average content in milk (% w/w)	Range (% w/w)
Water	87.1	85.3-88.7
Lactose	4.6	3.8-5.3
Fat	4.0	2.5-5.5
Protein	3.3	2.3-4.4
casein	2.6	1.7-3.5
Mineral substances	0.7	0.57-0.83
Organic acids	0.17	0.12-0.21
Miscellaneous	0.15	-

1.1.1. Lactose

Lactose is the major carbohydrate of milk and it is unique to milk; other carbohydrates are present in milk only in traces. Lactose is a disaccharide composed of glucose and galactose. It is the principal carbon source for most of the microorganisms that grow in milk. Lactose is metabolized to compounds of lower molecular weight by various microorganisms. The most important fermentations from the technological point of view are those that produce lactic acid, which is the basis for production of fermented dairy products (Walstra & Jenness, 1984).

The concentration of lactose in milk is the most constant compared with fat and protein content (Grimley et al., 2009; Heck et al., 2009; Lindmark-Månsson, 2003).

1.1.2. Milk fat

More than 98% of milk fat is made up of triglycerides. Other lipids include phospholipids (0.8%), cholesterol (0.3%), diglycerides (0.3%), free fatty acids (0.1%), and cerebrosides (0.1%). The fatty acids of milk lipids vary widely in chain length (4-20 carbon atoms) and in saturation (0-4 double bonds). The content of saturated fatty acid residues is about 63%. Milk fat contains a

relatively high proportion of short-chain fatty acid residues with 4-10 carbon atoms. Oleic acid is the most abundant of the unsaturated fatty acid residues (about 70%). The other unsaturated fatty acid residues are present in a wide variety of chain length, unsaturation and isomers (Walstra & Jenness, 1984). Majority of milk fat is included into fat globules (see below).

The concentration of fat in milk (Grimley et al., 2009; Heck et al., 2009; Lindmark-Månsson et al., 2003) and fatty acid composition (Heck et al., 2009) are substantially variable, and most important cause of variation is feeding pattern of cows, which varies with season, region, farming practice and so on.

1.1.3. Milk proteins

The main milk proteins are categorized as caseins and whey proteins, which comprise roughly 80% and 20% of total protein in milk, respectively (Walstra & Jenness, 1984). The concentrations and properties of milk principal proteins are summarised in Table 2.

Table 2. Concentrations and properties of major milk proteins (adapted from Farrel et al., 2004, Walstra & Jenness, 1984 and Walstra et al., 2006).

Properties	Caseins				Whey proteins	
	α_{s1}-CN	α_{s2}-CN	β-CN	κ-CN	α-LA	β-LG
Conc. in milk, g·kg^{-1}	10.7	2.8	8.6	3.1	1.2	3.2
Molecular weight, kD	23.6	25.2	24.0	19.0	14.2	18.3
Amino acid res./mol.	199	207	209	169	123	162
Pro, res./mol.	17	10	35	20	2	8
P-Ser, res./mol.	8-9	10-13	5	0-3	0	0
Cys, res./mol.	0	2	0	2	8	5
Carbohydrates	0	0	0	+	0	0
Isoelectric pH	4.5	5.0	4.8	5.6	4.3	5.2

Caseins are those phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20°C. Caseins are identified according to the homology of their primary structures (amino acid sequences) into the following families: α_{s1}-, α_{s2}-, β- and κ-casein (-CN), which in turn have several genetic variants (Farrel et al., 2004). Caseins have many prolines in their primary structure, which prevents the formation of secondary and tertiary structures. Lack of highly organized structure makes casein stable against denaturation but susceptible to proteolysis. The hydrophobic, polar and charged residues are not uniformly distributed throughout the molecular sequences but occur as hydrophobic or hydrophilic patches. α_{s1}-, α_{s2}- and β-CN are highly phosphorylated. The phosphate groups are esterified to serine residues of caseins and bind strongly divalent ions. α_{s1}-, α_{s2}- and β-CN tend to aggregate in the presence of Ca^{2+}. Unlike other caseins, κ-CN contains normally only one phosphoseryl residue and is insensitive to Ca^{2+}. Most of the κ-CN molecules are glycosylated to various extents, and they contain up to four tri- or tetrasaccharide

moieties attached to its C-terminal region increasing the hydrophilicity of that region (Fox et al., 2000).

The term *whey proteins* has been used to describe the group of milk proteins that remain soluble in milk serum or whey after precipitation of casein at pH 4.6 at 20°C. Traditionally, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin, immunoglobulins, and proteose-peptone fractions have been considered the major characterized components of this fraction (Farrel et al., 2004). In contrast to the caseins, the whey proteins possess high levels of secondary, tertiary, and quaternary structures. They are typical globular proteins, which are denatured upon heating. Whey proteins are not phosphorylated and are insensitive to Ca^{2+} (Fox et al., 2000). Denatured whey proteins may form aggregates with each other or with κ-CN (Vasbinder & de Kruif, 2003), which can markedly impair the rennet coagulation properties of milk during cheesemaking and alter the rheological properties of acid gel based fermented dairy products.

Milk protein composition is variable due to intrinsic genetic polymorphism of milk proteins, differences in their degree of phosphorylation and glycosylation (Farrel at al., 2004). In addition, protein content and relative concentrations of different fractions may vary with breed, stage of lactation and season (Jõudu et al., 2008; Wedholm et al., 2006).

1.1.4. Milk salts

Almost all of the salts are in the serum (diffusible fraction) or in the casein micelles, and very little is bound to the fat globules (Walstra & Jenness, 1984). Sodium, potassium and chloride ions are essentially diffusible, although calcium, inorganic phosphate and magnesium are partly bound to the casein micelles. About one-third of calcium, half the inorganic phosphate, two-thirds of magnesium and over 90% of citrate are in the aqueous phase of milk.

Ions in the diffusible fraction are not totally "free" in solution and different associations of ions occur. Calcium exists as ionic calcium (free ion) and a stable complex mainly with citrate (as the trivalent citrate), and to a lesser degree with inorganic phosphate (as a mixture of $H_2PO_4^-$ and HPO_4^{2-}) and with chloride. Sodium and potassium exist mainly as free ions, but a small part of these ions are associated with citrate, inorganic phosphate and chloride (Gaucheron, 2005).

A part of the salts in milk is present in or on the casein micelles. Part of those is considered as counterions. The casein molecules are negatively charged at the milk pH and are associated with positive counterions (Walstra et al., 2006), which comprise almost all of the K^+, Na^+, Mg^{2+}, and about 20% of the Ca^{2+} in the micelles (Walstra & Jenness, 1984). The rest of the ions, which is mainly calcium and phosphate together with a little citrate, is present in a state of colloidal calcium phosphate (CCP). The CCP consists of small, non-crystalline regions and is, moreover, bound to the proteins (Walstra et al., 2006). The colloidal calcium in milk can be defined as a mixture of calcium caseinate

(involving organic phosphate of phosphoserine residues) and calcium phosphate (which is an inorganic phosphate) (Gaucheron, 2005).

The salts of milk are in dynamic equilibrium: among themselves in solution, between solution and colloidal phosphate, and between solution and proteins (Walstra et al., 2006). Minerals play an important role in the structure and stability of the casein micelles. Small changes of the physico-chemical conditions can induce some repartition and consequently can alter the stability of casein micelles.

The mineral composition of milk is considered as relatively constant, but slight variations can still be observed. Calcium and phosphate contents are higher in milk rich in proteins. Also variations during different stages of lactation and in case of mastitis may occur (Gaucheron, 2005).

1.2. Milk structure

Milk is an aqueous solution of lactose, serum proteins, salts and other minor components. On the other hand, milk is a dispersion of fat globules and casein micelles. All particles exhibit Brownian motion and are negatively charged at natural milk pH (Walstra et al., 2006). Main structural elements and some of their properties are presented in Table 3.

Table 3. Main structural elements of milk and their properties (adapted from Walstra et al., 2006).

	Milk		
		Plasma	
			Serum
Structural element	Fat globules	Casein micelles	Globular proteins
Main components	Fat	Casein, water, salts	Serum proteins
Volume fraction	0.05	0.1	0.006
Particle diameter	0.1-10 μm	20-400 nm	3-6 nm
Number per mL	10^{10}	10^{14}	10^{17}
Surface area, $cm^2\ mL^{-1}$	700	40,000	50,000
Isoelectric pH	3.8	4.6	4-5

1.2.1. Milk fat globules

The particles of the largest size in milk are fat globules. Nearly all of the fat in milk exists in fat globules, only about 0.4% of the fat is found outside the globules (Walstra & Jenness, 1984). The milk fat globules vary in diameter from about 0.1 to 10 μm. Milk contains very many small globules, which comprise only a small fraction of the total fat: globules smaller than 1 μm make up about 75% of the total number of globules, but only about 2% of the total globular fat, and about 7% of the fat globule surface area (Walstra et al., 2006). Average fat globule size varies among breeds, among individual cows and with stage of

lactation (Walstra & Jenness, 1984). The positive relation was found between milk fat globule sizes and fat content in raw milk (Wiking et al., 2004).

Each fat globule is surrounded by a thin protective layer or membrane, which consists mainly of polar lipids and proteins, including enzymes. The mass of the membrane is about 2% of that of the total fat globules. Membrane prevents the fat globules from flocculation and coalescence, and protects the fat against lipolysis and oxidation (Walstra & Jenness, 1984). Milk handling during and after milk harvesting as air inclusion, agitation (pumping and stirring), cooling, heating, homogenisation and aging may alter fat globule membrane composition and properties (Evers, 2004).

1.2.2. Casein micelles

Despite their name, casein micelles are not the real "micelles" in terms of colloid science. A typical casein micelle is made up of nearly 20,000 individual protein molecules associated into complex colloidal particles, whose interior is formed by the highly phosphorylated caseins (α_s- and β-CN) interacting and aggregating with calcium phosphate, and whose sizes are controlled by the layer of κ-CN bound to their surfaces (Dalgleish, 2011). The average size of casein micelles in pooled milk was measured to be 120 nm (de Kruif et al., 2012), and is determined by the proportion of κ-CN in the mixture of caseins (Dalgleish, 2011). Average casein micelle size was found to be constant for a given cow during milking, lactation and over the years (de Kruif et al., 2012).

The structure of the casein micelle has been discussed for many years and has not been fully agreed until now. Among most widespread and debated models are the "submicelle model", the "nanocluster model" and the "dual-bonding model". The classical "submicelle model" (Walstra, 1990) suggests that a casein micelle is built of smaller units, which are of two main types, one primarily consisting of α_s- and β-CN, the other of α_s- and κ-CN, and which are linked together by CCP. The sub-micelles rich in κ-CN are located on the surface of the micelles and κ-CN protrudes out from the micelles core into the solvent as flexible "hairs". The "nanocluster model" was first introduced by Holt (1992) and considered the calcium phophate nanoclusters to be responsible for cross-linking individual proteins and holding the gel-like casein network together. This model was in some sense refined by "dual-bonding model" of Horne (1998), which takes into consideration two types of interactions keeping micelles structure together: a) binding of CCP to negatively charged phosphoserine clusters of caseins, reducing their charge, and b) hydrophobic interactions between the hydrophobic regions of the caseins.

The submicellar model has received much criticism and was shown to be nonconsistent with electron micrographs (Dalgleish et al., 2004; McMahon & McManus, 1998) and the experimental scattering based data (de Kruif et al., 2012). An advanced model based on calcium phosphate nanoclusters was considered to be the most relevant in the recent publications of Dalgleish (2011) and de Kruif et al. (2012).

De Kruif et al. (2012) considered the casein micelle as a homogeneous matrix of caseins in which the CCP nanoclusters are dispersed as very small "cherry stones" with the radius of 1.72 to 2.27 nm at an average distance of 18.6 nm (Figure 1). Based on the calculations, there are about 372 CCP nanoclusters per casein micelle in pooled milk. Attached to the surfaces of the nanoclusters are the centres of phosphorylation (3-5 nearby phosphorylated amino acid residues) of the caseins. The hydrophobic tails of the caseins, much larger than the CCP clusters, stick out. These tails are entangled with other hydrophobic tails forming small hydrophobic and denser protein regions of about 2 nm in radius. The association of the tails is driven by a collection of weak interactions, which engage hydrophobic interactions, hydrogen bonding, ion bonding, weak electrostatic Van der Waals attraction and other factors (but not the strong calcium phosphate interaction) leading to self-association. The κ-CN limits the process of self-association leading to stabilization of the native casein micelle.

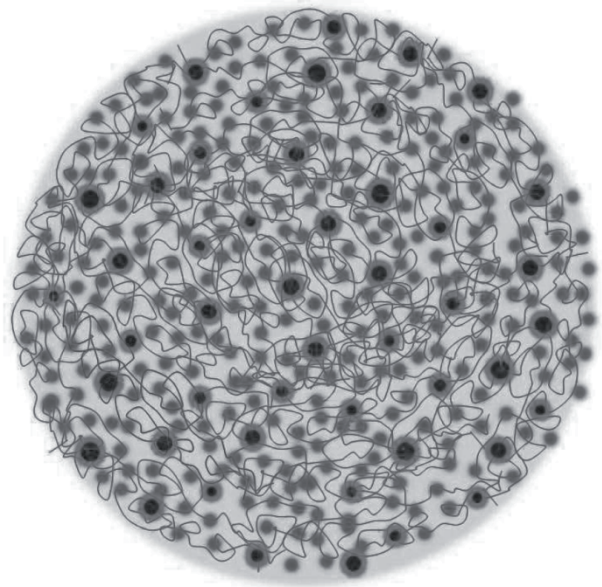

Figure 1. Schematic representation of the internal structure of casein micelle (by kind permission of C. G. de Kruif and Elsevier B.V.[1]). Black spheres are calcium phosphate nanoclusters, gray spheres are protein clusters.

While the internal structure of the casein micelles remains the matter of the debates for more than 50 years by now, the existence of the hairy layer of the micelles is universally accepted. However, the distribution of κ-CN on the

[1] Reprinted from Advances in Colloid and Interface Science, Vol. 171-172. De Kruif, C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V., Casein micelles and their internal structure, Pages No. 36-52, Copyright (2012), with permission from Elsevier.

surface of the casein micelles is not fully understood. It was proposed that the surface of the micelles is only partially covered by κ-CN, which is heterogeneously distributed (Dalgleish, 1998). The evidence for the organization of the surface of the casein micelles into tubular structures (bunches of κ-CN) was provided on the basis of the scanning electron micrographs, and it was proposed that the surface contains gaps between the substructures (Dalgleish et al., 2004). Eventually, it was proposed that the interior of the casein micelles is inaccessible for very large molecules and other casein micelles and only the outer layer of κ-CN will be detected as the particles approach each other. However, for smaller molecules, including individual proteins such as enzymes, the surface of the micelle will include also a part of the interior due to clefts or pores on the surface of the micelles (Dalgleish, 2011).

Milk can be moderately heated or cooled without significant aggregation of casein micelles or disruption of their basic structure. On the other hand, the micelles can be easily destabilized by treatment with proteolytic enzymes or by acidification resulting in the formation of coagulum, which is the basis of the production cheese and yoghurt-type products. In that sense, most of the important functional properties of the casein micelles are determined by the properties of the surface, rather than those of the interior. However, the interior of the casein micelles becomes important in postcoagulation rearrangements taking place in cheese curd or acid-induced gels (Dalgleish & Corredig, 2012).

1.3. Milk coagulation

Milk coagulation can be achieved with rennet or another proteolytic enzyme preparation, with acid, by heating, or by combinations of these factors. The manufacture of traditional ripened semi-hard and hard cheeses includes preculturing of the milk before rennet addition – rennet coagulation is facilitated by acid produced if a high percentage of starter bacteria is added. A combined acid and rennet coagulation is used in the manufacture of fresh and ripened soft cheeses; however, gel formation is mainly induced by acid. Queso Blanco cheese is made by heating milk to between 80 and 85°C and further acid addition (acetic, citric, phosphoric, etc., or simply lime juice) while gently stirring the milk, which leads to the formation of a coagulum (Walstra et al., 2006).

1.3.1. Rennet-induced coagulation

Casein micelles are stabilized by steric and electrostatic repulsion generated by the polyelectrolyte layer of κ-CN, which prevents micelles from aggregation (Dalgleish & Corredig, 2012; Dejmek & Walstra, 2004). Any process that removes the C-terminal part of κ-CN causes a decrease in the colloidal stability of the micelles (Dalgleish & Corredig, 2012).

Two stages, overlapping to some extend, can be raughly distinguished during renneting (Fox, et al., 2000):

1. κ-CN $\xrightarrow{enzyme}$ para-κ-CN + glycomacropeptide

2. para-κ-CN covered micelles $\xrightarrow{Ca^{2+}}$ gel

The primary phase of renneting involves the specific enzymatic modification of the casein micelles, and the second phase involves the aggregation of the rennet-altered micelles.

Several proteinases can coagulate milk, but most are too proteolytic (high level of non-specific proteolysis (Fox et al., 2000)). The main clotting enzyme used in cheesemaking is chymosin (E.C. 3.4.23.4., MW 30,700, pI 4.6-4.7). At milk pH 6.7, chymosin splits only κ-CN Phe_{105}-Met_{106} bond, resulting in para-κ-CN, which remains attached to casein micelles, and glycomacropeptide (GMP), which is soluble in the whey fraction. The positive charge of the region of the κ-CN chain accounts presumably for a rapid binding of the negatively charged active site of the enzyme molecule (Walstra & Jenness, 1984). The sequence κ-CN f98-111 around the Phe_{105}-Met_{106} bond, rather than the residues in the bond itself, determines the binding affinity of the enzyme (Fox et al., 2000).

During renneting, there is about one enzyme molecule per 100 casein micelles and a micelle contains in the order of 1000 κ-CN molecules (Dejmek & Walstra, 2004). After the addition to milk (Figure 2a), the enzyme approaches the micelles "gradually" by means of diffusion, part of the diffusion path being in the hairy layer of the micelle, as the Phe-Met bond to be split is quite close to the micelle surface. This increases the diffusion time, and hence, the enzyme molecules bind gradually onto the surface of casein micelles. The reaction velocity thus is considered diffusion-limited. At milk physiological pH the "hairs" are removed at random (Walstra et al., 2006) (Figure 2b). Removal of the "hairs" results in a decrease of the hydrodynamic diameter of the casein micelles and causes a slight minimum in the viscosity during the initial phase of renneting (Lucey, 2009).

The release of the GMP from the surface of the casein micelles leading to a decrease of the zeta potential by ~ 50%, from -10/-20 to -5/-7 mV (Fox et al., 2000), reduces electrostatic as well as steric repulsion between rennet-altered casein micelles (Lucey, 2009). Then, the micelles can approach closer to one another and flocculate. The flocculation does not proceed until nearly 70% of κ-CN "hairs" are hydrolysed; after this point, *para*-casein micelles interact increasingly strongly with an increase in the extent of proteolysis (Dejmek & Walstra, 2004; Lucey, 2009). The term flocculation refers to weak reversible aggregation and coagulation to irreversible aggregation (Walstra et al., 2006). Only after more extensive removal of the protective κ-CN layer does true aggregation occur (Lucey, 2009). At first irregular (Figure 2c), but then somewhat thread-like (Figure 2d) aggregates are formed, which grow to form large tenuous flocks and eventually a continuous network (Dejmek & Walstra, 2004). A three-dimensional particle gel formed as a result of aggregation is dynamic in its nature. Further rearrangements of the bonds (fusion of the

micelles), particles and clusters of the particles result in shrinkage and coarsening of the gel network and induce syneresis (Mellema et al., 2002).

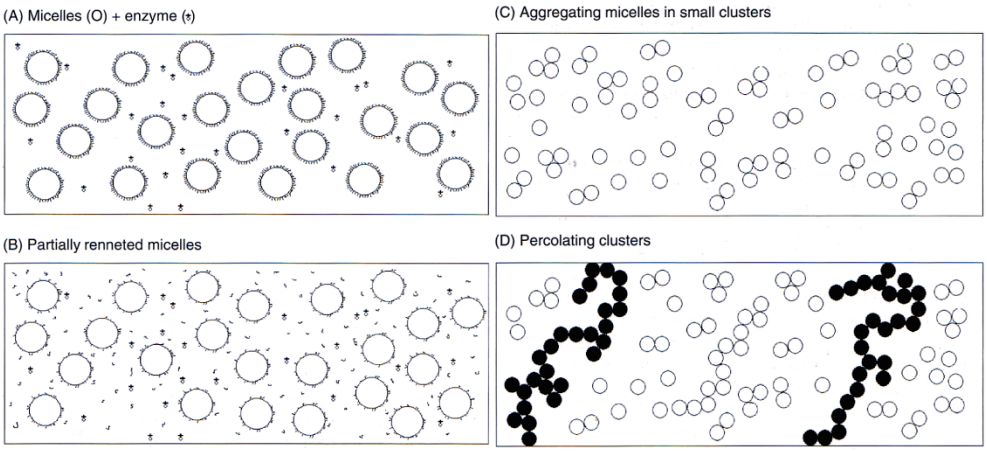

Figure 2. Schematic description of the various stages of enzymatic coagulation of milk (by kind permission of Elsevier B.V.[2]): a) initial mixture of casein micelles and enzyme, b) cleavage of κ-CN, c) initial aggregation into small clusters, d) reaching a gel point at percolation.

During cheesemaking, there are a number of compositional and environmental factors that affect the kinetics of rennet coagulation as well as the strength of the resultant gel (curd firmness): milk protein and fat content (Guinee et al., 1997), pasteurization temperature (Leaver et al., 1995; Singh & Waungana, 2001; Vasbinder et al., 2003), cooling and cold storage of milk (Malacarne et al., 2013; Raynal & Remeuf, 2000), renneting temperature (Ong et al., 2011) and pH (Leaver et al., 1995; Ong et al., 2012), addition of $CaCl_2$ (Bringe & Kinsella, 1986; Famelart et al., 1999; Sandra et al., 2012) etc.

1.3.2. Acid-induced coagulation

Acidification of milk can be induced by lactic acid bacteria (LAB), which produce lactic acid from lactose, by the addition of acids such as HCl, or by the use of glucono-δ-lactone (GDL), where the hydrolysis of GDL to gluconic acid results in a reduction of pH (Lucey & Singh, 1997). The final pH that is attained in GDL-induced gels depends on the amount initially added to milk, whereas starter bacteria can produce acid until they become inhibited by the low pH.

[2] Reprinted from Cheese: chemistry, physics and microbiology, Vol. 1 (3rd ed.), P. F. Fox, P. L. H. McSweeney, T. Cogan, & T. P. Guinee (Eds.). Horne, D. S., & Banks, J. M., Rennet-induced coagulation of milk, Pages No. 47-70, Copyright (2004), with permission from Elsevier.

Acidification causes a number of changes in casein micelles, which can be divided into three arbitrary pH regions during acidifying milk from pH 6.7 to 4.6. The decrease of pH from 6.7 to ~6.0 causes a decrease in the net negative charge on the casein micelles and reduces electrostatic repulsion. Further decrease of pH from ~6.0 to ~5.0 causes further decrease in the charge of κ-CN "hairs" on the micelles surface, so these "hairs" may shrink and steric stabilisation fails. The decrease of pH to ~5.0 is also accompanied by dissolution of CCP and consequent loosening of the molecular interactions between caseins. Aggregation of casein particles in unheated milk occurs at around pH 4.9. If acidification is performed at very high temperatures, a higher gelation pH is observed (Lucey, 2009). In heated milk, gelation occurs at higher pH (5.2-5.4), which can be attributed to the higher isoelectric pH (~5.2) of denatured whey proteins, associated with casein micelles upon heating, compared to that of caseins (pH 4.6) (Lucey, 2004b). In Publication IV, a novel approach – front-face fluorescence spectroscopy – was used for non-destructive dynamic monitoring of changes in the milk proteins during acidification by starter bacteria.

In dairy industry, cultured milk products are formed mainly as a result of the production of lactic acid during fermentation of lactose by the starter culture (Lucey, 2004a). Proteolytic enzymes of the bacteria can possibly be also engaged in this process because some of those can split κ-casein; however, the main mechanism is casein becoming insoluble near its isoelectric pH (Walstra et al., 2006). Acidification activity of milk is a very important characteristic of the quality of dairy starters. The methods used for the characterization of the acidification activity of starter bacteria are usually based on measuring pH changes or on determination of the accumulation of lactic acid per time unit (Zanatta & Basso, 1992). In addition to acid production, the LAB also play a key role in the formation of aroma, texture and flavour of the end-product. The metabolic activity of the bacteria can be characterized also by the heat produced during their growth, and this can be measured by microcalorimetry. In Publication II, an isothermal batch microcalorimeter was used to obtain the precise and detailed information on the growth of thermophilic LAB in continuous mode *in situ* (in small ampoules) during the formation of yoghurt.

1.4. Variation in composition and technological properties of milk

Cow's milk is rather constant in a qualitative sense, but there are considerable quantitative variations in chemical composition, size and stability of the structural elements, and properties of different lots of milk (Walstra & Jenness, 1984). These differences originate from a natural variation in milk composition (due to breed, individual cow etc.), contaminants entering milk via the cow from the feed or during and after milking (e.g. antibiotics, detergents and disinfectants) and various other changes occurring during and after milking and during further processing.

1.4.1. Natural variation

Three kinds of factors can lead to natural variation of milk composition: genetic factors (differences among breeds and individuals within breed), physiological conditions (stage of lactation, health state, age of the animal) and environmental factors (feed, weather conditions, treatment) (Walstra & Jenness, 1984).

Throughout the years, cows of Estonian Holstein-Friesian (EHF) breed were shown to have higher yield but lower protein and fat content than cows of Estonian Red (ER) or Estonian Native breed (Estonian Animal Recording Centre, 2012). In addition it has been shown that total CN, α_{s2}- and κ-CN, β-LG and the relative contents of α_{s1}- and κ-CN in total casein were lower in milk from cows of the EHF breed than those of the ER breed (Jõudu et al., 2008). Likewise, in spite of the higher milk productivity of Holstein-Friesian breed, milk from Italian Friesian cows was shown to have lower protein content, also lower curd firming rate, weaker coagulum and lower cheese yield than milk from Italian Brown breed (Malacarne et al., 2006). On the other hand, milk from Holstein-Friesian (HF) cows from Finnish herds was shown to have lower protein and fat content but better coagulation ability in comparison with Finnish Ayrshire (FA) cows (Tyrisevä et al., 2004).

Milk from about 30% of FA and 12% of HF cows was found categorized as poorly coagulating, while milk from 8.6% of FA and 1.3% of HF cows was non-coagulating (Tyrisevä et al., 2004). Similarly, more than 30% of the Swedish and Danish cows sampled produced poorly coagulating or noncoagulating milk (Wedholm et al., 2006) that was associated with a low concentration of κ-CN and a low proportion of κ-CN in relation to total casein.

Total protein content and ratios of different protein fractions have been reported to change during lactation (Caffin et al., 1985; Jõudu et al., 2008; Ostersen et al., 1997). The proportion of total casein was higher in mid-lactation; proportions of α_s- and κ-CN decreased and β-CN increased during lactation (Ostersen et al., 1997). A shift to higher pH of milk was also observed throughout the lactation (Tsioulpas et al., 2007a; Tyrisevä et al., 2004). The longest renneting time and lowest curd firmness were observed during the mid-lactation period (Ostersen et al., 1997; Tyrisevä et al., 2004).

The variability of milk composition of individual cows is greater than the variability of milk lots received at dairy factory – pooling of milk reduces the variations. The main differences of lots of pooled milk are assigned to seasonal differences and those between regions, which may differ in predominant breed or feeding practice (Walstra & Jenness, 1984).

Only few studies have been published which characterize the composition of cows' milk in a whole country with respect to seasonal, geographical or annual variations (Lindmark-Månsson et al., 2003; Heck et al., 2009). In milk collected from nine Swedish dairies, minimum protein content was observed in spring and maximum in autumn, fat content not showing any significant seasonal variation (Lindmark-Månsson et al., 2003). Heck et al. (2009) reported seasonal variations in the composition of average dairy milk in the Netherlands based on a single

pooled milk sample collected and analysed weekly within one year. Summer milk was characterized by low protein and low fat content, and the highest values were observed in winter in the Netherlands (Heck et al., 2009). Despite the clear variation in the total protein content during the year, the protein composition of Swedish and Dutch pooled milk was rather constant (Lindmark-Månsson et al., 2003; Heck et al., 2009). Unfortunately, these studies were performed without turning a special attention on the rennetability of milk.

The coagulation characteristics of Estonian raw bulk milk were evaluated in Publication I by direct measurement of rennet flocculation time (RFT) and curd firmness – correlations of these properties and milk composition were observed. Results of our study provide information on rennetability of bulk milk and its variability from a large number of farms representing the whole country.

1.4.2. Disinfectants

Effective cleaning and sanitizing of dairy equipment is a prerequisite for producing high quality dairy products with low bacterial counts. Disinfectant formulations including quaternary ammonium or halogen-containing compounds, and H_2O_2 are still used in dairy industry (Tamime & Robinson, 1999). Besides being not acceptable in European countries, the retardation of growth of unwanted bacteria in raw milk by adding substantial amounts of H_2O_2 is permitted in some developing countries with warm climate. The recommended quantities of H_2O_2 (if allowed to be added to milk) range from 100 to 800 mg L^{-1} (Björck, 1987). Yet, lower concentrations of disinfectant residues may get into the milk due to faulty cleaning systems, improper dosage, insufficient rinsing, unsuitable pipeline design, or in some cases can be deliberately added by producers into milk to 'improve' its bacterial quality.

Early studies of adding *high concentrations* of H_2O_2 (100 mg L^{-1} to 15 g L^{-1}) to milk prior to cheesemaking showed that the treatment resulted in softer cheese mass (Roundy, 1958; Kosikowski & Fox, 1968), higher solubility of casein, and higher susceptibility of casein to proteolysis by rennet (Fox & Kosikowski, 1967) and proteolytic enzymes of *Pseudomonas fluorescens* (Fish et al., 1969). Schmidt et al. (1969) reported reduced rate and lower completeness of casein clotting by rennet in the presence of high concentrations of H_2O_2.

Effects of *low concentrations* of H_2O_2 (< 10 mg L^{-1}) in milk have been commonly considered in scientific literature in conjunction with the lactoperoxidase (LPO) system, which prevents the growth of bacteria by catalyzing the oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) believed to possess antimicrobial properties, using H_2O_2 as the electron acceptor (Özer, 1999). The use of LPO system is recommended by FAO/WHO as a standard method for retarding bacterial growth in raw milk during collection and transportation to dairy plants in situations where refrigeration is not feasible (Codex Alimentarius Commission, 1991). Recommended concentrations of H_2O_2 added for activation of LPO system are up to 10 mg L^{-1} as noted. On the other hand, the activation of LPO system has been associated with inhibiting

effects on cheese and yoghurt starter cultures (reviewed by Seifu et al., 2005). Moreover, an impairment of rheological properties of yoghurt was reported, alterations being assigned to the oxidation of protein thiol groups (-SH) by $OSCN^-$ leading to decreased number and/or strength of cross-linking (Hirano et al., 1998a, b). Antimicrobial and oxidizing effects were not related in those studies to the action of H_2O_2 itself but to other components of LPO system.

Application of higher than recommended concentrations of H_2O_2 may cause irreversible inactivation of LPO, naturally occurring in raw milk (Björck, 1987; Kussendrager & van Hooijdonk, 2000; Fonteh et al., 2005). Some slight decrease in tyrosine content in milk, showing susceptibility of amino acids to oxidation, and significant increase in peroxide values, indicating lipid oxidation, have been reported in yoghurt produced from milk treated with considerably high levels of H_2O_2 (100-140 mg L^{-1}), which demonstrates the evidence of H_2O_2-promoted and apparently LPO-independent oxidation of milk proteins and lipids (Özer & Atamer, 1999). Nevertheless, the oxidative and inhibiting influences against starter bacteria of *intermediate concentrations* of H_2O_2 added to milk at higher levels (10-100 mg L^{-1}) than normally required for activation of LPO system, and independently of LPO has not been evaluated as yet and were addressed in Publication II.

1.4.3. Changes occurring during and after milking

Processes occurring in milk after it is drawn include creaming of fat globules and crystallization of part of the fat on cooling; inclusion of air bubbles, which may cause a disruption of fat globules; inclusion of oxygen, which may induce lipid oxidation; lipolysis and proteolysis initiated by milk or bacterial enzymes (Walstra & Jenness, 1984).

Deterioration of milk microbiological quality and decrease in curd yield during storage of milk in bulk milk tanks at the farm and in dairy silos have been reported (Leitner et al., 2008). A comprehensive information on the influence of different storage temperature/time combinations on the physico-chemical and microbiological characteristics as well as processing properties of milk was provided in the recent publication (Malacarne et al., 2013) – the storage of milk at the lowest observed temperature 4-6°C impaired the rennet coagulation properties of milk the most.

The differences between protein composition and the coagulation properties of milk sampled at the farms and the same milk received at the dairy (milk originated from the same milkings), which has undergone mechanical perturbations such as pumping and refrigerated transportation in trucks, were studied in Publication I.

1.5. Methods for monitoring milk coagulation

One of the earliest and simplest methods for the estimation of milk rennet coagulation properties is *the visual determination of the onset of flocculation* in a

glass tube (Berridge, 1952). In contrast to cheese manufacture, where milk is renneted under quiescent conditions to ensure gel formation, this method determines the time of coagulation under agitation. Yet, the effect of constant stirring on the enzyme action and aggregation kinetics is not clear, as well as this method is destructive towards the formation of milk coagulum. The registration of the onset of flocculation by human eye occurs later than optically determined onset of the aggregation of casein micelles (Tabayehnejad et al., 2012).

The most direct way to measure gel formation during milk coagulation is to monitor the evolution of rheological properties. *Dynamic low-amplitude rheometry* applies a low-amplitude oscillatory shear stress (τ_0) or strains (γ_0) and records the response of the developing gel (Horne & Banks, 2004). Under these conditions the gel strands of the gelling milk are strained to a fixed displacement (within their elastic limit) and recover instantaneously when the stress is removed (Fox et al., 2000). The measurements yield the elastic or storage modulus (G'), which is a measure of the energy stored per oscillation cycle and reflects how the sample behaves as an elastic solid, and the viscous or loss modulus (G''), which is a measure of the energy dissipated per cycle and indicates how much the sample behaves as a viscous liquid. The ratio of G'' to G' is tan δ, the tangent of the phase angle of the response to the applied stress or strain. The shear moduli are defined as follows:

$$G' = \left(\frac{\tau_0}{\gamma_0}\right)\cos\delta$$

$$G'' = \left(\frac{\tau_0}{\gamma_0}\right)\sin\delta$$

In uncoagulated milk $\delta > 45°$ and the viscous component dominates; when coagulation proceeds $\delta < 45°$ and the sample appears more like an elastic. Initially the G' lags behind growth of viscosity or viscous modulus, G'', but quickly crosses and dominates as gel elasticity rapidly develops (Horne & Banks, 2004). The stress required to achieve a fixed strain (displacement of a gel strand at a given position) increases as the gel strands become more elastic and firm; hence, measurement of stress energy provides a measure of the gel strength. The G' is a direct measure of curd firmness and is thus of high significance in cheese manufacture (Fox et al., 2000). The increase in the shear moduli after gelation reflects ongoing fusion of micelles, which results in an increase in the contact area between aggregated particles, and possibly the incorporation of additional particles into the gel network (Lucey, 2009). Low-amplitude dynamic rheometry gives a precise estimation of the rheological changes that occur during renneting without altering the process of gel formation; hence, it accurately reflects the changes in curd firmness that occur upon renneting milk in the cheese vat under quiescent conditions. However, limitations of such measurements include the high cost of instrumentation, the

high level of operator skill required for accurate measurement, the fact that only one sample can be analysed at a time, and instruments currently available cannot be used for online measurement of gel formation in the cheese vat (Fox et al., 2000).

Other instrumental methods with different principles have been used to monitor milk coagulation in laboratory scale research for better understanding of this process, and many attempts have been made to automate the cheese production by developing a variety of different on-line sensors for monitoring milk coagulation and prediction of cutting time. The last reviews of the *off-line* and *on-line* methods for assessment of milk coagulation – their operating principles, advantages and drawbacks were published a decade ago (Lucey, 2002; O'Callaghan et al., 2002). The techniques for monitoring of milk coagulation have been based mainly on a) measuring physical parameters related to the changes of rheological properties of milk using mechanical (e.g. "Formagraph"; McMahon & Brown, 1982), vibrational (e.g. "Sofraser", "Viscolite"; O'Callaghan et al., 2000), ultrasonic (Dwyer et al., 2005; Wang et al., 2007), thermal conductivity probes ("Hot-Wire"; O'Callaghan et al., 2000) or b) optical techniques detecting the changes in the sizes of micelles using near infrared (NIR) transmission or reflectance (e.g. "TxPro" and "CoAguLite"; Castillo et al., 2000; Klandar et al., 2007, O'Callaghan et al., 2000). Among the promising techniques to study coagulation of milk a diffusing wave spectroscopy was highlighted, which provides additional information on particle interactions and their dynamics during flocculation of casein micelles (Alexander & Corredig, 2007; Hemar et al., 2004; Huppertz & de Kruif, 2007).

Concerning the use of sensors in practice, the installation of mechanical and vibrational probes through the wall of a cheese vat is rather problematic; also proper cleaning of such systems is an issue. Furthermore, mechanically moving or vibrational action of the probe may be intrusive and thus only a limited degree of coagulation could be measured (O'Callaghan et al., 2002). Conversely, the optical methods have definite advantages as they are non-destructive, do not perturb coagulation, are integratable in-line and easily applicable on plant scale from the hygienic viewpoint. At the same time, the ability of optical methods to provide the information on the rheological properties of forming coagulum and prediction of resistance to cutting may be limited (Lucey, 2002). Variations in protein composition can also confound the results of optical measurements of curd firming (O'Callaghan et al., 2000).

Most recent research has been focussed specifically on the development of combined sensors for continuous non-invasive monitoring of milk coagulation and syneresis, based on ultrasonic measurements (Taifi et al., 2006) or a sensor utilizing a large field of view relative to curd particle size based on detecting NIR light backscatter (Fagan et al., 2007b, c; 2008). Recent publication of Fagan et al. (2011) has shown the potential applicability of front-face fluorescence spectroscopy to monitor both coagulation and curd syneresis phases in laboratory cheese vat.

Although many instrumental techniques have been developed for monitoring milk coagulation, the implementation of such systems in the dairy industry has been still modest overall (O'Callaghan et al., 2002). The objective of the Publication III was to study the potential of the front-face fluorescence spectroscopy to monitor the changes in milk proteins occurring during rennet-induced coagulation and to derive the coagulation time parameter, which could be used to monitor the coagulation ability of milk.

1.6. Front-face fluorescence spectroscopy in dairy research

After the absorption of light by a fluorescent molecule (*fluorophore*), electrons from the singlet ground electronic level S_0 move to an excited state S_n ($n > 1$). After that the molecule in S_n moves to the lowest excited state S_1 by dissipating a part of its energy in the surrounding environment, which is called internal conversion. The molecule returns from the lowest excited state S_1 to the ground state S_0 and emission of a photon may occur – this phenomenon is *fluorescence* (Valeur, 2002). The energy absorbed by a fluorophore is higher than the energy of the emitted photon. Thus, the emission spectrum has its maximum shifted to longer wavelengths compared to the maximum of absorption spectrum (Albani, 2007).

Each electronic state has several associated vibrational levels, which means that excitation does not occur at only one single wavelength, but rather over a distribution of wavelengths corresponding to different vibrational transitions. The deactivation of the excited state occurs only from the vibrational ground level, but emission also occurs at several wavelengths as it may reach different vibrational levels in the electronic ground state. Therefore, all fluorophores have specific spectral excitation and emission profiles characterizing their unique fluorescent properties (Christensen et al., 2006). These profiles can be measured as excitation and emission spectra separately or as a complete excitation-emission matrix, also known as fluorescence landscapes or spectral fluorescence signatures (SFS).

If absorbance is less than 0.1 optical density units, the intensity of the emitted light is proportional to fluorophore concentration and excitation and emission spectra are accurately recorded by classical right-angle fluorescence device. If the absorbance is higher than 0.1 optical density units, a dilution of samples is needed. But in this case the organisation of the food matrix is lost. To avoid these problems, the method of front-face fluorescence spectroscopy can be used when measuring turbid, powdered or solid samples (Karoui et al., 2003). In front-face illumination mode, the incident beam is set to the sample at an angle other than right angle, and the measurement of fluorescence is performed from the surface of the sample (Christensen et al., 2006).

Fluorophores are mainly aromatic or linear conjugated molecules, in which the transition of de-localized π-electrons to the excited state is observed (Valeur, 2002). Milk and milk products contain a wide variety of naturally occurring

intrinsic fluorophores as aromatic amino acids – tryptophan, tyrosine and phenylalanine in proteins, vitamin A and B_2, cofactors etc. (Karoui et al., 2003). In proteins, tryptophan fluorescence dominates; zero or weak tyrosine and phenylalanine fluorescence results from energy transfer to tryptophan and/or neighbouring amino acids (Albani, 2007). The major proteins in milk (α_{s1}-, α_{s2}-, β-, κ-CN, α-LA and β-LG) contain at least one tryptophan residue each (Fox et al., 2000).

Intrinsic tryptophan residues in proteins are sensitive fluorophores, those fluorescence depends on the polarity of the environment and existence of quenching molecules in the vicinity of tryptophan (Ladokhin, 2000). Polar amino acid side chains (Asp, Glu, Lys, Arg and protonated His), amide groups of Gln and Asn, phenol group of Tyr, as well as disulfide (Cys-Cys) bridges or single Cys can effectively quench tryptophan fluorescence (Chen & Barkley, 1998; Engelborghs, 2003; Ladokhin, 2000). Changes in microenvironment, induced for example by ligand binding or conformational changes in proteins may result either in enhancement or quenching of fluorescence and in shifts in the spectrum to shorter or longer wavelengths (Ladokhin, 2000). Several inherent advantages of fluorescence spectroscopy for characterisation of molecular interactions and reactions have been drawn out: 1) it is 100-1000 times more sensitive than other spectrophotometric techniques; 2) fluorescent compounds are highly sensitive to their environment; 3) fluorescence method is relatively rapid (Karoui et al., 2003).

At present there are only a few publications concerning the application of fluorescence spectroscopy to monitor coagulation of milk (Fagan et al., 2011; Herbert et al., 1999; Laligant et al., 2003; Lopez & Dufour, 2001). The specific feature of front-face fluorescence spectroscopy is that the technique is potentially able to reflect changes taking place with proteins at the molecular level during renneting, apart from the other optical methods (e.g. measurement of NIR absorbance (Klandar et al., 2007) or NIR light backscatter profile (Fagan et al., 2007b)) that rely on the changes in particle size. Herbert et al. (1999) have shown that front-face fluorescence spectroscopy was able to distinguish acid-induced coagulation of milk from rennet-induced process and allowed the detection of structural changes in casein micelles during coagulation. Laligant et al. (2003) used a dynamic approach for characterization of acid gelation of milk combining front-face fluorescence and other measurements. Lopez and Dufour (2001) successfully applied front-face fluorescence spectroscopy for investigation of the influence of the composition of fat globule surface on the rennet-induced coagulation of reconstituted milk, however, interpretation of the changes of tryptophan fluorescence intensity profiles recorded during the renneting was not provided by the authors. It is known that in the course of the manufacture of skimmed milk powder, the equilibrium of calcium between serum and colloidal phases as well as the size of casein micelles could be irreversibly altered (Martin et al., 2007), which was suggested to explain the differences in rennet-induced coagulation properties of raw and reconstituted

milk samples (Martin et al., 2008). Hence, analytical techniques applied for monitoring of reconstituted milk should be separately validated also in the case of natural bulk milk. Recently, Fagan et al. (2011) have shown that spectral changes of naturally occurring milk fluorophores in bulk milk allowed monitoring of both coagulation and curd syneresis phases by mounting a fluorescence probe into a laboratory cheese vat. The evolution of tryptophan fluorescence has been shown to have the largest response during coagulation compared with other intrinsic fluorophores of milk – riboflavin and vitamin A. A need for further study under a wider range of processing conditions was indicated by the authors (Fagan et al, 2011). In Publication III we aimed to provide a deeper insight into different phases of renneting during coagulation of different milk samples with various coagulation properties (reconstituted skimmed milk, whole and skimmed raw bulk milk) using front-face fluorescence spectroscopy together with reference methods.

2. AIMS OF THIS DISSERTATION

The aims of this dissertation were to investigate the indigenous and exogenous factors affecting technological properties of milk and to apply a novel front-face fluorescence spectroscopy method for monitoring of milk coagulation. The specific aims were as follows:

I. To determine the quality of raw bulk milk collected from individual farms in Estonia in relation to cheesemaking properties (Publication I) with the special emphasis on:

- seasonal variations;
- comparison between bulk milk from individual farms and pooled milk from dairy silos;
- verification of correlations of bulk milk composition and renneting properties.

II. To study the influence of a common disinfectant used in dairy industry, H_2O_2, on the growth of thermophilic starter bacteria and acid gelation of milk (Publication II).

III. To apply front-face fluorescence spectroscopy to study rennet- (Publication III) and acid- (Publication IV) induced coagulation of milk.

3. MATERIALS AND METHODS

The detailed descriptions of the materials and methods applied are available in the publications. The following sections are provided to make this dissertation more accessible.

3.1. Milk supply and treatment

3.1.1. Milk samples from farms, tank trucks and dairy silos (Publication I)

More than thousand raw bulk milk samples from over 170 individual dairy farms of geographically different locations in Estonia delivering milk to two large dairies were collected during the period February 2004 – June 2007. Samples were collected in 34 batches distributed randomly throughout all seasons, and each time approximately 30 milk samples were analysed. On most sampling days, samples were also taken from tank trucks and dairy silos where the milk from examined individual farms was pooled on that day. The details of the sampling procedure are provided in Publication I.

3.1.2. UHT milk samples (Publication II)

Utilization of ultra-high temperature treated (UHT) milk (Kalev Paide Tootmine AS, Paide, Estonia) with fat contents of 3.5% and 0.05% (referred as UHT 3.5% and UHT 0.05%, respectively) enabled the investigation of H_2O_2 oxidative or antimicrobial influence independently of LPO system, since high temperature is known to inactivate the natural antimicrobial LPO of milk (Kussendrager & van Hooijdonk, 2000).

To samples of UHT 3.5% and UHT 0.05% various dilutions of H_2O_2 (Riedel-de Haën, Seelze, Germany) were added to yield final concentrations of 0, 10, 25, 50, 75, 100, 150, 200 and 250 mg L^{-1} in milk. The treated milk samples were left at 40°C for one hour to promote chemical reaction between H_2O_2 and milk constituents before starter bacteria were added. To assure complete utilization of H_2O_2 before inoculation, the aliquots of UHT 3.5% samples incubated with H_2O_2 were treated with excess bovine liver catalase (Sigma-Aldrich, St. Louis, MO, USA) prior to the addition of starter culture.

3.1.3. Milk samples for monitoring coagulation by front-face fluorescence spectroscopy and reference methods (Publication III)

Coagulation experiments were performed with reconstituted skimmed milk (RSM) and both, whole and skimmed raw bulk milk samples.

RSM was prepared from low-heat skimmed milk powder (LHSMP; Valio Ltd., Helsinki, Finland) by dissolution in distilled water at 10% (w/v) milk solids, with addition of $CaCl_2$ to yield 5 or 10 mM final concentration. RSM without $CaCl_2$ addition was also used in some trials.

Nine samples of whole bulk milk with varying coagulation properties were collected from four dairy farms supplying milk for cheese production at Saaremaa Dairy (Kuressaare, Estonia) on different sampling days. Skimmed milk was prepared by centrifuging aliquots of the whole milk at 3,000 g for 10 min at 4°C.

3.1.4. RSM from untreated and irradiated LHSMP (Publication IV)

A part of LHSMP (Valio Ltd.) was irradiated by gamma rays at 10 kGy using a dosimetric system GEX WinDose (Centennial, CO, USA). Untreated LHSMP and irradiated LHSMP (irrLHSMP) were reconstituted in distilled water to yield a final concentration of 10% (w/v) milk solids, mixed thoroughly for one hour, heated at 90°C for 30 min and cooled to 40°C immediately before experiments, resulting in regular RSM or RSM prepared from irrLHSMP (irrRSM) ready for inoculation.

3.2. Starter cultures and inoculation rates (Publication II and IV)

Liquid bulk starter containing *Streptococcus* (*St.*) *thermophilus* and *Lactobacillus* (*Lb.*) *delbrueckii* subsp. *bulgaricus* (CH XY-11, Chr. Hansen, Hørsholm, Denmark) was used at inoculation rate of 1% in Publication II.

St. thermophilus ST12 (ST12; Chr. Hansen) was used in Publication IV. Milk samples were inoculated at 1% (v/v). Inoculation rate of 10^5 cfu mL^{-1} was used in dynamic rheological and front-face fluorescence measurements during acidification. Preparation of the inoculum is provided in detail in Publication IV.

3.3. Analysis of milk composition (Publication I)

3.3.1. General characteristics of milk

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 bacterial count (TBC) was determined using a BactoScan FC (Foss Electric) in Milk Analysis Laboratory of Estonian Animal Recording Centre (Tartu, Estonia). The pH of all milk samples was also measured.

3.3.2. Analysis of soluble cations and anions in rennet whey

Rennet whey was separated from the coagulated milk samples, prepared as described in section 3.6.4, after the measurements of curd firmness. Rennet whey was filtered and mixed 1:1 with isopropanol, centrifuged at 14,000 g for 5 minutes and diluted with the proper eluent before analysis. A high-performance liquid chromatographic 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 x 150 mm) at 25°C and eluent 3.0 mM nitric acid, flow rate 1.0 mL min^{-1}. Anions were separated using Waters IC-Pac A column (4.6 x 50 mm) at 25°C; eluent lithium borate/gluconate containing n-butanol and acetonitrile, flow rate 1.2 mL min^{-1}.

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

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 the literature (Otte et al, 1997; Miralles et al., 2003; Heck et al., 2008). The six major protein fractions (α-LA, β-LG and α_{s1}-, α_{s2}-, β-, κ-CN) were detected and quantified. Concentrations of the protein fractions were expressed via relative concentration (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. The κ-CN exists in milk in one major carbohydrate-free form (Farrel et al., 2004), which is separated well by CE and comprises ~50% of the total κ-CN peak area (Otte et al., 1997), and at least six minor fractions, 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 Publication I, therefore, to obtain an adequate relative protein composition of raw milk, the integrated areas of identified major peaks of κ- and α_{s2}-CN were multiplied by two.

3.4. Rennet coagulation of milk (Publication I and III)

Samples of milk were equilibrated in glass tubes at 35°C (Publication I) or at 30°C (Publication III) for at least 30 min using a circulating water bath, followed by addition of 1% (v/v) 10 g L^{-1} (Publication I) or 2.5, 5 or 10 g L^{-1} (Publication III) aqueous solutions of chymosin (Sigma, St. Louis, MO, USA).

After the addition of rennet, samples were mixed by rapid inversion of test tubes three-five times. The rennet flocculation time was determined by visual observation (Publication I and Publication III) or coagulated milk samples were transferred to the rheometer's cup or fluorimetric optical cell for immediate rheological or fluorescence measurements (Publication III). In Publication III, each milk sample was divided into aliquots and renneted separately for visual determination of rennet flocculation time, dynamic rheological or fluorescence measurements.

3.5. Dynamic characterisation of bacterial growth and milk coagulation

3.5.1. Microcalorimetry (Publication II)

Isothermal batch microcalorimeter TAM III (Thermometric, Järfälla, Sweden) was used to determine the starter growth-related heat evolution during fermentation at 40°C. After addition of the starter culture, samples were stirred and transferred into the autoclaved ampoules. In the exponential growth phase the relationship between biomass concentration (X) and specific growth rate (μ) may be described by the first order kinetics $dX/dt = \mu X$. Assuming that the rate of biomass formation (dX/dt) is proportional to the rate of heat production (dQ/dt), maximum specific growth rate (μ_{max}) of the starter was calculated from power-time curves as a slope of ln dQ/dt over time (t) (Maskow & Babel, 2003):

$$\ln \frac{dQ}{dt}_t = \ln \frac{dQ}{dt}_0 + \mu_{max} t$$

3.5.2. Dynamic rheological measurements (Publication II, III and IV)

Low-amplitude oscillatory measurements were conducted using a Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) with the direct strain oscillation option, the Peltier temperature control unit C-PTD200 and coaxial cylinder measuring system CC27 (outer and inner diameters 28.92 and 26.66 mm, respectively).

After the addition of starter (Publication II and Publication IV) or rennet (Publication III), milk samples were stirred and an appropriate volume was transferred into the measuring system. In Publication II and Publication IV the gelation assays were performed at 40°C and followed for up to 24 h. In

Publication III milk was coagulated at 30°C and rheological parameters were determined in 10 s intervals until coagulation occurred. Rheological parameters were determined in oscillation mode at a frequency of 0.1 Hz (Publication II and Publication IV) or 1 Hz (Publication III) and the strain of 0.01, which was within the linear viscoelastic region and did not perturb the coagulation process. The elastic modulus (G'), viscous modulus (G'') and loss tangent (tan δ) were recorded during the measurements.

3.5.3. Front-face fluorescence spectroscopy (Publication III and IV)

The Instant Screener® Compact spectrofluorimeter (LDI Ltd., Tallinn, Estonia) equipped with pulsed xenon lamp was used for the front-face fluorescence measurements of renneted or acidified milk samples. Measurements were performed in a 10-mL optical cell with quartz bottom window. The spectral fluorescence signatures (SFS) were recorded as a matrix of fluorescent intensities depending on excitation and emission wavelengths (λ_{ex} and λ_{em}, respectively) resulting in 3D records.

In Publication III the SFS were recorded at 30°C every 10-11 s after rennet addition throughout coagulation. The fluorescence signal was measured near maximum emission of tryptophan at λ_{ex} ranging from 280 to 283 nm and λ_{em} ranging from 325 to 328 nm with 1 nm slits.

In Publication IV the SFS were recorded at 40°C from milk samples inoculated with ST12 every 15 min throughout the fermentation. The SFS were recorded changing λ_{ex} in the range 230-400 nm and λ_{em} in the range 250-615 nm, with excitation and emission slits set at 5 nm.

3.6. Determination of coagulation time parameters and characterisation of rennet-induced gels

3.6.1. Rennet flocculation time (Publication I and III)

The rennet flocculation time (RFT) of milk was measured visually using a modified Berridge method (Berridge, 1952). After the addition of rennet, samples were gently stirred in a water bath at 35°C (Publication I) or at 30°C (Publication III) until RFT was determined as the time from rennet addition until the formation of the first visible flocks of coagulated protein on the walls of the glass tube.

3.6.2. Coagulation time parameters determined from dynamic rheological measurements (Publication III)

Based on dynamic rheological measurements, the time indicating the start of aggregation of casein micelles was derived from the time when G'' started to deviate from the baseline ($t_{G''}$) in coagulating milk; the gelation time (t_g) was defined as time needed to reach the crossover point of G' and G''.

3.6.3. Coagulation time derived from fluorescence measurements (Publication III)

For smoothing the raw tryptophan fluorescence intensity data and estimation of possible coagulation time parameters from the fluorescence patterns an algorithm was developed in MatLab version R2009a (The MathWorks, Inc., Natick, MA, USA), in which a Savitzky-Golay smoothing filter was used to reduce the amount of high frequency noise component in the signal (Savitzky & Golay, 1964). The Savitzky-Golay filter was chosen, as it tends to preserve features of the original distribution such as relative maxima, minima and width that are required for precise estimation of coagulation times. For details see Publication III.

The filtering was followed by calculating the derivatives of the filtered signals and finding local extreme points. Exact point corresponding to coagulation time was chosen by comparison of tryptophan fluorescence intensity data to coagulation time parameters determined by reference methods as described above. The time to the minimum of the second derivative of tryptophan fluorescence profile ($t_{2\min}$) was proposed to be used as a coagulation time parameter.

3.6.4. Curd firmness (Publication I)

Rennet gels were prepared by addition of 100 μL of a 10 g L^{-1} chymosin solution to 10 mL of skimmed milk (3,000 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.

3.7. Determination of gelation time and characterisation of acid-induced milk gelation (Publication II and IV)

Based on dynamic rheological measurements, the gelation time (t_g) was defined as the time necessary for the rheometer response (torque) to reach the value of 0.01 μNm, considered to be the first reliable increase in viscosity that exceeded the signal noise indicating that the milk started to gel. A gelation rate was obtained from the maximum rate of increase in G' over time, $(dG'/dt)_{max}$. The structure of acid gels formed was described by the values of G', G'' and tan δ measured 6 h after the start of gelation.

4. RESULTS AND DISCUSSION

The results of this dissertation are presented and discussed in four sections, which correspond to four publications this work is based on.

4.1. Technological quality of raw bulk milk in Estonia (Publication I)

A survey on raw milk microbiological quality (Stulova et al., 2010) and chemical composition together with renneting characteristics (Publication I) was conducted in 2004-2007 using the bulk milk samples collected from farms located in 7 of the 15 counties of Estonia, covering more than a half (53%) of the total land area, and thus, was regarded as representing milk quality in Estonia.

The study on the microbiological quality (Stulova et al., 2010) showed that although overall bacterial numbers in raw milk had decreased significantly in the past decades, the numbers of technologically harmful bacteria (psychrotrophic and sporeforming species) increased over lactic acid bacteria (LAB), which might potentially cause problems in case of usage of milk for cheese production.

The primary objective of Publication I was to evaluate the rennetability of Estonian raw bulk milk by the direct measurement of coagulation characteristics (rennet flocculation time – RFT and curd firmness), to establish seasonal and non-seasonal variations and to find correlations between these properties and milk composition. The comparison of the properties of milk sampled at the farms and the same milk received at the dairy, which had undergone mechanical perturbations as pumping and refrigerated transportation in tank trucks, was also carried out.

4.1.1. Milk renneting properties

Milk pH is known to affect the first stage of milk renneting – an enzyme-catalyzed reaction as well as nonenzymatic phase of rennet coagulation (Fox et al., 2000). The mean pH of Estonian bulk milk obtained from individual farms has become 0.1 units higher in the past two decades: in 2004-2007 the mean pH was 6.76 ± 0.04 (Table 4) and in 1984-1991 – 6.67 ± 0.03. The mean pH of raw bulk milk in Estonia in 2004-2007 was also 0.05-0.1 units higher than reported for milk from individual cows in UK (Tsioulpas et al, 2007a) or bulk milk analysed in France, Sweden and Italy (Martin et al, 1997; Lindmark-Månsson et al, 2003; Malacarne et al., 2006). Taking into account rather strong negative correlation between milk pH and curd firmness, r = -0.49 (see section 4.1.5), higher losses of curd fines in whey during vat process and lower cheese yield could be expected with the increase in the pH of raw bulk milk.

Higher pH of milk has been associated in the literature with the increased somatic cell count (SCC) of over 800,000 cells mL^{-1} (Moslehishad et al., 2010). Only one sample exceeded that boundary and the mean SCC (277,000 cells mL^{-1}) was three times lower than the critical level in bulk milk collected from

Estonian farms in 2004-2007. Indeed, the microbiological quality of milk has definitely improved in the last two decades in Estonia (Stulova et al., 2010). However, even two decades ago the raw milk quality was not so poor that this could have been assumed to be the reason for observing lower pH than in 2004-2007. Dairy farms in Estonia have been traditionally rather big and raw milk has been usually stored at the farm not more than 24 h after milking. Hence, the LAB had not have sufficient time to grow to considerable numbers in cooled milk to produce enough lactic acid to decrease milk pH by 0.1 units in the past in comparison with the situation in 2004-2007. Furthermore, we did not find any correlation between pH and total bacterial count (TBC) in bulk milk. The LAB counts were also determined in all received samples during the survey (data not included in Publication I) and no correlation was found between pH and LAB counts. Thus, it was assumed that the observed increase of average milk pH could be associated with the changes in feeding strategies of cows (increased share of concentrates) as a result of industrialisation of dairy farming.

Table 4. The renneting properties of raw bulk milk samples collected from individual farms and pooled milk samples collected from trucks and dairy silos in 2004-2007 in Estonia. The *p*-values characterize 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
	Mean ± SD	Range		Mean ± SD	
		Min value	Max value		
pH	6.76 ± 0.04	6.56	6.86	6.76 ± 0.05	NS
RFT (min)	5.45 ± 1.06	2.07	10.35	6.48 ± 1.11	<0.001
Curd firmness (g)	21.5 ± 4.5	9.7	36.4	23.5 ± 5.1	<0.01

The direct milk renneting characteristics of the individual milk samples analysed in 2004-2007 varied in a wide range: values of RFT varied five-fold and curd firmness – more than three-fold (Table 4). Although curd firmness of the milk samples from different farms varied to a great extent, pooling of milk decreased the variability a lot, i.e. firmness of the curd obtained from the milk from dairy silos varied from 19 to 27 g depending on the sampling occasion. Only one bulk milk sample from one sampling did not result in any measurable coagulum during the survey; milk from that farm produced coagulum with satisfactory time and of good firmness in all other sampling days.

The mean values of renneting properties characterizing individual farms were calculated for the dairy farms sampled at least three times during the observed period. The greatest variation was observed in case of curd firmness: the mean values for bulk milk of individual farms differed two-fold from 14 to 29 g. The mean RFT for milk of different farms varied from 4.5 to 6.8 min, and mean pH varied from 6.68 to 6.81.

4.1.2. Seasonal variations in milk general composition and renneting properties

Remarkable seasonal variations in milk composition and renneting properties were found in Estonian farm-tank milk (Table 5). The mean protein and fat contents in Estonian milk were the lowest in summer and the highest in autumn. Although the climate, calving patterns and feeding strategies are relatively similar in Estonia compared to the Netherlands and Sweden (Heck et al., 2009; Lindmark-Månsson et al., 2003), Estonian milk showed somewhat different seasonal changes in the two main components, protein and fat. Regarding the entire data set presented in Table 5, milk with the best cheese-making properties (low pH, low TBC, high protein and fat, firmer coagulum) was produced in autumn. On the other hand, the shortest mean RFT in Estonian milk was recorded in summer; the lowest mean urea and the highest mean SCC were also observed in summer (Table 5). The highest mean urea was observed in spring, which is in line with the results of Grimley et al. (2009) showing elevated urea concentrations in milk during turn-out from dry feed to spring pasture.

Table 5. The mean ± SD values of the general characteristics and renneting properties of raw bulk milk samples collected from individual farms through seasons. The *p*-values characterize the difference between the mean values obtained in case of milk samples collected during different seasons.

Parameter	Season when raw bulk milk samples were collected				*p*-value
	Winter	Spring	Summer	Autumn	
General characteristics					
Protein (g 100 g^{-1})	3.31 ± 0.13	3.27 ± 0.16	3.25 ± 0.13	3.41 ± 0.17	<0.001
Fat (g 100 g^{-1})	4.17 ± 0.25	4.14 ± 0.27	3.98 ± 0.20	4.30 ± 0.27	<0.001
Lactose (g 100 g^{-1})	4.73 ± 0.10	4.78 ± 0.08	4.77 ± 0.07	4.67 ± 0.10	<0.001
Urea (mg L^{-1})	254 ± 58	268 ± 65	220 ± 53	231 ± 68	<0.001
Freezing point (°C)	-0.528 ± 0.005	-0.528 ± 0.005	-0.526 ± 0.004	-0.527 ± 0.005	NS
TBC (10^3 cfu mL^{-1})	33 ± 96	33 ± 79	34 ± 81	19 ± 15	NS
SCC (10^3 cells mL^{-1})	266 ± 89	273 ± 105	300 ± 93	287 ± 134	<0.05
Renneting properties					
pH	6.77 ± 0.04	6.77 ± 0.04	6.74 ± 0.03	6.72 ± 0.05	<0.001
RFT (min)	5.65 ± 0.99	5.37 ±1.08	5.35 ± 0.93	5.54 ± 1.23	<0.01
Curd firmness (g)	20.0 ± 4.1	20.6 ± 3.2	22.0 ± 4.3	24.0 ± 4.9	<0.001

Principal component analysis (PCA) was applied to visualise the differentiation of farm milk quality by seasons. 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 observed in the plot of principal component two (PC2, 13.4% of the total variation) versus principal component three (PC3, 12.7% of the total variation) (Figure 3). 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. Hence, autumn milk was characterized by higher fat, protein, longer RFT, and lower pH; conversely, spring samples were discriminated due to higher pH. Summer milk showed lower pH, lower fat, protein, and RFT.

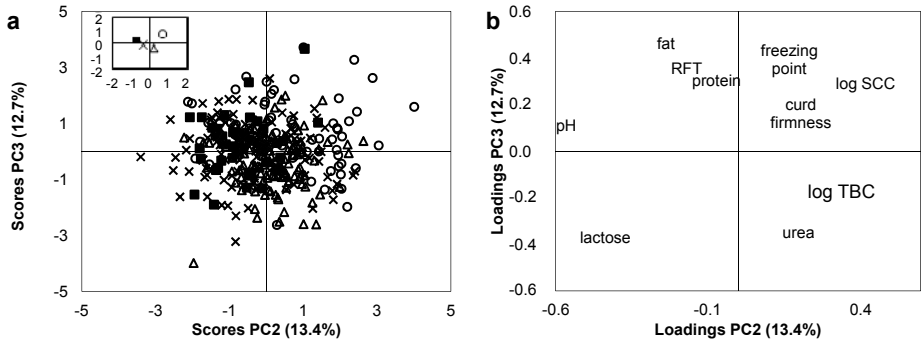

Figure 3. Scores (a) and loadings (b) of the second (PC2) and third (PC3) principal components obtained by PCA of general composition and renneting properties of raw bulk milk samples collected from individual farms in spring (■), summer (Δ), autumn (○) and winter (x). Averages ("centres of gravities") of the PCA scores of milk collected in different seasons are presented in the insert.

What are the possible reasons of seasonal differences in milk composition and the renneting properties? The calving of cows is distributed in Estonia evenly throughout the year (Estonian Animal Recording Centre, 2010). Hence, changes in milk composition could be explained by certain changes in cows' diet in warm and in cold months – more concentrates in winter and more fresh fodder in summer. 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.

It was clearly shown in Publication I for the first time that the renneting properties of milk also displayed a seasonal pattern – the seasonal variability has been reported earlier only for the chemical composition of milk (Heck et al., 2009; Lindmark-Månsson et al., 2003).

4.1.3. Stability of the quality of milk produced in Estonia

Cheese producers can take into account seasonal changes in milk quality, but random fluctuations in milk composition, not related to season, cannot be forecasted. Farms engaged in the survey and sampled at least three times in the period 2004-2007 were evaluated for the stability of the composition and

renneting characteristics of the milk they produced. The quality of milk was considered variable if the values of standard deviation (SD) calculated 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 and rennet 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 content and curd firmness; 13% of the farms were variable in protein and 7% in lactose content of milk. Large variations in the composition of milk produced by the same farm indicated most probably the lack of the proper management of cows feeding.

4.1.4. 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 significantly different in milk from tank trucks and dairy silos in comparison with the samples from individual farms ($p < 0.05$) (Table 6).

Table 6. 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*-values characterize 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
	Mean ± SD	Range		Mean ± SD	
		Min. value	Max. value		
α-LA (%)	2.8 ± 0.4	1.7	3.7	2.9 ± 0.6	NS
β-LG (%)	8.8 ± 1.2	6.9	12.0	10.1 ± 1.3	<0.05
α_{S1}-CN (%)	26.5 ± 1.4	23.7	29.9	26.7 ± 1.4	NS
α_{S2}-CN[a] (%)	5.3 ± 0.7	4.0	7.1	6.2 ± 0.4	<0.001
κ-CN[b] (%)	10.0 ± 1.0	7.9	12.1	9.5 ± 0.6	<0.05
β-CN (%)	37.1 ± 1.4	30.9	38.9	35.3 ± 2.1	<0.05
WP (%)	11.7 ± 1.4	9.3	15.5	13.0 ± 1.8	<0.05
CN (%)	78.9 ± 1.4	75.1	81.3	77.6 ± 1.8	<0.05
WP:CN	0.148 ± 0.021	0.115	0.206	0.168 ± 0.028	<0.05

[a] recalculated assuming that the major fraction comprises 50% of the total α_{S2}-CN area
[b] recalculated assuming that the major fraction comprises 50% of the total κ-CN area

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, however, the difference was not statistically significant ($p > 0.05$). Psychrotrophic bacteria have been reported as predominant in Estonian raw milk (Stulova et al., 2010). These bacteria also possess considerable proteolytic activity on milk proteins

(Ercolini et al., 2009), and they can activate plasmin activators under cheese-making conditions (Frohbieter et al., 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 if incubated with artificial micelles (Koka & Weimer, 2000). Lower relative total casein content in pooled milk from tank trucks and dairy silos and essentially lower content of κ- and β-CN fractions (see Table 6) refer 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, causing an overestimation of the β-LG fraction in milk if proteolysis had 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 had one minute longer mean RFT ($p < 0.001$), which is almost 20% longer than the mean RFT value measured for the bulk milk from individual farms (see Table 4). 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, the 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 of 27 and 23 g, respectively), and among milk from dairy silos (difference of 8 g), the difference of nearly 2 g between the mean curd firmness of farm milk and the 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 the means in the present study (the mean values were not weighted), the conclusions drawn could not be considered completely impeccable.

Differences in the relative protein composition and properties of bulk milk obtained from individual farms and pooled milk from tank trucks and dairy silos provides us with awareness that, in addition to the original milk composition and microbiological quality, the factors encountered after milking at the farm and before processing at dairy plant, such as refrigerated transportation of milk to dairy silos, milk vigorous pumping, occurrence of disinfectant residues etc., may become also important in determining milk coagulability.

4.1.5. Correlation between chemical composition and renneting properties of bulk milk

The Pearson's coefficients between the renneting properties, milk composition and ions in rennet whey are shown in Table 7. The pH of milk correlated negatively with curd firmness and protein content and positively with RFT ($p <$

0.001). The pH of milk also correlated significantly with ions determined in rennet whey: negative correlations with Na^+, K^+ and Cl^- were observed. 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. However, this was probably due to the close relationship of fat, lactose and protein contents. The positive correlation between curd firmness and K^+ content in rennet whey was also established ($p < 0.05$). The relative κ-CN composition correlated with curd firmness even more than the total protein content, $r = 0.45$ ($p < 0.001$). These results obtained in case of bulk milk correspond to those reported by Wedholm et al. (2006) based on milk from individual cows, which showed the association of low concentration of κ-CN with poor clotting properties of milk, resulting in weak curd and lower cheese yield. The results of Publication I have shown that correlations between milk composition and technological properties exist not only in milk from individual cows but also in pooled milk utilised for processing.

Table 7. Correlation matrix of Pearson's coefficients of milk renneting properties, general and relative protein composition, and ions in rennet whey.

	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	-0.23	-0.07	0.04
κ-CN	-0.10	-0.23	0.45**
β-CN	-0.09	0.29	-0.18
WP:CN	0.04	-0.02	-0.05

* correlation is significant at the 0.05 level; ** correlation is significant at 0.01 level; *** correlation is significant at 0.001 level

4.2. Effect of H_2O_2 on the growth of starter bacteria and structure of acid gels (Publication II)

The retardation of the growth of starter bacteria by H_2O_2 may take place via the following paths: 1) direct action on microbial cells; 2) activation of lactoperoxidase (LPO) system; 3) oxidation of substrates – oxidation of vitamins or amino acids, cross-linking of proteins making them unavailable for bacteria, or accumulation of toxic compounds, e.g. lipid oxidation products. The direct action of H_2O_2 on the cells of LAB can be precluded or at least largely alleviated by incubating milk treated with H_2O_2 at elevated temperatures for adequate time, or using catalase before inoculation – the approaches applied in Publication II to degrade H_2O_2. The natural LPO system of milk is inactivated during high temperature treatment (Kussendrager & van Hooijdonk, 2000); hence, any LPO-related oxidative or antimicrobial action is not likely to occur in ultra-high temperature treated (UHT) milk used in Publication II. Therefore, oxidation of milk proteins, fats etc., and not the direct action of H_2O_2 on the bacterial cells was assumed to be the main cause of inhibition of the growth of starters and formation of gel in UHT milk. A proof for this hypothesis was sought by exploring the growth of thermophilic starter bacteria in UHT milk by using isothermal batch microcalorimetry and comparing the results with the rheological profiles of milk gelation during acidification.

4.2.1. Inhibition of growth of starter bacteria by H_2O_2

A typical power-time curve of UHT milk samples inoculated with thermophilic starter containing *St. thermophilus* and *Lb. bulgaricus* showed two peaks corresponding to two growth phases of the starter bacteria (Figure 4). *St. thermophilus* may exhibit diauxic growth in milk, displaying two exponential growth phases separated by a nonexponential phase, during which proteinase is synthesized; during the first exponential growth phase free amino acids and small peptides and during second phase caseins are utilized as the source of nitrogen (Letort et al., 2002). *Lb. bulgaricus* grows in milk in the presence of *St. thermophilus* exhibiting no diauxia (Courtin et al., 2002). Hence, the occurrence of two peaks on power-time curves could be explained by diauxic growth of *St. thermophilus*.

All power-time curves of UHT 3.5% and UHT 0.05% milk samples, treated with H_2O_2 at concentrations from 0 to 100 mg L^{-1} and one hour afterwards inoculated with thermophilic starter, retained the two-stage growth pattern (Figure 4a and b). However, increasing the concentrations of H_2O_2 led to the prolongation of the lag phase. The maximum specific growth rate (μ_{max}) of the thermophilic starter decreased significantly in both the first and the second exponential growth phases ($p < 0.05$) with the increase of H_2O_2 concentration at which milk was treated (see Table 1 in Publication II). In case of 150 mg L^{-1} H_2O_2 the heat production associated with the growth of starter bacteria was initiated only on the second day of experiment in UHT 3.5% and UHT 0.05%.

No heat production was recorded during 5 days in case of 200 and 250 mg L^{-1} H_2O_2, indicating total inhibition of the growth of the starter bacteria.

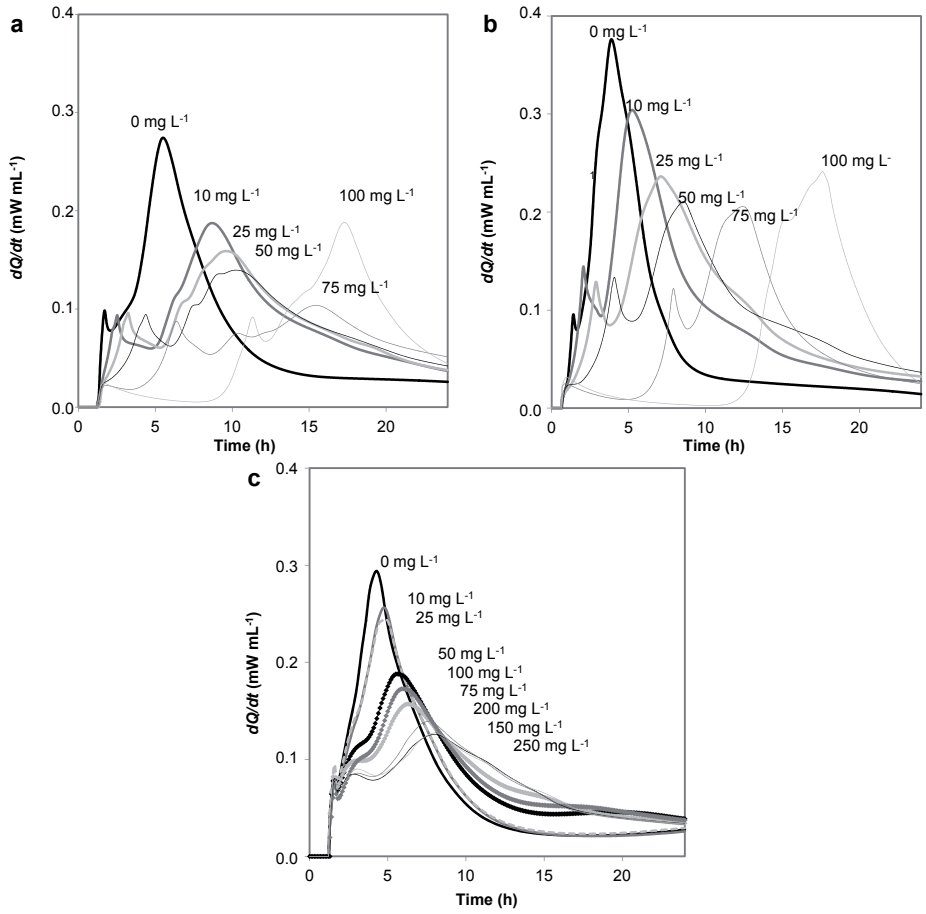

Figure 4. Mean power-time curves of UHT 3.5% (a) or UHT 0.05% (b) inoculated milk samples pre-treated with H_2O_2 at different concentrations one hour before starter addition. Some aliquotes of UHT 3.5% milk pre-treated with H_2O_2 were also treated with catalase before starter addition (c).

Nevertheless, looking carefully at power-time curves of milk samples treated with higher amounts of H_2O_2 (over 50 mg L^{-1}), one can see some heat production in the beginning of the curves, before the first and second peaks associated with the growth of the bacteria (Figure 4a, b). This comparatively small descending heat production was also recorded from milk samples containing H_2O_2 but with no starter added and correlated with the amount of H_2O_2. Hence, abovementioned heat evolution should be an indication of H_2O_2 decomposition proceeding further in milk even after inoculation with starter. In contrast to our initial assumption, incubation of milk for one hour at 40°C was not enough for added H_2O_2 to undergo complete decomposition. Moreover, in UHT 3.5% milk

samples, treated with catalase one hour after H_2O_2 addition and prior to inoculation, the starter had the same lag-phase (Figure 4c) and no change in μ_{max} in the first exponential growth phase ($p > 0.05$) compared to the control samples without treatment with H_2O_2, in contrast to milk with added H_2O_2 but lacking catalase treatment. It was obvious from calorimetrical data that bacteria started to grow in milk samples not treated with catalase only after H_2O_2 had been fully decomposed (Figure 4a, b). Therefore, inhibition of growth of starter bacteria and prolongation of their lag phase in samples with H_2O_2 added to milk and not subsequently treated with catalase was assigned to *the direct inhibitory effect of residual H_2O_2*.

Still, power-time curves of inoculated milk samples, pre-treated for one hour with H_2O_2 at different concentrations and followed by catalase treatment before inoculation, showed rather modified patterns compared to control ones (Figure 4c). A significant decrease of the μ_{max} ($p < 0.05$) in the second exponential growth phase from 0.8 h^{-1} in control sample to 0.5-0.6 h^{-1} after H_2O_2 pre-treatment at 150-250 mg L^{-1} in milk was observed. In other words, the elimination of residual H_2O_2 from milk with catalase removed the inhibitory effect on the first exponential growth phase, but retained the inhibition in the second exponential growth phase of starter bacteria. Relying on calorimetrical data, we assumed that the oxidative action of H_2O_2 in UHT milk was directed presumably on the components essential for microbial growth particularly during the second exponential growth phase. Milk contains a wide variety of components, including proteins that can act like antioxidants and can serve as the primary target for H_2O_2 action (Østdal et al., 2000). Protein oxidation may occur through the oxidation of amino acid side chains or protein backbone resulting in protein-protein cross-linkage (e.g. through the formation of dityrosine) or cleavage of the peptide bond (Berlett & Stadtman, 1997; Giulivi et al., 2003; Takasaki et al., 2005). Recently, it was demonstrated that the treatment of milk with transglutaminase had a growth-slowing effect on yoghurt starter bacteria, and it was proposed that low molecular weight peptides and/or amino acids were cross-linked by the enzyme and became partially unavailable for bacteria (Özer et al., 2007). Similarly, we assumed that H_2O_2 caused oxidative changes in proteins, used as a source of nitrogen during the second exponential growth phase, diminishing their availability for starter bacteria. Therefore, also in samples with H_2O_2 added to milk and not treated with catalase before starter addition, the retardation of bacterial growth might occur also *via the oxidation of substrate* in addition to primary inhibitory effect – direct bacteriostatic influence of H_2O_2.

Comparison of the power-time curves recorded in case of UHT 3.5% and UHT 0.05% milk fermented with thermophilic starter (Figure 4a and b) and also the data from Table 1 in Publication II showed that the duration of the lag phase and growth in the first exponential phase were more severely affected in skim milk than in 3.5% milk. Conversely, second exponential growth phase was more prolonged on increasing concentrations of H_2O_2 in 3.5% milk compared to skim

milk. The rate of decomposition of H_2O_2 was reported to be higher in whole milk than in skimmed milk (Amin & Olson, 1967). Significant increase in peroxide values in yoghurt produced from H_2O_2-treated raw milk (Özer & Atamer, 1999) and in H_2O_2-treated raw cream (Özer et al., 2000) refer to possible stimulation of lipid oxidation in high-fat samples. Produced lipid hydroperoxides may be subsequently decomposed to yield the reactive aldehyde species that can amplify damage to proteins by alternation of amino acids side chains (Uchida, 2003). Therefore, the retardation of bacterial growth due to the oxidation of substrate initiated by H_2O_2 is more evident to be the case in milk containing fat than in low-fat milk.

4.2.2. Milk gelation and small deformation rheological properties of yoghurts prepared from milk treated with H_2O_2

Gelation curves of UHT 3.5% and UHT 0.05% milk samples, treated with H_2O_2 at concentrations from 0 to 100 mg L^{-1} and one hour later inoculated with thermophilic starter are shown in Figure 5. No change in viscoelastic moduli (no gelation) was observed in milk samples with H_2O_2 added in concentrations over 150 mg L^{-1} during 24 hours.

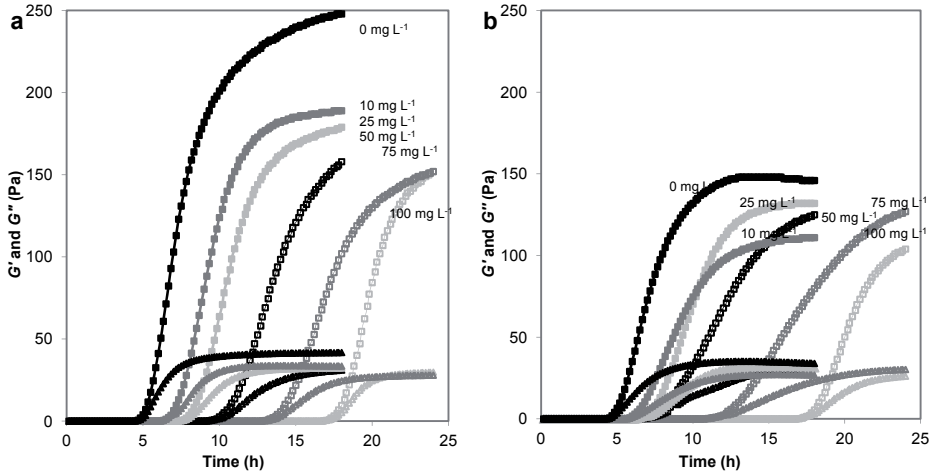

Figure 5. Mean curves of elastic (G') (■) and viscous (G'') moduli (▲) as a function of time in inoculated milk samples pre-treated with H_2O_2 (0 to 100 mg L^{-1}) one hour before starter addition: a) UHT 3.5% and b) UHT 0.05% milk samples.

A significant prolongation of the time of initiation of gel formation (t_g) was caused by increasing H_2O_2 concentration in the case of both, UHT 3.5% and UHT 0.05% milk samples (Table 8). Already the lowest concentration of H_2O_2 utilized (10 mg L^{-1}) prolonged the lag phase of gel formation for at least one hour. The t_g of milk treated at 100 mg L^{-1} of H_2O_2 reached approximately 17 hours, extending the onset of gel formation fourfold compared to the control samples. Treatment with H_2O_2 led to the decrease of gelation kinetics (decrease of $(dG'/dt)_{max}$) and the decrease of the solid-like character of the gels formed

(decrease of elastic modulus – G' and increase of loss tangent – tan δ). We assumed that the remarkably affected growth of starter bacteria due to residual H_2O_2 in milk (Figure 4a and b) was the primary reason for delayed and slowed down acid production, which consequently postponed the onset of milk gelation and also might have influenced the texture of yoghurts.

Table 8. The means ± SD of gelation time (t_g), storage modulus (G'), loss tangent (tan δ) and gelation rate ($(dG'/dt)_{max}$) for control and H_2O_2 pre-treated UHT 3.5% or UHT 0.05% samples treated or not with catalase before starter addition.

Milk	H_2O_2 (mg L^{-1})	Catalase	t_g (h)	G' (Pa)	tan δ	$(dG'/dt)_{max}$ (Pa h^{-1})
UHT 3.5%	0	-	4.6^{a}	209^{a}	0.191^{a}	64.9^{a}
	10	-	6.4^{b}	173^{ab}	0.194^{a}	51.2^{b}
	25	-	7.4^{c}	156^{bc}	0.200^{a}	43.7^{bc}
	50	-	9.4^{d}	128^{c}	0.221^{b}	33.3^{cd}
	75	-	12.7^{e}	115^{c}	0.224^{b}	29.7^{d}
	100	-	16.9^{f}	145^{bc}	0.203^{a}	45.2^{bc}
UHT 0.05%	0	-	4.3^{a}	135^{a}	0.250^{a}	37.9^{a}
	10	-	5.7^{b}	95^{a}	0.265^{a}	22.7^{a}
	25	-	6.4^{c}	115^{a}	0.261^{a}	29.3^{a}
	50	-	7.9^{d}	98^{a}	0.273^{a}	23.7^{a}
	75	-	11.2^{e}	68^{a}	0.301^{b}	17.4^{a}
	100	-	17.2^{f}	100^{a}	0.256^{a}	28.0^{a}
UHT 3.5%	0	+	3.2^{a}	237^{a}	0.182^{a}	89.6^{a}
	75	+	3.3^{a}	239^{a}	0.186^{a}	79.2^{a}
	100	+	3.6^{a}	226^{a}	0.187^{a}	72.6^{a}
	150	+	3.8^{a}	242^{a}	0.187^{a}	82.4^{a}
	200	+	3.8^{a}	233^{a}	0.186^{a}	79.3^{a}
	250	+	4.0^{a}	232^{a}	0.188^{a}	74.7^{a}

The $(dG'/dt)_{max}$ and G' decreased in UHT 3.5% samples to the minimum value on increasing the H_2O_2 concentration in milk up to 75 mg L^{-1}, and resulted in almost twofold weaker gels compared to control sample (Table 8). Milk samples pre-treated with H_2O_2 at the level of 100 mg L^{-1} one hour before starter addition exhibited slightly higher G' values again, but still did not reach the values of the control sample. Both, high and low fat milk samples exhibited an increase in tan δ at 75 mg L^{-1} of H_2O_2 added into milk one hour before inoculation, and then diminished at concentration of 100 mg L^{-1} up to the level in control gels.

The dynamic rheological properties of gels produced from low fat samples were less influenced by H_2O_2 addition compared to milk with higher fat content (Figure 5, Table 8). The $(dG'/dt)_{max}$ and G' 6 h after onset of gelation showed in UHT 0.05% milk similar dependence on H_2O_2 concentration as in 3.5% UHT samples, but the differences were not statistically significant according to ANOVA ($p = 0.084$ and $p = 0.068$, respectively).

It could be assumed that the stronger negative influence of H_2O_2 pre-treatment of milk exerted on the rheological characteristics of gel observed in case of UHT 3.5% could be explained by more intensive protein oxidation taken place in high-fat milk which might hinder proper gel structure formation in addition to 'simple' deterioration of gel formation because of reduced rate of acid production by starter bacteria. Nevertheless, UHT 3.5% milk samples incubated with H_2O_2 (10 to 250 mg L^{-1}) for one hour and subsequently treated with catalase prior to inoculation, i.e. milk samples free of residual H_2O_2, showed a negligible increase in t_g compared to the control ones ($p = 0.146$) and did not exhibit any significant effect on $(dG'/dt)_{max}$ or rheological characteristics of gels (G', G'', tan δ) 6 hours after the onset of gelation ($p > 0.05$) (Table 8). Thus, our assumption, considering enhanced weakening of gel structure in H_2O_2-treated high-fat milk due to the changes in milk proteins participating in gel matrix formation, was not supported.

It was shown in Publication II that even low concentrations of H_2O_2 led to the prolongation of initiation time of gelation and deterioration of gel quality, acted presumably mainly through the direct inhibition of starter bacteria by residual H_2O_2. Milk samples pre-treated with H_2O_2 and then with catalase, ensuring complete removal of the oxidant, showed nearly the same gelation times and overall rheological characteristics as in the control indicating lack of influence of pre-treatment on the formation of protein gel matrix. The data obtained indicated that H_2O_2 did not alter the structure of milk proteins determining coagulation processes but rather changed the bioavailability of amino acids included in the peptides and proteins for starter bacteria. Nevertheless, in milk treated with H_2O_2 followed by catalase, modified growth profile of yoghurt starter bacteria recorded during second exponential growth phase may potentially result in yoghurt with e.g. altered flavour or other defects, even though gel properties would not be affected.

4.3. Front-face fluorescence spectroscopy monitoring rennet-induced coagulation (Publication III)

A novel front-face fluorescence spectroscopy method was introduced and applied to investigate the rennet coagulation of reconstituted skimmed milk (RSM) and raw bulk milk. Tryptophan fluorescence intensity patterns allowed us to distinguish different phases during the enzymatic coagulation of milk, and determine the specific time corresponding to the onset of aggregation of casein micelles.

4.3.1. Characteristic tryptophan fluorescence intensity profiles during rennet coagulation

Typical tryptophan fluorescence intensity profiles recorded during rennet coagulation of different types of milk (RSM, skimmed and whole raw bulk milk) are presented in Figure 6. The general pattern of the evolution of tryptophan

fluorescence during rennet coagulation of RSM (Figure 6a) was rather similar to that reported by Lopez and Dufour (2001). However, the experiments performed with RSM with addition of different amounts of $CaCl_2$ and with different concentrations of enzyme used to induce coagulation indicated that the fluorescence profiles can look quite different from those reported earlier. Moreover, the behaviour of tryptophan fluorescence observed in bulk milk (Figure 6b and c) was clearly different from that measured in RSM – instead of an overall descending character of the signal in RSM, ascending profile was observed in bulk milk during coagulation within the time span of the measurements. However, if the fluorescence response during renneting of bulk milk was recorded for longer period, a decline in tryptophan emission could also be observed similarly to that obtained in RSM. Instead of initially descending signal observed in skimmed bulk milk (as well as in RSM), tryptophan fluorescence rose quickly in the whole milk during the first minutes of renneting, but the further profile was very similar to that of skimmed bulk milk.

In Publication III we divided the tryptophan fluorescence profiles recorded during rennet-induced coagulation into four stages and tried to relate the changes in measured signal with combined result of release of tryptophan fluorescence and factors that quench the effect, as briefly described below.

1. After the addition of rennet, a decrease of tryptophan fluorescence is seen (but only in the case of skimmed milk – Figure 6a and b).

   The quenching substances – Cys-Cys bridges of chymosin and Asp residues located in the active site of the enzyme (Gilliland et al., 1990) – get closer to the tryptophans of κ-CN located on the surface of the micelles and therefore can reduce their fluorescence. The process is gradual and sequential because, firstly, the number of enzyme molecules is far less than potential cutting sites on the surface of the casein micelles, and secondly, the enzyme approaches the micelles by diffusion, whose path being partly located in the hairy layer, which increases the diffusion time (Walstra et al., 2006).

   In non-homogenized whole milk, the creaming effect causes a decrease in the concentration of fat globules at the bottom of the optical cell, from there the fluorescence emission is measured in the front-face mode. Photons emitted by tryptophan can be absorbed by vitamin A contained in fat globules in the course of resonance energy transfer (Lopez & Dufour, 2001; Peterson & Rask, 1971) explaining the depression of tryptophan fluorescence in milk containing fat. During the first 2-3 minutes of measurement in the whole milk, the increase in tryptophan fluorescence was observed presumably due to the gradual depletion of vitamin A content at the bottom of the optical cell with time, not depending whether milk was being renneted or not. Hence, a decrease in tryptophan fluorescence caused by successive binding of coagulating enzyme could not be seen in the whole milk because of counteracting and overbalancing effect of creaming (Figure 6c).

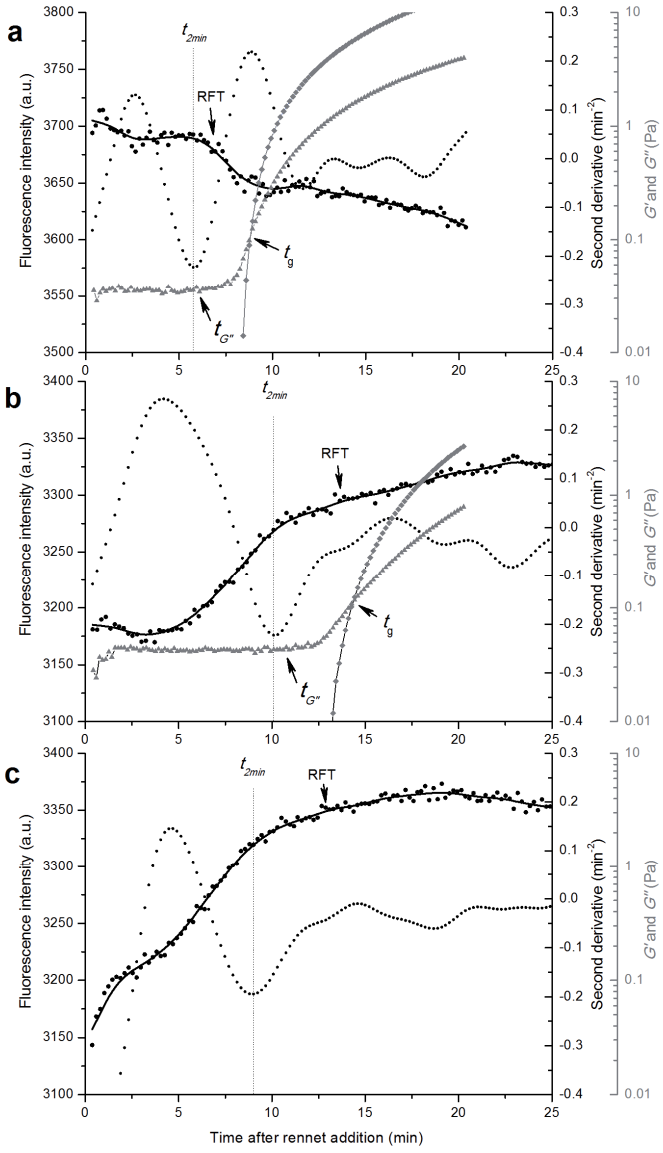

Figure 6. Tryptophan fluorescence data (●) recorded during rennet coagulation of milk and their corresponding second order derivatives: a) RSM enriched with 0.01 M $CaCl_2$, b) skimmed or c) whole raw bulk milk. Renneting was induced by addition of 5 (a) or 10 (b, c) mg mL^{-1} solution of chymosin at 1% (v/v). Smoothed curves and their second derivatives are indicated with black solid or dotted lines, respectively. The times to the minimum of the second order derivative (t_{2min}) are indicated with vertical dashed lines. Evolution of elastic (G') (♦) and viscous (G'') (▲) moduli during rennet coagulation RSM and skimmed milk are also presented. Time corresponding to initial increase in G'' is defined as $t_{G''}$, time to crossover point of G' and G'' is defined as gelation time (t_g). Approximate time of visually determined rennet flocculation time (RFT) is also indicated.

2. Some time later the initial decrease in tryptophan fluorescence stops (if any occurred) and signal starts to increase (see Figure 6).

   The C-terminal part of κ-CN (GMP), removed from the surface of the micelles by the enzyme action, does not contain any aromatic amino acids but includes twelve polar (Asp, Glu and Lys) and five amide side chain groups (Asn and Gln) – potential quenchers of tryptophan fluorescence. In contrast, the N-terminal part of κ-CN (para-κ-CN), containing one Trp, nine Tyr and four Phe residues, remains attached to the micelle. Thus, the release of GMP can cause an increase of fluorescence of aromatic amino acid residues retained in the structure of para-κ-CN. In addition, the dissociation of the enzyme-substrate complex after the reaction should also counterbalance an initial decrease in tryptophan fluorescence.

   Eventually, during enzymatic phase of renneting, there are two counteracting effects influencing fluorescence signal that occur simultaneously: absorption of chymosin onto the surface of the micelles causing a decrease, and removal of GMP and release of enzyme resulting in an increase of tryptophan emission. In skimmed milk, the increase in tryptophan fluorescence becomes noticeable when the enhancing effect caused by cleavage overcomes the depression induced by enzyme binding.
3. At some specific point of time, the fluorescence signal starts to decline as in RSM (Figure 6a), or the slope of the ascending fluorescence curve decreases markedly as in bulk milk (Figure 6b and c).

   If the majority of κ-CN is cleaved, para-κ-CN micelles can approach each other and start to form small chain-like aggregates (Fox et al., 2000). The fluorescence of tryptophan residues located near the surface of micelles can be suppressed by the appearance of additional protein-protein interactions between aggregating particles. The fluorescence signal showed clear decrease in case of RSM enriched with $CaCl_2$ and only a reduction of the slope of the ascending signal in case of bulk milk, which suggests more intensive aggregation of micelles in RSM which contained more calcium than raw bulk milk usually did.
4. After reaching the maximum, the fluorescence signal starts to decrease continuously (in case of bulk milk), or the decline of fluorescence initiated at the previous stage continues with a different slope (in RSM).

   Three-dimensional gel, which is dynamic in its nature, is formed in the result of aggregation. After the onset of gel formation, further changes in protein matrix – incorporation of additional caseins, fusion and rearrangements of particles result in the thickening and hardening of the gel (Mellema et al., 2002) accompanied by lasting decrease in fluorescence, which supports a dynamic character of the gel.

4.3.2. Determination of coagulation time parameter by fluorescence measurements

For better understanding of the changes in the tryptophan fluorescence signal taking place during renneting, the dynamic rheological measurements were conducted in parallel with recordings of fluorescence profiles.

The time indicating the start of aggregation of casein micelles was derived from the time when G'' started to deviate upwards from the baseline ($t_{G''}$). In case of RSM samples the moment when the fluorescence started to descend practically coincided with the $t_{G''}$ (Figure 6a). In case of skimmed bulk milk the $t_{G''}$ roughly coincided with the turning point on the fluorescence curve after which an ascending signal continued to increase with a slower rate (Figure 6b). As for the whole bulk milk, the dynamic rheological measurements failed to show the profile typical to skimmed milk – G' was initially higher than G'' and their respective curves did not have a crossover, as well as viscoelastic moduli increased from the onset of the measurements and the point at which the aggregation of the micelles might proceed was not detectable on rheological profiles. Nevertheless, taking into account the evident similarity of the overall shapes of the fluorescence profiles of skimmed and whole bulk milk (except the very beginning of the curves), the moment of change in the slope of ascending fluorescence signal was suggested to correspond to the onset of aggregation likewise in whole milk (Figure 6c).

In case of RSM samples the moment when the change in the slope of the descending fluorescence signal occurred coincided with the time of the crossover of G' and G'' curves (t_g in Figure 6a), meaning that the three-dimensional gel was established at this point. The gelation of bulk milk occurred approximately when tryptophan fluorescence intensity reached its maximum value, but a specific point analogous to rheological gel point was not clearly discernible on fluorescence profiles, as the curves did not have well-marked transition from ascending to descending behaviour.

The data obtained in Publication III showed that the changes in tryptophan fluorescence intensity were different during coagulation of RSM, skimmed and whole bulk milk. Nevertheless, the universal approach for the determination of the coagulation time parameters utilising the minimum of the second order derivative ($t_{2\min}$) of fluorescence signal was introduced, as also illustrated in Figure 6. In RSM samples, the $t_{2\min}$ designated the time point when fluorescence curve started to decrease (Figure 6a). In the bulk milk samples the $t_{2\min}$ perfectly fitted the time point when tryptophan fluorescence intensity curve changed the slope.

In contrast to our findings, Fagan et al. (2011) proposed an approach utilizing the time to the maximum of the first derivative of tryptophan fluorescence profile ($t_{1\max}$) for monitoring milk coagulation. The comparison of the fluorescence and rheological patterns recorded in the present study indicated that a measurable aggregation of casein micelles proceeded later than $t_{1\max}$ typically

occurred. On the contrary, the t_{2min} parameter may potentially refer to the start of aggregation of casein micelles, irrespectively of the nature of the milk sample.

4.3.3. Coagulation time parameters determined by different methods

The enrichment of RSM with different amounts of $CaCl_2$ and the addition of rennet at different concentrations resulted in a wide range of coagulation kinetics – estimated t_{2min} displayed nearly a tenfold difference between the fast and slowly coagulating samples. In skimmed and whole bulk milk the t_{2min} parameter ranged from 8.7 ± 0.5 to 12.8 ± 1.0 min and from 7.4 ± 0.8 to 10.6 ± 0.6 min, respectively. As the fluorescence profiles obtained during coagulation of RSM and bulk milk samples were substantially different, the Pearson's correlation coefficients (r) between coagulation time parameters derived from fluorescence or reference measurements were calculated separately for different types of milk (Table 8).

Table 8. Pearson's correlation matrix of milk coagulability estimating parameters, obtained by different methods, measured in RSM, skimmed or whole raw bulk milk.[a]

Milk	Variable	t_{2min}	RFT	$t_{G''}$	t_g
RSM	t_{2min}	1			
(n = 7)	RFT	0.98^{***}	1		
	$t_{G''}$	0.98^{***}	0.95^{**}	1	
	t_g	0.94^{**}	0.92^{**}	0.99^{***}	1
Skimmed raw bulk milk	t_{2min}	1			
	RFT	0.77^{*}	1		
(n = 9)	$t_{G''}$	0.98^{***}	0.78^{*}	1	
	t_g	0.96^{***}	0.73^{*}	0.94^{***}	1
Whole raw bulk milk	t_{2min}	1			
(n = 9)	RFT	0.12	1		
	$t_{G''}$	-	-	-	
	t_g	-	-	-	-

[a] Parameters in the table are defined as follows:
t_{2min} – time to the minimum of the second derivative of tryptophan fluorescence intensity signal,
RFT – visually determined rennet flocculation time,
$t_{G''}$ – time to the initial increase of G'' derived from dynamic rheological measurements,
t_g – time to the crossover of G' and G'' derived from dynamic rheological measurements.
* significant at the 0.05 level; ** significant at 0.01 level; *** significant at 0.001 level.

In RSM the t_{2min} had the strongest relationship with rennet flocculation time (RFT) and $t_{G''}$ (r = 0.98). The parameters determined by reference methods (visual and rheological) appeared to correlate with each other less well than with t_{2min} determined by front-face fluorescence spectroscopy. The strongest relationship in the case of skimmed bulk milk was observed between t_{2min} and rheologically determined parameters, particularly $t_{G''}$ (r = 0.98). Noteworthy, the

correlations between t_{2min} and RFT and between RFT and rheological coagulation parameters were notably poorer, but still significant. Unfortunately, no significant correlation between t_{2min} and RFT was observed in the case of whole bulk milk, and rheological parameters could not be derived due to the altered patterns of rheological profiles (see above). Possible explanation of the different relationships obtained between t_{2min} and RFT when measured in different types of milk is given below.

The visual determination of RFT is very subjective, and the specific moment of coagulation process, which RFT value corresponds to, is not well defined. The estimation of RFT in different types of milk may reflect a different degree of flocculation due to differences in viscosity and turbidity of the samples. Our results indicated that the strongest correlations between RFT and coagulation parameters determined by other methods were obtained if measured in the case of RSM samples. Considerably poorer correlation of RFT with t_{2min}, $t_{G''}$ and t_g in skimmed bulk milk samples may be associated with its thicker nature compared to RSM, which might influence the reliability of RFT determination. At the same time coagulation parameters other than RFT still demonstrated strong correlations with each other in skimmed bulk milk. The determination of RFT value in case of the whole bulk milk was quite difficult due to adherence of flakes of milk fat to the glass walls during stirring of the milk samples in thin test tubes, which could be confused with the first signs of protein flocculation. Nevertheless, the shape of tryptophan fluorescence pattern was very characteristic and determination of t_{2min} in the whole milk samples was as simple as in skimmed bulk milk samples. Hence, only the determination of RFT, rather than fluorescence measurements in whole milk was considered unsuccessful in Publication III. Further research is still needed for proving the capability of the fluorescence method to assess coagulation time characteristics in whole bulk milk.

4.4. Front-face fluorescence spectroscopy monitoring milk acidification (Publication IV)

The application of front-face fluorescence spectroscopy in the study of the differences of the growth of *St. thermophilus* ST12 in milk reconstituted from the untreated or irradiated at 10 kGy low-heat skim milk powder (RSM and irrRSM, respectively) is reported in Publication IV.

Firstly, the comparison of spectral fluorescence signatures (SFS) of the control and irradiated milk powders and the reconstituted milk samples provided the first insight into the changes which had taken place with milk proteins during irradiation. The fluorescence intensity corresponding to tryptophan emission maximum was threefold lower in irradiated LHSMP (irrLHSMP) than in LHSMP and the coordinates of the maxima $\lambda_{ex}/\lambda_{em}$ were 285/330 and 285/325 nm, respectively. After reconstitution, almost a twofold difference in the fluorescence intensity between the spectra of irrRSM and RSM were observed.

We suggested that the decreased tryptophan fluorescence in irrLHSMP and irrRSM was associated with the oxidation of aromatic amino acids or production of other oxidation products (Stadtman & Levine, 2003), which could quench the fluorescence, or with conformational changes of proteins caused by irradiation (Cieśla at al., 2004). A distinctive additional fluorescence signal with a maximum at $\lambda_{ex}/\lambda_{em}$ of 350/410-420 nm, which corresponded most probably to the oxidation or Maillard reaction products (Becker et al., 2003; Liu & Metzger, 2007), was observed on the SFS spectra of irrLHSMP (see Fig. 1 in Publication IV) and irrRSM samples.

The changes in maximum fluorescence intensity of tryptophan recorded during fermentation of RSM and irrRSM by ST12 were similar, except for the fact that these occurred much faster in irrRSM, which was in line with the faster drop of pH in irrRSM sample (Figure 7a). Changes in fluorescence intensity corresponded presumably to the modifications of the environment of the tryptophan residues caused by pH decrease during acidification, whereas changes in the protein network associated with gelation phenomenon were not clearly distinguishable. The gelation points of RSM and irrRSM determined by the rheological measurements (marked with an arrow in Figure 7a) did not coincide with the same specific points on the tryptophan fluorescence intensity profiles, but were different for the two milk samples. The gelation point in acidified RSM sample occurred between the maximum and the local minimum and in irrRSM – coincided with the local minimum on the fluorescence pattern. The tryptophan fluorescence intensity patterns obtained in this study during fermentation with ST12 were similar to those shown in the case of milk acidified by glucono-δ-lactone (Lopez & Dufour, 2001). This suggested that the profile presented may be more or less universal and independent of whether acidification was induced by addition of glucono-δ-lactone or growth of starter culture.

The SFS spectral data recorded during fermentation of RSM and irrRSM with ST12 were pooled in one matrix and analysed by PCA (Figure 7b). Two fermentations were fully discriminated along PC1 axis. Changes of SFS in time were observed along PC1 and PC2 axes. The discrimination of the SFS spectra during fermentation was associated with PC1 and PC2 due to specific λ_{ex} of tryptophan fluorescence peak at maximum λ_{em} of near 330 nm. Considering PC1, the fluorescence intensity increased after excitation at 295-300 nm during the first phase of fermentation, and after the turning point a change in the opposite direction was observed. Considering PC2, the fluorescence intensity mainly decreased after excitation at 275 nm during the time course of fermentation. Earlier the tryptophan fluorescence signal measured in processed cheese samples was reported to be composed of two tryptophan components with $\lambda_{ex}/\lambda_{em}$ of 300/347 and 280/339 nm, which were assigned to the fluorophores in different protein structures (Christensen et al., 2003). Comparing our results with those cited above, two types of tryptophan residues included in

different protein structures could also be differentiated in milk during fermentation.

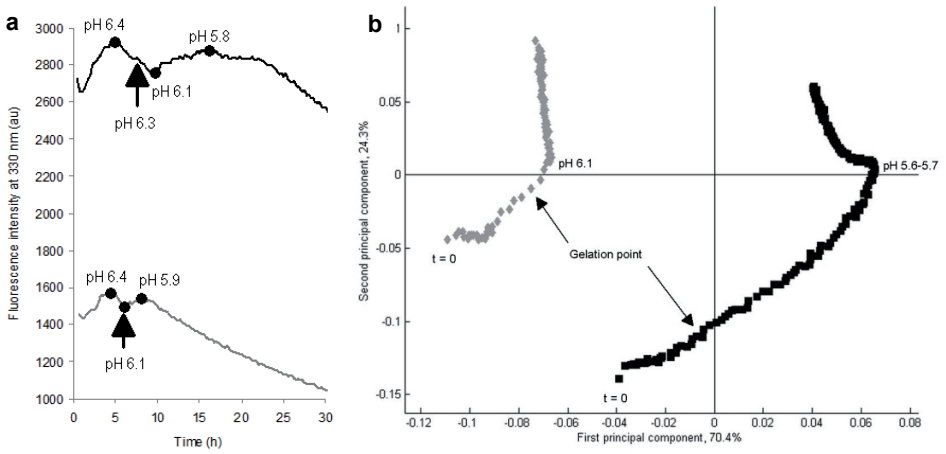

Figure 7. a) Development of the maximum fluorescence intensity of tryptophan during fermentation of RSM (black) and irrRSM (grey) with *Streptococcus thermophilus* ST12 at inoculation rate of 10^5 cfu mL^{-1}. b) The PCA similarity map defined by two first principal components for the SFS data recorded during fermentation of RSM (■) and irrRSM (♦). Arrows mark the mean gelation times determined rheologically.

The layout of the scores on both axes during the time span of fermentations of RSM and irrRSM samples followed rather different profiles. Two phases with turning point at pH 5.6-5.7 and pH 6.1 in the case of RSM and irrRSM, respectively, were observed on the similarity map. These data showed that the turning point separating two phases on the similarity map did not necessarily correspond to the gelation point. From the data obtained we can conclude that depending on milk used as a substrate for ST12 growth the comparable reduction of pH could lead to different changes in protein structures depending on the history of the samples.

Based on the results presented in Publication IV, we suggest that front-face fluorescence spectroscopy could be potentially applicable and useful to elucidate the peculiarities of acidification processes in milk. Front-face fluorescence spectroscopy can provide additional structural information in dynamic mode, which is not readily obtainable by other commonly used experimental techniques.

5. CONCLUSIONS

The two main aims of the dissertation were: a) to gain better insight into the factors affecting technological properties of milk, including original composition of bulk milk and external factors as transportation, pooling of milk, ingress of contaminants i.e. H_2O_2, and b) to apply front-face fluorescence spectroscopy for monitoring enzymatic and acid-induced coagulation of milk.

The main results of this dissertation could be summarized as follows:

I. The renneting properties together with the composition of bulk milk from a representative number of Estonian dairy farms were determined and compared with those of pooled milk from tank trucks and dairy silos for the first time. It was shown that
   - pH of the Estonian bulk milk has increased 0.1 units during the past decades, and it is higher than that of milk produced in other countries in Europe;
   - the renneting characteristics of bulk milk of different farms varied in a wide range; moreover, clear seasonal changes in milk rennetability and firmness of coagulum were observed;
   - although pooling of milk reduces the variations, the important correlations between the composition and renneting characteristics of bulk milk were found to correspond to those reported earlier for milk from individual cows. Milk pH correlated statistically significantly with curd firmness and RFT; curd firmness correlated also with protein content and with κ-casein fraction in particular ($r = 0.45$);
   - changes taking place in milk after milking at the farm and before processing at the dairy plant influence the technological properties of milk. The ratio of whey protein to casein was higher, due to partial degradation of κ- and β-casein fractions, and RFT was longer in pooled milk from tunk trucks and dairy silos. These aspects deserve further investigation.

II. Application of microcalorimetry enabled comprehensive evaluation of the action of a common disinfectant H_2O_2 on the growth of thermophilic starter bacteria in milk. It was shown that
   - the residual H_2O_2 caused the direct inhibition of bacterial growth that resulted in prolonged gelation time and deteriorated gel quality at concentrations starting from 10 mg L^{-1} even if added into milk one hour before starter inoculation;
   - the pre-treatment of raw milk with H_2O_2 for one hour followed by its complete decomposition by catalase did not interfere with acid-induced coagulation, but changed presumably the bioavailability of nutrients for starter bacteria affecting their growth.

III. A novel front-face fluorescence spectroscopy method was applied for detailed investigation of rennet coagulation of reconstituted skimmed milk (RSM) and raw bulk milk. The method could be applied in a laboratory-scale research of milk coagulation, and a non-destructive sensor for in-line monitoring of milk coagulation during cheesemaking or for off-line evaluation of milk suitability for cheese production in dairies could be developed. It was shown that
    - the changes in tryptophan fluorescence intensity patterns recorded during renneting depended on the nature of milk, presence of milk fat and enrichment with $CaCl_2$;
    - tryptophan fluorescence intensity patterns allowed to distinguish different phases of enzymatic coagulation, and to determine a specific point (t_{2min}), which corresponded to the moment when aggregation of casein micelles started;
    - t_{2min} was found to correlate with coagulation time parameters obtained by reference methods in RSM and skimmed bulk milk; the characteristic shape of fluorescence pattern also allowed the determination of t_{2min} in the case of whole bulk milk, but applicability of the method for the determination of the coagulation characteristics of whole milk needs additional verification.

IV. Front-face fluorescence spectroscopy allowed to differentiate milk samples undergone different treatments (e.g. irradiation) and to trace acid-induced changes occurring with milk during fermentation with starter. It was shown that
    - tryptophan fluorescence intensity patterns recorded during acidification corresponded to changes in milk proteins caused primarily by pH decrease and not by gelation phenomenon as such;
    - depending on the nature of milk samples the comparable reduction of milk pH led to different changes in the structures of proteins during acid gel formation.

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ABSTRACT

The ability of milk to coagulate is essential for manufacturing of cheese and fermented dairy products, which provides the added value to milk.

First of all, this dissertation aimed to evaluate the rennetability of Estonian raw bulk milk by direct measurement of rennet flocculation time (RFT) and curd firmness and to find correlations between these properties and milk composition. The direct information on seasonal and non-seasonal variations in rennetability of bulk milk collected from a large number of farms was provided. The results showed that the pooling of milk at the farms, i.e. averaging of milk composition, did not change basic correlations between milk composition and cheesemaking properties reported earlier in case of milk of individual dairy cows. Pooled milk from tank trucks and dairy silos originating from the same milkings was also analysed. Changes in whey proteins to casein ratio due to partial degradation of κ- and β-casein fractions in pooled milk from tank trucks and dairy silos in comparison with the farm milk were observed. Considering changes in the technological properties of milk subjected to mechanical perturbations as pumping and refrigerated transportation in trucks, renneting times of farm milk samples were in average one minute shorter than those of samples from dairy silos. Further, the influence of disinfecting agent, hydrogen peroxide (H_2O_2), on the diauxic growth of starter bacteria and formation of acid-induced gel was investigated by applying microcalorimetry and dynamic low-amplitude rheology, respectively. Even the lowest concentrations of H_2O_2 (starting with 10 mg L^{-1}) added into milk one hour before inoculation caused the direct inhibition of thermophilic bacteria, retarded the onset of milk gelation, and resulted in formation of weaker gels. In milk samples pre-treated with H_2O_2 and subsequently with catalase, ensuring complete removal of the oxidant, the first exponential growth phase of starter bacteria was not affected, and hardly any difference in gelation and no changes in rheological characteristics of mature gels were observed. Nevertheless, clear bacteriostatic influence of H_2O_2 pre-treatment on the second exponential growth phase was observed in milk treated with H_2O_2 followed by catalase, which may potentially result in the end-product with altered flavour or other defects, even though gel properties would not be affected. These studies highlighted that in addition to the original milk composition the external factors encountered between milking and 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.) may also affect enzymatic and/or acid-induced coagulation and properties of the end-product.

Another aim of this dissertation was to apply a novel front-face fluorescence spectroscopy method for detailed investigation of rennet coagulation as well as acid coagulation induced by starter bacteria. Tryptophan fluorescence intensity patterns allowed distinguishing different phases of enzymatic coagulation, starting with first interactions of enzyme with casein micelles and ending with

gel aging. Furthermore, this study showed the ability of fluorescence method to estimate coagulation properties of different milk substrates: reconstituted skimmed milk with wide range of coagulation kinetics as well as skimmed bulk milk samples. A characteristic shape of fluorescence pattern was also obtained during coagulation of whole milk, but the applicability of this method to determine coagulation characteristics in whole milk is still to be verified. Considering the application of front-face fluorescence spectroscopy to trace acid-induced changes occurring with milk proteins during fermentation with starter, tryptophan fluorescence was shown to be highly sensitive to the reduction of milk pH and clearly showed differences in acidification profiles between different reconstituted skimmed milks.

KOKKUVÕTE

Piima kalgendumisvõime on esmatähtis omadus, millele põhineb juustu ja fermenteeritud piimatoodete valmistamine ja mis annab piimale lisandväärtuse.

Käesolevas töös hinnati Eesti farmide segupiima kalgendatavust kalgendumisaja (RFT) ja kalgendi tugevuse määramise kaudu ja leiti seosed segupiima koostise ja kalgendatavuse vahel. Töös analüüsiti paljudest farmidest võetud segupiima proove läbi nelja aasta, mis võimaldas kirjeldada ka sesoonseid kõikumisi Eesti farmipiima kalgendumisomadustes. Saadud tulemuste põhjal näidati, et üksikute lehmade piima kokkusegamine farmis (piima koostise keskmistamine) ei muuda olulisi seoseid piima koostise ja kalgendatavuse vahel (piima pH ja kalgendi tugevuse ja RFT vahel, kalgendi tugevuse ja valgusisalduse, eriti κ-kaseiini sisalduse vahel), mida oli näidatud üksikute lehmade piima puhul. Samuti võrreldi üksikute farmide segupiima ja samade farmide kokku segatud piimade (proovid võetud piimakogumisautodest ja tööstuste silotankidest) koostist ja kalgendatavust. Tööstusesse jõudnud ja silotangi piimas oli keskmiselt kõrgem vadakuvalkude ja kaseiinide suhe, mis võib viidata β-kaseiini osalisele hüdrolüüsile. Piim, mida oli pumbatud farmimahutist piimakogumisautosse, veetud tööstusesse ja pumbatud tööstuse silotanki, kalgendus keskmiselt minuti võrra kauem võrreldes farmist võetud segupiima proovidega. Käesolevas töös uuriti ka desinfektandina kasutatava vesinikperoksiidi (H_2O_2) mõju termofiilsete juuretisebakterite kasvule ja happekalgendi moodustumisele, kasutades mikrokalorimeetriat ja dünaamilist reomeetriat. Juba väga madalate H_2O_2 kontsentratsioonide (alates 10 mg L^{-1}) lisamine piima tund enne inokuleerimist põhjustas juuretisebakterite otsese inhibeerimise, piima geelistumise aeglustamise ja nõrgema geeli struktuuri moodustumise. H_2O_2 lisamine, millele järgnes katalaasiga töötlemine vahetult enne inokuleerimist kindlustamaks oksüdeeriva aine täieliku eemaldamise, ei mõjutanud juuretisebakterite kasvu esimese eksponentsiaalse faasi jooksul ja ei mõjutanud valmis geeli reoloogilisi omadusi. Samas oli bakterite kasvudünaamika teises eksponentsiaalses faasis selgelt mõjutatud, mis võib tähendada muutusi lõpp-produkti aroomi- või maitseprofiilis või muid defekte, vaatamata sellele, et geeli struktuur ei muutunud. Käesoleva töö tulemused näitavad, et lisaks piima algsele koostisele võivad piima ensümaatilist ja/või happelist kalgendamist ning valmistoote kvaliteeti mõjutada ka sellised esmapilgul minoorsed välised mõjud nagu lüpsijärgne jahutamine, pumpamine, transportimine, desoainete jääkide sattumine piima jms.

Doktoritöö teiseks eesmärgiks oli välja selgitada pinna-fluorestsentsspektroskoopia meetodi kasutatavust piima ensümaatilise kalgendamise ja hapendamise jälgimiseks. Trüptofaani fluorestsentsi intensiivsuse muutuste järgi eristati erinevaid kalgendumise faase, alustades ensüümi seostamisega kaseiini mitsellidega ja lõpetades kalgendi vananemisega. Lisaks sellele oli fluorestsentsmeetodi abil võimalik määrata piima kalgendumisomadusi, mida kinnitati nii taastatud piima kui ka kooritud

toorpiima katsetes. Rasvase toorpiima kalgendamise puhul registreeriti samuti iseloomulik fluorestsentsi profiil, siiski vajab fluorestsentsmeetodi kasutatavus täispiima kalgendumisaja määramiseks edasist valideerimist. Samuti näidati, et trüptofaani fluorestsents on väga tundlik juuretisebakterite poolt esile kutsutud piima pH languse suhtes. Hapendamise jooksul registreeritud fluorestsentsi muutuste profiilide järgi oli võimalik selgelt eristada erinevalt töödeldud taastatud piimas toimuvaid protsesse.

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Composition and renneting properties of raw bulk milk in 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

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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

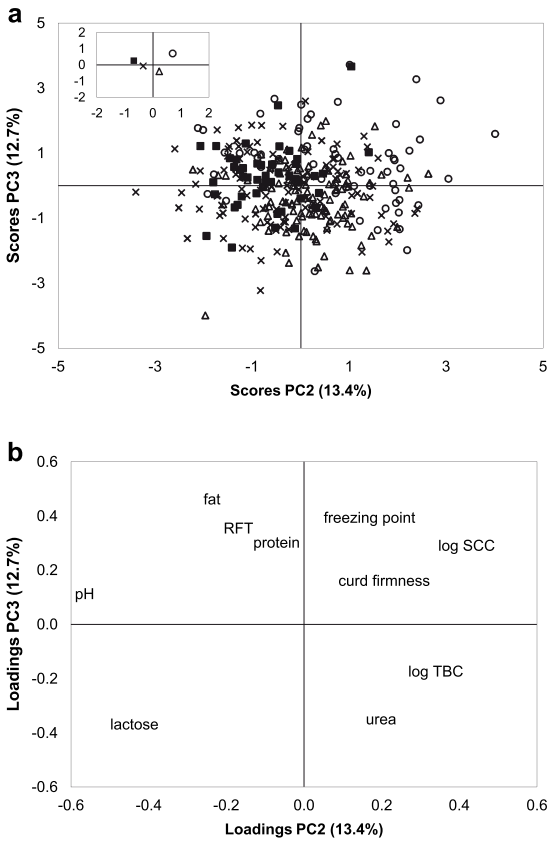

Fig. 1. Scores (a) and loadings (b) of the second (PC2) and third (PC3) principal components obtained by PCA of milk general composition and renneting properties of raw bulk milk samples collected from individual farms: milk samples collected in spring (■), summer (△), autumn (○) and winter (×). Averages ("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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PUBLICATION II

Kriščiunaite, T., Stulova, I., Kabanova, N., Laht, T.-M., and Vilu, R.

The effect of hydrogen peroxide on the growth of thermophilic lactic starter and acid gelation of UHT milk

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The effect of hydrogen peroxide on the growth of thermophilic lactic starter and acid gelation of UHT milk

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ABSTRACT

The influence of H_2O_2 pre-treatment (10–250 mg L^{-1}) of ultra-high temperature treated (UHT) milk devoid of lactoperoxidase activity on the growth of thermophilic starter in the process of yoghurt production was studied using isothermal batch microcalorimetry and dynamic rheological measurements. Typical dual-peak power-time curves of diauxic growth of starter bacteria in milk were registered. Even the lowest concentrations of H_2O_2 added into milk 1 h before inoculation hindered the growth of thermophilic bacteria, retarded the onset of milk gelation, and resulted in formation of weaker gels. UHT milk samples treated with H_2O_2 and subsequently with catalase showed certain bacteriostatic influence of H_2O_2 pre-treatment on the second exponential growth phase. However, hardly any difference in gelation and no changes in rheological characteristics of mature gels in milk free of residual H_2O_2 due to catalase treatment prior to inoculation in comparison with H_2O_2-free control samples were observed.

1. Introduction

Effective cleaning and sanitizing of dairy equipment is a prerequisite for producing high quality dairy products with low bacterial counts. Disinfectant formulations including quaternary ammonium or halogen-containing compounds and hydrogen peroxide (H_2O_2) are commonly used in dairy industry for sanitizing purposes (Tamime & Robinson, 1999). Sodium dichloroisocyanurate, sodium hypochlorite, and H_2O_2 are main active substances of disinfectants used also in dairies and farms in Estonia (Henno & Olkonen, 1998). Being unacceptable in European countries, the retardation of unwanted bacteria in raw milk by addition of substantial amounts of H_2O_2 is permitted in some developing countries with warm climate. The recommended quantities of H_2O_2 to be added to milk range from 100 to 800 mg L^{-1} (Björck, 1987). Yet, low concentrations of disinfectant residues may get into the milk due to faulty cleaning systems, improper dosage, insufficient rinsing, unsuitable pipeline design, or in some cases can be deliberately added by producers into milk to 'improve' its bacterial quality.

Early studies of adding high concentrations of H_2O_2 (100 mg L^{-1} to 15 g L^{-1}) to milk prior to cheese making showed that the treatment led to softness of cheese mass (Kosikowski & Fox, 1968; Roundy, 1958), higher solubility of casein, and higher susceptibility of casein to proteolysis by rennet (Fox & Kosikowski, 1967) and *Pseudomonas fluorescens* proteolytic enzymes (Fish, Pinkston, Claydon, & Mickelsen, 1969). Schmidt, Morris, and Morr (1969) reported reduced rate and completeness of casein clotting by rennet in presence of high concentrations of H_2O_2.

Effects of low concentrations of H_2O_2 in milk have been commonly considered in scientific literature in conjunction with the lactoperoxidase (LPO) system, which prevents the growth of bacteria by catalyzing the oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) believed to possess antimicrobial properties, using H_2O_2 as the electron acceptor (Özer, 1999). The use of LPO system is recommended by FAO/WHO as a standard method for retarding bacterial growth in raw milk during collection and transportation to dairy processing plants in situations when refrigeration of the raw milk is not feasible (Codex Alimentarius Commission, 1991). Recommended concentrations of H_2O_2 added for activation of LPO system are up to 10 mg L^{-1}.

Inhibiting effects of LPO system on cheese and yoghurt starter cultures have been reviewed by Seifu, Buys, and Donkin (2005). Weakening of gel structure, softening and lower viscosity of yoghurt were reported in LPO activated samples, alterations being assigned to the oxidation of thiol groups (−SH) of proteins by $OSCN^-$ leading to decreased number and/or strength of cross-linking (Hirano et al., 1998a; Hirano, Hirano, Oooka, & Hatanaka, 1998b). As well, Østdal, Bjerrum, Pedersen, and Andersen (2000) have shown that changes in milk proteins can occur through the transition of radicals formed

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on LPO during its reaction with H_2O_2 to other milk proteins, and thereby are not necessarily mediated by $OSCN^-$. In these studies antimicrobial and oxidizing effects were not related to the action of H_2O_2 itself but to other components of LPO system.

It has been shown that higher than recommendable concentrations of H_2O_2 may cause irreversible inactivation of LPO (Björck, 1987; Fonteh, Grandison, & Lewis, 2005; Kussendrager & van Hooijdonk, 2000). Some slight decrease in tyrosine content in milk, showing susceptibility of amino acids to oxidation, and significant increase in peroxide values, indicating lipid oxidation, have been reported in yoghurt produced from raw milk treated with considerably high levels of H_2O_2 (100–140 mg L^{-1}) and subsequently heated before inoculation, which demonstrate the occurrence of H_2O_2-promoted and apparently LPO-independent protein and lipid oxidation in milk (Özer & Atamer, 1999). Nevertheless, the oxidative capacity of intermediate concentrations of H_2O_2 added to milk at higher levels (10–100 mg L^{-1}) than normally required for activation of LPO system, and independently of LPO, has not been evaluated as yet.

Retardation of starters by H_2O_2 may take place via the following paths: 1) direct action on microbial cells; 2) activation of LPO system; 3) oxidation of substrates – oxidation of vitamins or amino acids, cross-linking of proteins, making them unavailable for bacteria; or accumulation of toxic compounds, e.g., lipid oxidation products. Direct action of H_2O_2 on the cells of lactic acid bacteria can be potentially precluded or at least largely alleviated by incubating milk samples treated with H_2O_2 at elevated temperatures for adequate time to allow its degradation, or using catalase before inoculation. The LPO system is inactivated during high temperature treatment (Kussendrager & van Hooijdonk, 2000); hence, any LPO-related oxidative or antimicrobial action is not likely to occur in UHT milk. At the same time, lower rate of spontaneous H_2O_2 decomposition should be expected in heat-treated milk in comparison with raw milk (Gilliland, 1969). Thus, oxidation of milk proteins, fats, etc., and not the direct action of H_2O_2 on the bacterial cells has been expected to be the main cause of inhibition of the growth of starters and processes of gel formation in UHT milk. In the present study, the proof for this statement was sought exploring the growth of thermophilic starter bacteria in UHT milk by isothermal batch microcalorimetry and comparing the results with the rheological profiles of milk gelation during yoghurt production.

Microcalorimetry is a useful method to obtain easily kinetic and thermodynamic information on microbial growth that can be barely obtained by conventional bacteriological methods (see Kabanova, Kazarjan, Stulova, & Vilu, 2009). Gardea et al. (2002) compared heat evolution by bacteria with traditional plate counts assessing microbiological quality of milk undergone different treatments. To the best of our knowledge no published information can be found so far on the utilization of microcalorimetry for the study of the growth of lactic starters in milk.

The specific objectives of the present work were as follows: 1) to apply microcalorimetry to study the influence of low to high concentrations of H_2O_2 (10–250 mg L^{-1}) independently of LPO system on the growth of thermophilic starter bacteria in UHT milk; and concomitantly 2) to investigate gelation and determine rheological properties of resulting yoghurt using low amplitude oscillatory measurements.

2. Materials and methods

2.1. Milk and starter supply

Commercial UHT milk with 3.5% and 0.05% fat content (Kalev Paide Tootmine AS, Paide, Estonia) was used in all experiments. Liquid bulk starter containing *Streptococcus thermophilus* (*Str. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) (Chr. Hansen XY-11) was purchased from a small dairy (Nõmmiku Talu, Estonia) and used at inoculation rate of 1% in all experiments.

2.2. Preparation of milk samples

Samples of milk (UHT 3.5% and UHT 0.05%) were equilibrated for 1 h at 40 °C before H_2O_2 addition. Dilutions were prepared from stock 30% H_2O_2 solution (Riedel-de Haën, Seelze, Germany), and 75 μL of corresponding dilution were added to 50 mL of UHT milk to yield final concentrations of 0, 10, 25, 50, 75, 100, 150, 200 and 250 mg L^{-1}. Then, milk samples were left at 40 °C for at least 1 h before starter addition to promote chemical reaction between H_2O_2 and milk constituents.

To assure complete utilization of H_2O_2 before inoculation, parallel samples of UHT 3.5% milk samples incubated with different concentrations of H_2O_2 at 40 °C for 1 h were treated in excess with bovine liver catalase (Sigma–Aldrich, St. Louis, MO, USA) prior to the addition of starter culture. Some samples were also treated with nitrogen gas for removing oxygen prior to the measurements of power-time curves in microcalorimeter.

2.3. Microcalorimetry

Isothermal batch microcalorimeter TAM III Thermal Activity Monitor (Thermometric, Järfälla, Sweden) with thermal power detection limit of 0.5 μW was used for the determination of thermophilic starter growth-related heat evolution during yoghurt production in control and H_2O_2-treated UHT 3.5% and UHT 0.05% samples, and also milk samples treated with catalase and N_2. After the addition of starter at 40 °C, samples were stirred and then 2 mL were transferred into the autoclaved ampoules. At least two ampoules were filled per sample and milk without starter was used as a reference. The experiments were run at 40 °C, and power-time curves were registered until the signal returned back to the baseline.

Taking into account that in the exponential growth phase the relationship between biomass concentration (X) and specific growth rate (μ) may be described by the first order kinetics

$$\frac{dX}{dt} = \mu X \tag{1}$$

and assuming that the rate of biomass formation (dX/dt) is proportional to the rate of heat production (dQ/dt), maximum specific growth rate (μ_{max}) of thermophilic starter was calculated from power-time curves as a slope of $\ln dQ/dt$ over time (t) (Maskow & Babel, 2003):

$$\ln\frac{dQ}{dt_t} = \ln\frac{dQ}{dt_0} + \mu_{max}t \tag{2}$$

The total heat produced during fermentation (Q_{tot}) and the heat evolved until the gelation point ($Q_{t_{gel}}$) were determined using TAM Assistant software version 0.9.1012.40 (Thermometric).

2.4. Dynamic rheological measurements

Low amplitude oscillatory measurements were conducted using a Physica Modular Compact Rheometer MCR 301 (Anton Paar GmbH, Graz, Austria) with the direct strain oscillation (DSO) option, the Peltier temperature control unit C-PTD200 and coaxial cylinder measuring system CC27 (outer and inner diameters 28.92 and 26.66 mm, respectively).

After the addition of starter at 40 °C, milk samples were stirred and an appropriate volume was transferred into the measuring

system. To prevent evaporation, vegetable oil was added to the surface of milk sample. Gelation assays were performed at 40 °C, and were followed for up to 24 h. Samples were oscillated every 10 min after inoculation with starter during the whole experimental run at a frequency of 0.1 Hz and the strain applied was 0.01, which was within the linear viscoelastic region.

Measurements were replicated in three individual milk samples. The storage (elastic) modulus (G'), loss (viscous) modulus (G'') and loss tangent (tan δ) were determined. The gelation time (t_{gel}) was defined as the time necessary for the rheometer response (torque) to reach the value of 0.01 μNm, considered to be the first reliable increase in viscosity that exceeded the signal noise indicating that the milk started to gel. G' curves were smoothed with 1% relative smoothing range and differentiation against time (dG'/dt) was performed with Rheoplus/32 V2.66 software (Anton Paar GmbH). A gelation rate was obtained from the maximum rate of increase in G' over time, $(dG'/dt)_{max}$.

2.5. Statistical analysis

The experimental data were submitted to single-factor analysis of variance (ANOVA), and the differences among means were determined by Fisher's least significant difference (LSD) test. Paired Student's t tests were run to compare the rheological properties of samples with different fat content. Differences were considered significant when p values were less than 0.05.

3. Results and discussion

3.1. Growth of thermophilic starters in milk

A typical power-time curve of inoculated milk samples contained two peaks corresponding to two growth phases of the starter bacteria: first a smaller one and after that the main larger peak (Fig. 1a). The first, smaller peak did not change on the catalase and N_2 treatment (Fig. 1b, c). However, the lag-phase between the first and the second growth phases disappeared on the treatments, and the calculated μ_{max} of the bacteria increased in the second growth phase from 0.64 ± 0.01 h^{-1} in control to 0.79 ± 0.05 and 1.06 ± 0.31 h^{-1} in catalase and N_2-treated milk samples, respectively. The amount of the bacteria formed during fermentation expressed by Q_{tot} did not change upon aforementioned treatments according to ANOVA (data not shown). Milk may contain about 10 mg L^{-1} of oxygen introduced during milking and further processing (Siegenthaler & Kosikowski, 1969). Gilliland & Speck (1969) have shown that oxygen incorporated into milk during aeration slowed down the acid production by lactic acid bacteria. Sparging with N_2 removes molecular oxygen from milk enforcing the anaerobic conditions of growth. Catalase treatment can also lead to the utilization of O_2 participating in a two-stage reaction in cooperation with milk enzymes (e.g. xanthine oxidase or sulfhydryl oxidase), in a similar way as shown for the glucose oxidase − catalase system (Parpinello, Chinnici, Versari, & Riponi, 2002), but indigenous milk enzymes are not likely to sustain UHT process. On the other hand, some lactobacilli can produce various amounts of H_2O_2 during their growth (Ito et al. 2003; Juffs & Babel, 1975) that may turn self-inhibitory for them (Reiter & Härnulv, 1984); and thus, removal of produced H_2O_2 by catalase could be another reason for stimulated growth of starter bacteria. In accordance with aforementioned studies, we assume that removal of oxygen by N_2 and elimination of starter lactobacilli produced H_2O_2 by catalase were the reason for faster growth of thermophilic starters in our experiments.

However, it should be emphasized that presence or absence of oxygen in the milk samples does not change the two-phase pattern of growth (diauxy of the growth). We assume that the occurrence of two peaks on power-time curves is explained by a switch in metabolism of starter bacteria from growth on free amino acids present in milk to growth on amino acids and peptides released by the enzymes synthesized by the bacteria. Indeed, Letort, Nardi, Garault, Monnet, and Juillard (2002) have shown that *Str. thermophilus* demonstrates a diauxic growth in milk, displaying two exponential growth phases separated by a nonexponential phase, during which proteinase is synthesized. During the first exponential growth phase free amino acids and small peptides and during second phase caseins are utilized as the source of amino acids (Letort et al., 2002).

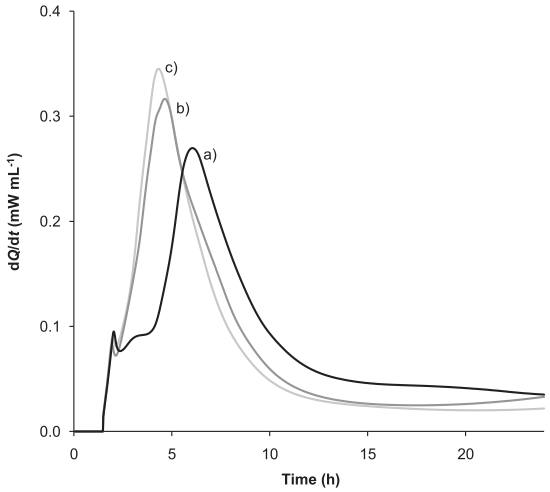

Fig. 1. Characteristic thermal profiles of milk inoculated with thermophilic lactic starter. Mean power-time curves ($n = 3$) of a) UHT 3.5% control samples, b) UHT 3.5% samples treated with catalase and c) UHT 3.5% samples sparged with N_2 before inoculation.

According to our data (Fig. 1), the first exponential growth phase of thermophilic starter containing *Str. thermophilus* and *Lb. bulgaricus* was normally shorter and the calculated μ_{max} was higher than in the second phase (Table 1). Also it was shown for *Str. thermophilus* by Letort et al. (2002) that the growth rate of the cells decreased during the second exponential phase in comparison with the first growth phase due to the limitation in peptide transport when caseins were used as the source of amino acids. *Lb. bulgaricus* had been shown to grow steadily in milk in the presence of *Str. thermophilus*, exhibiting no diauxia until the end of fermentation (Courtin, Monnet, & Rul, 2002). It is noteworthy that the second peak on the power-time curves corresponded to the approximate time when bacteria reached the late exponential or the beginning of stationary phase indicated by the plate counts (data not shown). Taking into account the possibilities of microcalorimetry in detailed description of growth of the starters, further study of quantitative peculiarities of growth of the mixed yoghurt cultures deserves certainly a future attention.

3.2. Inhibition of growth of starter bacteria by H_2O_2

Mean power-time curves of UHT 3.5% and UHT 0.05% milk samples, treated with H_2O_2 at concentrations from 0 to 100 mg L^{-1} and 1 h afterwards inoculated with thermophilic starter, are shown in Fig. 2. It is seen that all power-time curves retained the two-stage growth pattern, but increasing concentrations of H_2O_2 led to the prolongation of the lag-phase of growth of the starter culture, as well as both of the growth stages were shifted further in time.

Table 1
Means ± SD[a] of time at maximum heat production rate ($t_{(dQ/dt)_{max}}$), maximum specific growth rate (μ_{max}), heat[b] produced by starter bacteria throughout the entire fermentation (Q_{tot}) and before the onset of gel formation ($Q_{t_{gel}}$) obtained from microcalorimetric power-time curves for control and H_2O_2 pre-treated UHT 3.5% or UHT 0.05% samples treated or not with catalase before starter addition.

Milk	H_2O_2 (mg L^{-1})	Catalase	$t_{(dQ/dt)_{max}}$ (h)		μ_{max} (h^{-1})		Q_{tot} (J mL^{-1})	$Q_{t_{gel}}$ (J mL^{-1})
			1st exponential phase	2nd exponential phase	1st exponential phase	2nd exponential phase		
UHT 3.5%	0	–	1.71 ± 0.05^{a}	5.51 ± 0.22^{a}	3.10 ± 0.31^{a}	0.64 ± 0.01^{a}	12.83 ± 3.12^{a}	1.27 ± 0.18^{a}
	10	–	2.43 ± 0.18^{b}	8.82 ± 0.35^{b}	1.49 ± 0.12^{b}	0.74 ± 0.05^{b}	12.88 ± 0.27^{a}	1.28 ± 0.03^{a}
	25	–	3.15 ± 0.17^{c}	9.54 ± 0.38^{bc}	1.03 ± 0.05^{c}	0.59 ± 0.02^{c}	12.08 ± 0.17^{a}	1.64 ± 0.15^{b}
	50	–	4.25 ± 0.26^{d}	10.05 ± 0.90^{c}	0.78 ± 0.00^{d}	0.48 ± 0.04^{d}	11.43 ± 0.85^{a}	2.22 ± 0.12^{c}
	75	–	6.39 ± 0.11^{e}	15.56 ± 0.44^{d}	0.78 ± 0.02^{d}	0.27 ± 0.00^{e}	10.35 ± 0.49^{a}	2.00 ± 0.07^{d}
	100	–	11.33 ± 0.03^{f}	17.26 ± 0.19^{e}	1.25 ± 0.04^{c}	0.37 ± 0.01^{f}	9.73 ± 2.28^{a}	2.59 ± 0.06^{e}
UHT 0.05%	0	–	1.44 ± 0.02^{a}	3.90 ± 0.04^{a}	2.70 ± 1.60^{a}	1.43 ± 0.08^{a}	7.79 ± 2.00^{a}	2.70 ± 0.43^{a}
	10	–	2.10 ± 0.03^{b}	5.21 ± 0.20^{b}	1.76 ± 0.09^{b}	0.91 ± 0.13^{b}	10.72 ± 0.09^{b}	2.82 ± 0.15^{a}
	25	–	2.93 ± 0.04^{c}	7.11 ± 0.01^{c}	1.06 ± 0.02^{c}	0.56 ± 0.01^{c}	11.13 ± 0.22^{b}	2.15 ± 0.03^{b}
	50	–	4.13 ± 0.00^{d}	8.61 ± 0.01^{d}	0.90 ± 0.01^{c}	0.45 ± 0.01^{c}	10.77 ± 0.05^{b}	2.40 ± 0.03^{ab}
	75	–	7.94 ± 0.05^{e}	12.43 ± 0.12^{e}	1.14 ± 0.01^{b}	0.47 ± 0.03^{c}	7.15 ± 0.29^{a}	1.96 ± 0.16^{b}
	100	–	15.19 ± 0.13^{f}	17.75 ± 0.28^{f}	1.41 ± 0.04^{b}	ND	6.47 ± 0.31^{a}	2.75 ± 0.24^{a}
UHT 3.5%	0	+	1.58 ± 0.08^{a}	4.28 ± 0.00^{a}	3.17 ± 0.05^{a}	0.84 ± 0.02^{a}	19.20 ± 4.82^{a}	0.85 ± 0.03^{a}
	10	+	1.62 ± 0.06^{a}	4.78 ± 0.08^{b}	3.28 ± 0.12^{a}	0.82 ± 0.04^{a}	19.40 ± 3.09^{a}	0.76 ± 0.08^{a}
	25	+	1.62 ± 0.05^{a}	4.87 ± 0.07^{b}	3.14 ± 0.06^{a}	0.74 ± 0.02^{ab}	16.61 ± 2.46^{ab}	0.77 ± 0.03^{a}
	50	+	1.60 ± 0.05^{a}	5.63 ± 0.07^{c}	2.96 ± 0.96^{a}	0.77 ± 0.12^{ab}	12.44 ± 2.63^{b}	0.83 ± 0.10^{a}
	75	+	1.59 ± 0.12^{a}	6.29 ± 0.22^{d}	3.07 ± 0.14^{a}	0.57 ± 0.17^{bc}	12.65 ± 3.13^{b}	0.70 ± 0.08^{a}
	100	+	1.63 ± 0.06^{a}	5.95 ± 0.14^{e}	3.55 ± 0.92^{a}	0.65 ± 0.10^{abc}	12.07 ± 1.83^{b}	0.77 ± 0.15^{a}
	150	+	1.63 ± 0.10^{a}	8.03 ± 0.06^{f}	3.23 ± 0.25^{a}	0.50 ± 0.11^{c}	13.27 ± 1.27^{b}	0.79 ± 0.14^{a}
	200	+	1.63 ± 0.05^{a}	7.60 ± 0.05^{g}	3.27 ± 0.24^{a}	0.54 ± 0.14^{bc}	13.55 ± 1.39^{b}	0.86 ± 0.28^{a}
	250	+	1.65 ± 0.11^{a}	8.05 ± 0.05^{f}	3.17 ± 0.26^{a}	0.59 ± 0.34^{bc}	12.98 ± 1.36^{b}	1.16 ± 0.48^{a}

[a] Means of the same milk samples treated with various concentrations of H_2O_2 followed by a different superscript letter are significantly different at $p < 0.05$ (ANOVA followed by Fischer's LSD test); ND indicates that accurate μ_{max} during 2nd exponential phase could not be unambiguously determined from power-time curve due to altered shape.
[b] Values are corrected by subtracting heat evolved as a result of residual H_2O_2 degradation in milk before the production of metabolic heat.

Times at maximum heat production rate ($t_{(dQ/dt)_{max}}$) obtained from microcalorimetric power-time curves mark in practice the end of exponential growth phase – it can be seen from Fig. 2 and Table 1 that the end of first exponential phase was shifted almost ten times from 1.71 ± 0.05 h to 11.33 ± 0.03 h in the case of 3.5% milk, and from 1.44 ± 0.02 h to 15.19 ± 0.13 h in the case of 0.05% milk with an increase of H_2O_2 concentrations from 0 to 100 mg L^{-1}. Higher fat content somewhat diminished the effect of H_2O_2 on bacterial growth. Essentially, the same behaviour was observed also in case of the second exponential growth phase – addition of increasing concentrations of H_2O_2 moved the end of the exponential phase further in time (Fig. 2, Table 1), and higher fat content of the milk samples reduced the effect. It can be assumed that H_2O_2 had more sites to react with in UHT 3.5% samples, and thus less residual inhibitory H_2O_2 remained in milk prior to inoculation influencing the growth of starter bacteria.

The μ_{max} of the thermophilic starters decreased in both the first and the second exponential growth phases ($p < 0.05$), when H_2O_2 had been added compared to no addition (Table 1). Total heat (Q_{tot}) produced during the growth of bacteria in milk corresponds to overall biomass formed during fermentation. Although growth kinetics of thermophilic starter changed markedly in milk samples after H_2O_2 treatment, the total number of bacteria in yoghurt was not affected so notably in samples with H_2O_2 concentration up to 100 mg L^{-1} (see total heat production data in Table 1). Heat production associated with the growth of starter bacteria was initiated only on the second day of experiment in UHT 3.5% and UHT 0.05% milk samples if H_2O_2 was added at concentration of 150 mg L^{-1}. No heat production was recorded during 5 days of observation in case of H_2O_2 concentrations of 200 and 250 mg L^{-1}, indicating total inhibition of starter bacteria. These results are similar to those reported for different lactic acid bacteria strains using the similar H_2O_2 levels (see for example Subramanian & Olson, 1968).

Nevertheless, looking carefully at power-time curves of milk samples treated with higher amounts of H_2O_2 (over 50 mg L^{-1}), one can see some heat production in the beginning of the curves, before the first and second peaks associated with the growth of the bacteria (Fig. 2). This comparatively minor descending heat production also occurs on power-time curves recorded from the samples containing H_2O_2 but no starter added (data not shown). In the latter case it was also observed that higher initial H_2O_2 concentrations in milk corresponded to higher amounts of produced heat. Hence, abovementioned heat evolution should be assigned to H_2O_2 decomposition in milk.

Thus, in contrast to our initial assumption, incubation of milk for 1 h at 40 °C was not enough for added H_2O_2 to undergo complete decomposition. Moreover, our results showed that in UHT 3.5% milk samples, treated with catalase 1 h after H_2O_2 addition prior to inoculation, the starter had the same lag-phase (Fig. 3) and no change in μ_{max} (Table 1) in the first exponential growth phase ($p > 0.05$) compared with the control samples without H_2O_2 added in contrast to milk with added H_2O_2 but lacking catalase treatment. Hence, in samples with H_2O_2 added to milk and not subsequently treated with catalase, inhibition of growth of starter bacteria and prolongation of their lag-phase could be assigned mainly to the direct inhibitory effect of residual H_2O_2. Looking carefully at calorimetrical data, it becomes obvious that bacteria start to grow in milk samples not treated with catalase only when H_2O_2 has been practically fully decomposed (Fig. 2).

Still, power-time curves of inoculated milk samples treated with catalase 1 h after incubation with H_2O_2 showed rather modified patterns compared to control ones (Fig. 3). Increase in $t_{(dQ/dt)_{max}}$ as well as decrease of the value of μ_{max} in the second exponential growth phase were observed ($p < 0.05$) (Table 1). Together with modified growth characteristics of starter bacteria during the second exponential phase, Q_{tot} produced during the growth became markedly lower in milk samples that have previously undergone H_2O_2 treatment at concentrations over 50 mg L^{-1} (Table 1); in other words, less biomass was produced on substrate substantially oxidized by H_2O_2.

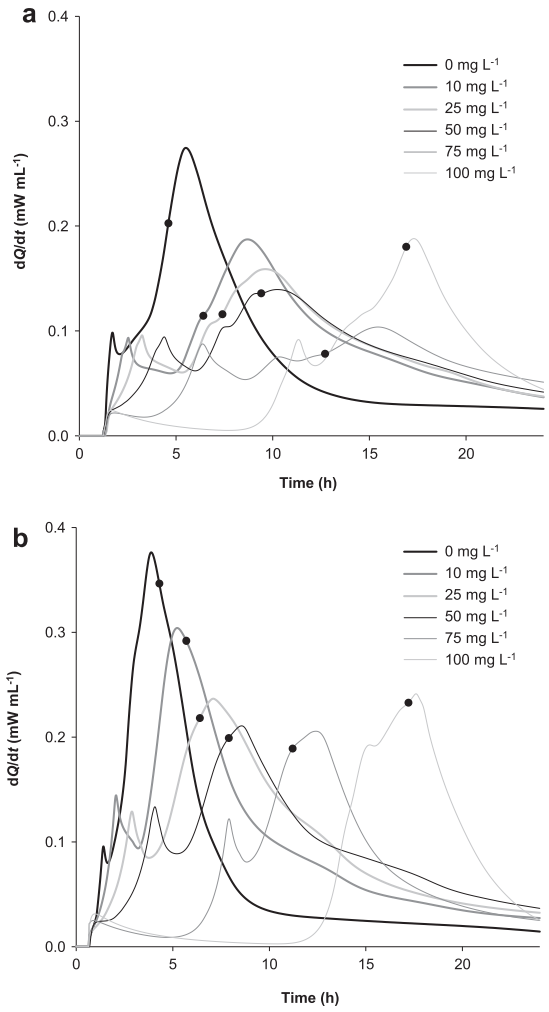

Fig. 2. Mean power-time curves of inoculated milk samples pre-treated with H_2O_2 (0–100 mg L^{-1}) 1 h before starter addition: a) UHT 3.5% ($n = 3$) and b) UHT 0.05% ($n = 2$) milk samples. Mean ($n = 3$) gelation times (●) of the samples are marked on the power-time curves.

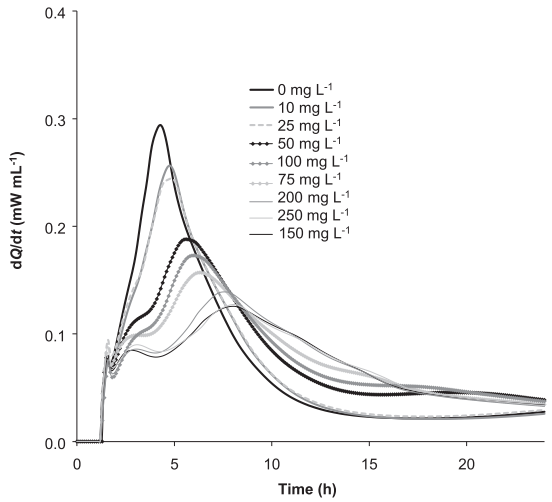

Fig. 3. Mean power-time curves ($n = 3$) of UHT 3.5% inoculated milk samples, incubated with H_2O_2 (0–250 mg L^{-1}) for 1 h and subsequently treated with catalase before starter addition.

Relying on calorimetrical data, it could be assumed that oxidative action of H_2O_2 in UHT milk was most noticeable on the components essential for microbial growth particularly during the second exponential growth phase. Milk contains a wide variety of components, including proteins that can act like antioxidants and can serve as the primary target for H_2O_2 action (Østdal et al., 2000). Protein oxidation may occur through the oxidation of amino acid side chains or protein backbone resulting in protein–protein cross-linkage (e.g., through the formation of dityrosine) or cleavage of the peptide bond (Berlett & Stadtman, 1997; Giulivi, Traaseth, & Davies, 2003; Takasaki, Kato, Murata, Homma, & Kawakishi, 2005). Recently, Özer, Kirmaci, Oztekin, Hayaloglu, and Atamer (2007) have demonstrated that the treatment of milk with microbial transglutaminase had a growth-slowing effect on yoghurt starter bacteria, and proposed that low molecular weight peptides and/or amino acids were cross-linked by the enzyme and became partially unavailable for bacteria. Similarly, we assume that H_2O_2 could cause oxidative changes in proteins, required as a source of nitrogen during the second exponential growth phase, diminishing their availability to starter bacteria. As a result, the retarded growth of the starter bacteria was observed in milk treated with H_2O_2 and subsequently with catalase, and the impaired rheological properties of gels formed could be expected as well.

3.3. Milk gelation and small deformation rheological properties of yoghurts

Gelation curves, which show the evolution of G' and G'' throughout the fermentation, were acquired using dynamic rheological measurements (Fig. 4). The determined t_{gel}, the calculated parameter that characterizes the kinetics of gelation ($(dG'/dt)_{max}$), and the values of the viscoelastic moduli (G' and G'') and tan δ measured 6 h after the start of gelation, describing the structure of mature gels formed, are presented in Table 2. No change in viscoelastic moduli (no gelation) was observed in milk samples with H_2O_2 added in concentrations over 150 mg L^{-1} during 24 h of fermentation, the data being consistent with microcalorimetrical measurements that did not show any growth of starter bacteria during the same period.

A significant prolongation in t_{gel} was caused by increasing H_2O_2 concentration in milk samples (Table 2): the lowest observed concentration (10 mg L^{-1}) already prolonged the lag-phase of yoghurt formation for at least 1 h. The t_{gel} of milk with 100 mg L^{-1} of H_2O_2 reached approximately 17 h, extending the onset of gel formation fourfold compared to the control samples. The t_{gel} as a function of H_2O_2 concentration for UHT 3.5% and UHT 0.05% milk samples is presented in Fig. 5; the data were fitted to linear regression model and a straight line was obtained from t_{gel} versus H_2O_2 concentration (x) plot ($t_{gel,\ UHT3.5\%} = 0.12\ x + 4.58$, $r = 0.99$ and $t_{gel\ UHT0.05\%} = 0.12\ x + 3.73$, $r = 0.96$). According to the calculations, the time of initiation of gel formation in milk with higher fat content was about 1 h longer (4.58 h versus 3.73 h respectively), but the influence of increasing concentrations of H_2O_2 on t_{gel} was characterized with the same slope.

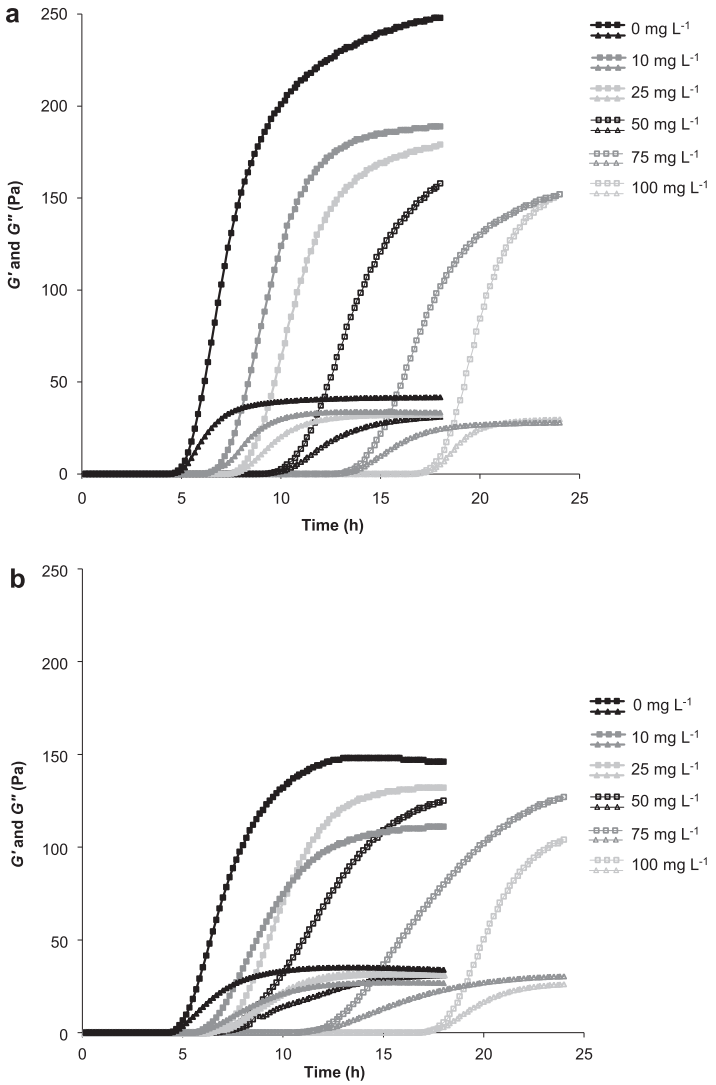

Fig. 4. Mean curves of storage (G') (■) and loss (G'') moduli (▲) as a function of time in inoculated milk samples pre-treated with H_2O_2 (0–100 mg L^{-1}) 1 h before starter addition: a) UHT 3.5% and b) UHT 0.05% milk samples. Curves are means of three replicates.

The t_{gel} values were marked on the growth curves of starter bacteria (see Fig. 2), and the heats produced by the bacteria up to the starting points of gelation ($Q_{t_{gel}}$) were calculated (Table 1). As seen from the data, full exponential growth of the bacteria was required to produce enough lactic acid to ensure the initiation of gelation in UHT 0.05% milk samples, while UHT 3.5% milk samples gelled generally before the transition from exponential to stationary phase. A significant increase of $Q_{t_{gel}}$ with the increase of H_2O_2 concentration was observed in the case of UHT 3.5% samples ($p < 0.001$), while in case of UHT 0.05% milk virtually no effect was seen (Table 1).

The $(dG'/dt)_{max}$ were higher, and the rheological properties of gels (G' and G'') were better in the case of milk samples containing higher amounts of fat ($p < 0.05$) (Fig. 4). Treatment with H_2O_2 led to the decrease of $(dG'/dt)_{max}$ and elastic nature of gels formed (value of G'), and increase of fragility (decrease of G'' value together with the increase of tan δ value) (Table 2). In UHT 3.5% samples, G' and G'' as well as $(dG'/dt)_{max}$ decreased to the minimum value on increasing the H_2O_2 concentration in milk up to 75 mg L^{-1} and resulted in almost twofold weaker gels ($G' = 115 \pm 37$ Pa, $G'' = 25.6 \pm 6.9$ Pa) compared to control sample ($G' = 209 \pm 8$ Pa, $G'' = 40.0 \pm 0.9$ Pa). Milk samples pre-treated with H_2O_2 at the level of 100 mg L^{-1} 1 h before starter addition exhibited slightly higher G' and G'' values again, but still did not reach the values of the control sample. Both high and low fat milk samples exhibited an increase in tan δ up to 75 mg L^{-1} of H_2O_2 added into milk 1 h before inoculation, and then diminished at concentration of 100 mg L^{-1} up to the level in control gels (Table 2). We assume that largely affected

Table 2
Means ± SD[a] of gelation time (t_{gel}), storage modulus[b] (G'), loss modulus[b] (G''), loss tangent[b] (tan δ) and gelation rate ($(dG'/dt)_{max}$) for control and H_2O_2 pre-treated UHT 3.5% or UHT 0.05% samples treated or not with catalase before starter addition.

Milk	H_2O_2 (mg L^{-1})	Catalase	t_{gel} (h)	G' (Pa)	G'' (Pa)	tan δ	$(dG'/dt)_{max}$ (Pa h^{-1})
UHT3.5%	0	–	4.6 ± 0.3^{a}	209 ± 8^{a}	40.0 ± 0.9^{a}	0.191 ± 0.004^{a}	64.9 ± 11.5^{a}
	10	–	6.4 ± 0.6^{b}	173 ± 13^{ab}	33.6 ± 1.7^{ab}	0.194 ± 0.005^{a}	51.2 ± 4.0^{b}
	25	–	7.4 ± 0.3^{c}	156 ± 24^{bc}	31.1 ± 3.8^{bc}	0.200 ± 0.007^{a}	43.7 ± 5.5^{bc}
	50	–	9.4 ± 0.7^{d}	128 ± 34^{c}	28.1 ± 6.5^{bc}	0.221 ± 0.013^{b}	33.3 ± 7.6^{cd}
	75	–	12.7 ± 0.3^{e}	115 ± 37^{c}	25.6 ± 6.9^{c}	0.224 ± 0.011^{b}	29.7 ± 8.1^{d}
	100	–	16.9 ± 0.4^{f}	145 ± 1^{bc}	29.4 ± 0.4^{bc}	0.203 ± 0.003^{a}	45.2 ± 3.1^{bc}
UHT0.05%	0	–	4.3 ± 0.0^{a}	135 ± 8^{a}	33.9 ± 1.8^{a}	0.250 ± 0.001^{a}	37.9 ± 2.2^{a}
	10	–	5.7 ± 0.0^{b}	95 ± 9^{a}	25.1 ± 2.1^{a}	0.265 ± 0.004^{b}	22.7 ± 4.1^{a}
	25	–	6.4 ± 0.5c	115 ± 32^{a}	30.0 ± 7.1^{a}	0.261 ± 0.011^{c}	29.3 ± 10.7^{a}
	50	–	7.9 ± 1.4^{d}	98 ± 41^{a}	26.5 ± 9.6^{a}	0.273 ± 0.019^{d}	23.7 ± 10.8^{a}
	75	–	11.2 ± 1.0^{e}	68 ± 24^{a}	20.0 ± 5.9^{a}	0.301 ± 0.023^{e}	17.4 ± 4.8^{a}
	100	–	17.2 ± 0.5^{f}	100 ± 12^{a}	25.5 ± 2.7^{a}	0.256 ± 0.005^{f}	28.0 ± 6.6^{a}
UHT3.5%	0	+	3.2 ± 0.0^{a}	237 ± 4^{a}	43.1 ± 0.7^{a}	0.182 ± 0.001^{a}	89.6 ± 4.9^{a}
	75	+	3.3 ± 0.0^{a}	239 ± 6^{a}	44.4 ± 1.5^{a}	0.186 ± 0.001^{a}	79.2 ± 0.5^{a}
	100	+	3.6 ± 0.4^{a}	226 ± 6^{a}	42.3 ± 0.4^{a}	0.187 ± 0.003^{a}	72.6 ± 7.6^{a}
	150	+	3.8 ± 0.2^{a}	242 ± 24^{a}	45.1 ± 3.9^{a}	0.187 ± 0.002^{a}	82.4 ± 17.2^{a}
	200	+	3.8 ± 0.1^{a}	233 ± 11^{a}	43.2 ± 1.7^{a}	0.186 ± 0.002^{a}	79.3 ± 7.4^{a}
	250	+	4.0 ± 0.7^{a}	232 ± 11^{a}	43.6 ± 1.3^{a}	0.188 ± 0.004^{a}	74.7 ± 9.8^{a}

[a] Means of the same milk samples treated with various concentrations of H_2O_2 followed by a different superscript letter are significantly different at $p < 0.05$ (ANOVA followed by Fischer's LSD test).
[b] Rheological parameters of the gels were measured 6 h after the onset of gelation.

growth of starter bacteria, due to residual H_2O_2 present in milk without further catalase treatment, was the primary reason for delayed and slowed down acid production, consequently, postponed gelation and influenced texture of yoghurts.

The dynamic rheological properties of gels produced from low fat samples were less affected by H_2O_2 addition compared to milk with higher fat content (Fig. 4, Table 2). In UHT 0.05% milk, gelation rate and viscoelastic moduli 6 h after onset of gelation showed similar dependence on H_2O_2 concentration like in 3.5% UHT samples, but the differences were not statistically significant according to ANOVA ($p = 0.068$, $p = 0.084$ and $p = 0.136$ for $(dG'/dt)_{max}$, G' and G'', respectively) (Table 2). It is known that the rate of decomposition of H_2O_2 is higher in whole milk than in skimmed milk (Amin & Olson, 1967). Significant increase in peroxide values in yoghurt produced from H_2O_2-treated raw milk (Özer & Atamer, 1999) and H_2O_2-treated raw cream (Özer, Kirim, & Atamer, 2000) have been reported referring to stimulation of lipid oxidation. Lipid hydroperoxides produced may be subsequently decomposed to yield the reactive aldehyde species that can amplify damage to proteins by alternation of amino acids side chains (Uchida, 2003). Therefore, in milk containing fat, enhanced oxidative influence of H_2O_2 on protein structure, affecting their further availability as a nutrient for bacteria, and thus, more pronounced effect of H_2O_2 on the rate of acid production by starter, gelation of milk and properties of yoghurt could be expected.

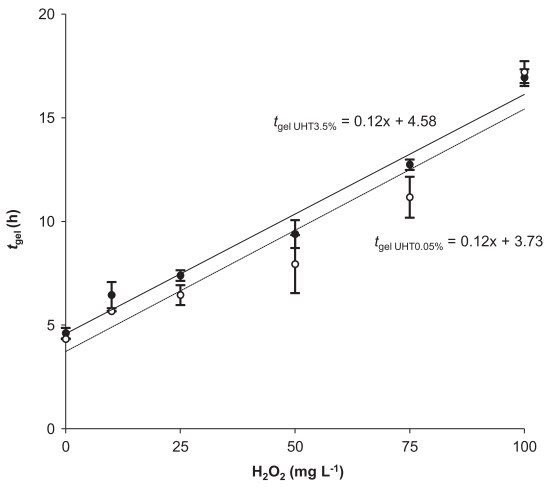

Fig. 5. Gelation initiation times of UHT 3.5% (●) and UHT 0.05% (○) milk samples as a function of H_2O_2 concentration. Values are means of three replicates with vertical bars for the standard deviations.

Treatment of milk with catalase before starter addition improved the gelation of UHT 3.5% milk without H_2O_2 added, resulting in slightly shorter t_{gel} ($p < 0.05$), higher $(dG'/dt)_{max}$ ($p < 0.01$) and higher G' and G'' ($p < 0.005$) (Table 2). Results fit with calorimetrical data that showed that catalase treatment favoured the growth of thermophilic starter, especially in the second exponential growth phase (Fig. 1); hence, enhanced acid production by bacteria contributed to improved gelation and resultant gel structure. On the contrary, samples incubated with H_2O_2 (10–250 mg L^{-1}) for 1 h and subsequently treated with catalase prior to inoculation, i.e. milk samples free of residual H_2O_2, showed a negligible increase in t_{gel} compared to control ones ($p = 0.146$) (Table 2). Heat produced by starter bacteria up to the gelation point (Table 1) and consequently the amount of lactic acid leading to the onset of milk gelation was virtually the same and independent of H_2O_2 concentration ($p > 0.05$). Our results are in agreement with other studies that report that no prolongation of incubation was needed to reach a certain pH value in acidified milk samples, previously incubated with H_2O_2 at 100 or 140 mg kg^{-1} for 6 h and then heated at 85 °C for 20 min and lacking residual H_2O_2 activity prior to inoculation (Özer & Atamer, 1999). However, if treated with H_2O_2 in the concentration range 100–400 mg kg^{-1} somewhat extended incubation periods were reported by Özer, Grandison, Robinson, and Atamer (2003). The altered kinetics of gelation were assumed to be caused by –SH group oxidation in milk proteins (Özer et al., 2003), and inhibition of bacterial growth was not taken into consideration in the latter study.

According to our data, incubation of milk with H_2O_2 for 1 h and further treatment with catalase, i.e., samples that lack residual H_2O_2 prior to inoculation, did not exhibit any visible effect on $(dG'/dt)_{max}$ or rheological characteristics of mature gels (G', G'',

tan δ) 6 h after the onset of gelation ($p > 0.05$). In contrast to our results, yoghurts produced from milk pre-treated with H_2O_2 for 6 h and then heated at 85 °C for 20 min were reported previously to show a decrease in firmness, increased whey separation, lower viscosity (Özer & Atamer, 1999) and impaired dynamic rheological properties (Özer et al., 2003). It was concluded that altered properties of resultant yoghurt in addition to changed kinetics of gelation were also caused by oxidation of −SH groups by H_2O_2 (Özer & Atamer, 1999; Özer et al., 2003). To the best of our knowledge, available −SH groups of milk proteins are likely to be previously oxidized as a result of high temperature applied during the production of UHT milk (Cosio, Mannino, & Buratti, 2000). Thus, we propose that oxidation of −SH groups by H_2O_2 was not taking place, but other functional groups of milk proteins were affected in UHT milk.

4. Conclusions

The present study has shown that even low concentrations of H_2O_2 led to the prolongation of initiation time of gelation and deterioration of gel quality, caused mainly by the direct inhibition of starter bacteria by residual H_2O_2. Milk samples pre-treated with H_2O_2 and then with catalase, ensuring complete removal of the oxidant, showed nearly the same gelation time and overall rheological characteristics indicating lack of influence of pre-treatment on the formation of protein gel matrix. Nevertheless, in milk treated with H_2O_2 followed by catalase, modified growth profile of yoghurt starter bacteria recorded during second exponential growth phase may likely result in yoghurt with altered flavour, even though gel properties would not be affected. The data obtained indicated that H_2O_2 did not alter the structure of milk proteins determining coagulation processes but changed bioavailability of amino acids included in the peptides and proteins.

It has been shown in the present study that microcalorimetry is an excellent method for monitoring the details of growth of lactic acid bacteria in food matrices, but also chemical and physical changes of solid state matrices. Detection of heat production associated with H_2O_2 degradation in milk should be mentioned as a good example of the latter.

Acknowledgements

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PUBLICATION III

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Front-face fluorescence spectroscopy studying rennet coagulation of milk

Manuscript

Front-face fluorescence spectroscopy studying rennet coagulation of milk

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Abstract

A novel front-face fluorescence spectroscopy method was applied for detailed investigation of rennet coagulation of reconstituted skimmed milk (RSM) and raw bulk milk. The changes in fluorescence response at tryptophan maximum emission recorded during renneting were dependent on the nature of milk sample, presence of milk fat and enrichment with $CaCl_2$. Tryptophan fluorescence intensity patterns allowed distinguishing different phases of enzymatic coagulation, as well as determination of specific point (t_{2min}), which corresponded to the moment when aggregation of casein micelles proceeded. Fluorimetric determination of coagulation time parameter was validated with two reference methods: visual observation of flocculation time and dynamic rheological measurements. In RSM and skimmed bulk milk, t_{2min} was found to correlate with coagulation parameters obtained by reference methods. In whole bulk milk, the characteristic shape of fluorescence pattern also allowed determination of t_{2min}, but its applicability to determine coagulation characteristics in whole milk is still to be verified.

1. Introduction

Milk coagulation is an essential process in cheese making which defines in large extent the quality and yield of the final product (Fagan, Castillo, Payne, O'Donnell, & O'Callaghan, 2007a; Johnson, Chen, & Jaeggi, 2001; Riddell-Lawrence & Hicks, 1989). Assessment of milk coagulation characteristics should be of high interest to the dairy industry due to straightforward economic reasons.

Different instrumental methods to monitor milk coagulation have been used in laboratory scale research for better understanding of this process, and many attempts have been made during the last decades to aid the automation of the cheese production by developing a variety of different techniques for monitoring milk coagulation and prediction of cutting time. The last reviews of the off-line and on-line methods for assessment of milk coagulation – their operating principles, advantages and drawbacks were published a decade ago (Lucey, 2002; O'Callaghan, O'Donnell, & Payne, 2002). The performance of several on-line techniques simultaneously or in comparison with reference methods was evaluated by O'Callaghan, O'Donnell, and Payne (1999), O'Callaghan, O'Donnell, and Payne (2000) and Klandar, Lagaude, and Chevalier-Luci (2007). The techniques for monitoring of milk coagulation have been based mainly on a) measuring physical parameters related to the changes of rheological properties of milk using mechanical, vibrational, ultrasonic or thermal conductivity probes or b) detecting changes in the sizes of the micelles using near infrared (NIR) transmission or reflectance, or dynamic light scattering (O'Callaghan et al., 2002). Among the promising techniques for the study of milk coagulation a diffusing wave spectroscopy method was highlighted, which provides additional information on particle interactions and their dynamics during flocculation of casein micelles (Alexander & Corredig, 2007; Hemar, Singh, & Horne, 2004; Huppertz & de Kruif, 2007). The optical methods for monitoring milk coagulation have

66 certain advantages as they are non-destructive, do not perturb coagulation, are integratable in-
67 line and easily applicable on plant scale from the hygienic viewpoint. Recent research has
68 been focussed specifically on the development of combined sensors for continuous non-
69 invasive monitoring of milk coagulation and syneresis, based on ultrasonic or NIR light
70 backscatter probe (Taifi at al., 2006; Fagan et al., 2007b, 2007c, 2008).
71 At present there are only a few publications concerning the application of fluorescence
72 spectroscopy for monitoring milk enzymatic coagulation. Herbert, Riaublanc, Bouchet,
73 Gallant, and Dufour (1999) have shown that front-face fluorescence was able to distinguish
74 acid-induced coagulation of milk from rennet-induced process and allowed the detection of
75 structural changes in casein micelles during coagulation. Lopez and Dufour (2001)
76 successfully applied front-face fluorescence for investigation of the influence of the
77 composition of fat globule surface on the rennet-induced coagulation of reconstituted milk,
78 however, interpretation of the changes of tryptophan fluorescence intensity profiles recorded
79 during the renneting was not provided by the authors. It is known that in the course of the
80 manufacture of skimmed milk powder, the equilibrium of calcium between serum and
81 colloidal phases as well as the size of casein micelles could be irreversibly altered (Martin,
82 Williams, & Dunstan, 2007), which was suggested to explain differences in rennet-induced
83 coagulation properties of raw and reconstituted milk samples (Martin, Williams, Choong, Lee,
84 & Dunstan, 2008). Hence, analytical techniques applied for monitoring of reconstituted milk
85 should be separately validated also in the case of natural bulk milk. Recently, in bulk milk,
86 Fagan et al. (2011) have shown that spectral changes of naturally occurring milk fluorophores
87 allowed monitoring both coagulation and curd syneresis phases, mounting a fluorescence
88 probe into a laboratory cheese vat. The evolution of tryptophan fluorescence has been shown
89 to have the largest response during coagulation compared with other intrinsic fluorophores of

milk – riboflavin and vitamin A. At the same time, a need for further study under a wider range of processing conditions was indicated by the authors (Fagan et al, 2011).

In the present study front-face fluorescence was used to record the evolution of tryptophan fluorescence during renneting of a) reconstituted skimmed milk samples and of b) raw bulk milk (whole and skimmed) with differing coagulation properties. Profiles of tryptophan fluorescence obtained during coagulation were related to different phases of renneting employing reference methods.

2. Materials and methods

2.1. Milk samples

Reconstituted skimmed milk (RSM) was prepared from low-heat skimmed milk powder (34.5% protein, 53% lactose, 8% ash, 3.8% moisture, 0.5% fat; Valio Ltd., Helsinki, Finland) by dissolution in distilled water at 10% (w/v) milk solids, with addition of $CaCl_2$ to yield 5 or 10 mM final concentration or without $CaCl_2$ addition. RSM samples were mixed by magnetic stirrer bar for 1 h at room temperature to assure full reconstitution, left overnight at 4°C and used for coagulation experiments on the next day.

Raw whole bulk milk was collected from four dairy farms supplying milk for cheese production at Saaremaa Dairy (Kuressaare, Estonia) on different sampling days, resulting in nine milk samples with varying coagulation properties. Skimmed milk was prepared by centrifuging aliquots of whole milk at 3000 g for 10 min at 4°C. Coagulation experiments were performed with both whole and skimmed milk samples.

2.2. Rennet coagulation

Coagulation of RSM samples enriched with $CaCl_2$ at the level of 0.01 M was induced by the addition of 10, 5 or 2.5 g L^{-1} dilutions of rennet from calf stomach (type I, contains approximately 80% NaCl; Sigma, St. Louis, MO, USA). The RSM samples enriched with $CaCl_2$ at 0.005 M were coagulated by the addition of 10 and 5 g L^{-1} dilutions of rennet. To RSM without calcium enrichment 10 g L^{-1} dilution of rennet was added, which did not result in coagulation within one hour of observation. The coagulation trials of RSM samples enriched with $CaCl_2$ at 0.01 M induced by 10 and 5 g L^{-1} dilutions of rennet were also repeated with different batch of low-heat skimmed milk powder on another day resulting in altered coagulation characteristics, and these coagulations were considered as separate coagulation trials. Coagulation of raw bulk milk samples was induced by the addition of 10 g L^{-1} dilution of rennet. Hence, a total number of eight different coagulation trials with RSM, nine coagulation trials with skimmed raw bulk milk and nine coagulation trials with whole bulk milk were performed. For determination of coagulation time parameters by three different methods (visual determination of rennet flocculation time, dynamic rheological and fluorescence measurements), each of these trials was coagulated in triplicate separately for each method used. The volumes of aliquots used for the determination of flocculation time and fluorescence measurements were 7.5 mL and for rheological measurements 19 mL.

Coagulation of milk samples was induced by the addition of 1% (v/v) of appropriate rennet dilution to the milk pre-equilibrated for one hour at 30°C. After the addition of rennet, samples were mixed by rapid inversion of test tubes three-five times. Then, rennet flocculation time was determined by visual observation, or samples were quickly transferred to the rheometer's cup or fluorimetric optical cell for immediate rheological or fluorescence measurements.

2.3. Rennet flocculation time

The rennet flocculation time (RFT) was measured visually in milk samples using a modified Berridge method (Berridge, 1952). After the addition of rennet as described in Section 2.2, the samples were gently stirred immersed in a water bath at 30°C until RFT was determined as the time from rennet addition until the formation of the first visible flocks of coagulated protein on the walls of the glass tube.

2.4. Dynamic rheological measurements during renneting

Low amplitude oscillatory measurements were conducted using a Physica MCR301 rheometer (Anton Paar GmbH, Germany) with a direct strain oscillation option, a Peltier temperature control unit C-PTD200 and a coaxial cylinder measuring system CC27 (outer and inner diameters 28.92 and 26.66 mm respectively). After the addition of rennet, milk samples were stirred and an appropriate volume was transferred into the measuring system. While milk was coagulated at 30°C, rheological parameters were determined every 10 s in an oscillation mode at a frequency of 1 Hz and a strain of 0.01, which did not perturb the coagulation process. The gelation time (t_g) was defined as a time to a crossover point of the elastic (G') and viscous (G'') moduli. In addition, the time point corresponding to the initial increase of G'' ($t_{G''}$) in coagulating milk, preceding the gelation point, was determined.

2.4. Front-face fluorescence spectroscopy

During milk coagulation, fluorescence spectra were recorded at 30°C using Instant Screener® Compact spectrofluorimeter (LDI Ltd., Tallinn, Estonia). After rennet addition, milk samples

were placed in a 10-mL optical cell with quartz bottom window and a fluorescence signal was recorded every 10-11 s throughout coagulation. The fluorescence signal was measured within a small window near maximum emission of tryptophan at excitation wavelengths ranging from 280 to 283 nm and emission wavelengths ranging from 325 to 328 nm with 1 nm slits. The small measuring window used allowed shortening of the time needed for an acquisition of every spectrum, retaining the opportunity to detect possible shifts in the fluorescence maximum while renneting proceeded.

Raw tryptophan fluorescence intensity data showed relatively high degree of scatter. For smoothing the data and estimation of possible coagulation time parameters from the fluorescence patterns an algorithm was developed in MatLab version R2009a (The MathWorks, Inc., Natick, MA, USA). The developed algorithm applied a Savitzky-Golay smoothing filter to reduce the amount of high frequency noise component in the signal (Savitzky & Golay, 1964). The Savitzky-Golay filter was chosen, as it tends to preserve features of the original distribution such as relative maxima, minima and width that are required for precise estimation of coagulation times. Parameters for filtering were selected empirically after running several tests. The filter applied first degree least squares polynomial and frame of seven datapoints, which roughly corresponded to 1-1.5 min window. Filtering was repeated three times. The filtering was followed by calculating the derivatives of the filtered signals and finding locations of local extremes. Exact point corresponding to coagulation time was chosen by comparison tryptophan fluorescence intensity data to coagulation time parameters determined by reference methods as described above.

2.6. Statistical analyses

Pearson correlation was used to determine correlations between parameters estimating milk coagulation ability obtained by visual, rheological and fluorescence methods. Significance of correlations was determined, taking into account the number of samples and the strength of relationships.

3. Results

Typical tryptophan fluorescence intensity profiles recorded during rennet coagulation of different types of milk (RSM, skimmed and whole raw bulk milk) are presented in Fig. 1. Data obtained showed that the changes in tryptophan fluorescence intensity during coagulation were different for different milk samples. Nevertheless, the universal approach for determination of coagulation time parameters utilising the minimum of the second order derivative ($t_{2\min}$) of fluorescence signal was introduced, as also illustrated in Fig. 1. The relationships between coagulation time parameters obtained by fluorescence, visual and rheological methods were evaluated using Pearson's correlation coefficients (r) separately for each type of milk, and are summarized in Table 1. More detailed data is presented below.

3.1. Rennet coagulation of RSM

The general pattern of the evolution of tryptophan fluorescence during rennet coagulation of RSM is presented in Fig. 1a and is rather similar to that reported by Lopez and Dufour (2001) for reconstituted milk containing coated fat globules. For better understanding of the changes in tryptophan fluorescence signal taking place during renneting, dynamic rheological measurements were conducted in parallel with recordings of fluorescence profiles and are superimposed on the fluorescence data in Fig. 1. Comparing the character of the changes of

tryptophan fluorescence curves with rheological profiles, we found that the time when the fluorescence clearly started to descend (Fig. 1a) overlapped approximately with the moment when G'' started to increase ($t_{G''}$). This moment was considered as an indication of the onset of the aggregation of casein micelles. Correspondingly, the time when the change in the slope of the descending fluorescence signal occurred coincided with the time of the crossover of G' and G'' curves (t_g), meaning that the three-dimensional gel was established at this point.

Nevertheless, the experiments performed with RSM at different levels of $CaCl_2$ enrichment and with different concentrations of enzyme solutions used to induce coagulation indicated that the fluorescence profile can look quite different from those presented in Fig 1a and reported previously by Lopez and Dufour (2001). For this reason, the change in the slope of descending fluorescence signal, referred as gelation point, was not so easily distinguishable during renneting in many cases. Hence, the former point on tryptophan fluorescence intensity curve corresponding to the start of aggregation was suggested to be used as the milk coagulation time parameter.

The time to the minimum of the second order derivative of tryptophan fluorescence profile (t_{2min}) was chosen to designate the time point when fluorescence curve started to decrease (Fig. 1a) and was calculated for all RSM coagulations following the smoothing procedure. The application of different levels of RSM enrichment with $CaCl_2$ and the addition of rennet at different concentrations resulted in the wide range of coagulation kinetics – estimated t_{2min} displayed nearly a tenfold difference between fast and slowly coagulating samples. The coagulation time parameters obtained by fluorescence, visual and rheological measurements showed good correlations with each other (Table 1). Fluorimetrically derived parameter t_{2min} had the strongest relationship with RFT and $t_{G''}$ (r = 0.98). The parameters determined by reference methods (visual and rheological) appeared to correlate with each other less well than with t_{2min} determined by front-face fluorescence spectroscopy.

3.2. Rennet coagulation of raw bulk milk

The behaviour of tryptophan fluorescence observed in bulk milk during rennet coagulation (Fig. 1b and c) was clearly different from that measured in RSM enriched with $CaCl_2$ (Fig. 1a). Instead of an overall descending character of the signal in RSM, bulk milk displayed generally ascending profile during coagulation within the time span the measurements were performed. However, measuring the fluorescence response during renneting of bulk milk samples for longer period resulted finally in a decline in tryptophan emission, similarly to that obtained in RSM.

In general, the evolution of tryptophan fluorescence during rennet coagulation of skimmed and whole bulk milk was very similar, except during the first minutes of measurements. Instead of initially descending signal observed in skimmed bulk milk (as well as in RSM), tryptophan fluorescence rose quickly in whole milk during the first minutes of renneting, but further profile was very similar to that of skimmed bulk milk.

The comparison of rheological and fluorescence profiles recorded during coagulation of skimmed bulk milk samples (Fig. 1b) has shown that the $t_{G''}$, corresponding to the moment when aggregation of micelles had started, roughly coincided with a turning point on the fluorescence curve after which an ascending signal continued to increase with a slower rate. As for the whole bulk milk, dynamic rheological measurements failed to show a standard profile typical to skimmed milk (data not shown in Fig. 1c) – G' was initially higher than G'' and their respective curves did not have a crossover, as well as viscoelastic moduli increased from the onset of the measurements and the point at which the aggregation of the micelles might proceed was not detectible on rheological profiles. Nevertheless, taking into account the evident similarity of the overall shapes of the fluorescence profile of skimmed and whole

bulk milk (except the very beginning of the curves), the change in the slope of ascending fluorescence signal was suggested to correspond to the onset of aggregation likewise in whole milk (Fig. 1c). A specific point analogous to rheological gel point was not discernible on fluorescence profiles, and the curves did not have well-marked transition from ascending to descending behaviour.

Raw tryptophan fluorescence intensity patterns recorded during renneting of bulk milk samples were treated mathematically in the same way as described for RSM. The t_{2min} parameter perfectly fitted the time point when tryptophan fluorescence intensity curve changed the slope. Recently, Fagan et al. (2011) proposed an approach utilizing the time to the maximum of the first derivative of tryptophan fluorescence profile (t_{1max}) for monitoring milk coagulation. The comparison of the fluorescence and rheological patterns recorded in the present study indicated that a measurable aggregation of casein micelles proceeded later than t_{1max} typically occurred. On the contrary, the t_{2min} parameter may potentially refer to the start of aggregation of casein micelles, irrespectively of the nature of the milk sample.

The t_{2min} parameter ranged in bulk milk samples from 8.7 ± 0.5 to 12.8 ± 1.0 min and from 7.4 ± 0.8 to 10.6 ± 0.6 min in case of skimmed and whole milk, respectively. As the fluorescence profiles obtained during coagulation of RSM and bulk milk samples were substantially different, the Pearson's correlation coefficients between coagulation time parameters derived from fluorescence or reference measurements were calculated separately for different types of milk (Table 1). With regard to skimmed bulk milk, the strongest relationship was observed between t_{2min} and rheologically determined parameters, particularly $t_{G''}$ (r = 0.98). Noteworthy, the correlations between t_{2min} and RFT and between RFT and rheological coagulation parameters were notably poorer, but still significant. Unfortunately, in whole bulk milk, no significant correlation between t_{2min} and RFT was obtained, and rheological parameters could not be derived due to the altered patterns of rheological profiles. Possible

289 explanation of the different relationships obtained between t_{2min} and RFT when measured in
290 different types of milk is given below.
291
292 **4. Discussion**
293
294 *4.1. Coagulation time parameters determined by different methods*
295
296 One of the earliest and simplest methods for the estimation of milk coagulation properties is
297 the visual determination of the onset of flocculation in a glass tube (Berridge, 1952). Yet, the
298 effect of constant stirring of the sample on the enzyme action and aggregation kinetics is not
299 clear, as well as this method is destructive towards the formation of milk coagulum. The time
300 registered as the onset of flocculation by human eye occurs later than micelles aggregation
301 actually commences. In the present study, the time indicating the start of aggregation of
302 casein micelles was alternatively derived from the time when G'' started to deviate upwards
303 from the baseline ($t_{G''}$). The $t_{G''}$ parameter allowed the indication of aggregation much earlier
304 than the drop of loss tangent (tan δ), which had been used elsewhere as an early indicator of
305 milk coagulation (Klandar et al., 2007), and occurred earlier than RFT. Whereas, the
306 fluorimetrically determined parameter (t_{2min}) indicated milk coagulation also earlier than RFT,
307 and was more or less equal to $t_{G''}$. Hence, the time associated with t_{2min} was suggested to
308 coincide with the start of aggregation of casein micelles – the second phase of renneting.
309 Consequently, the rheologically determined t_g, corresponding to the establishment of three-
310 dimensional gel, was considerably longer than t_{2min} and visual RFT measurements. Inevitably,
311 the following coagulation parameters: t_{2min}, RFT and t_g, corresponded to different moments of
312 multistage process of milk coagulation.

The visual determination of RFT is very subjective, and the specific moment of coagulation process, which estimated RFT corresponds to, is not well defined. The estimation of RFT in different types of milk may assign a different degree of flocculation to RFT, due to differences in viscosity and turbidity of the samples. Our results indicated that the strongest correlations between RFT and coagulation parameters determined by other methods were obtained when measured in RSM. Considerably poorer correlation of RFT with $t_{2\text{min}}$, $t_{G''}$ and t_{g} in skimmed bulk milk may be associated with its thicker nature compared with RSM which might influence the reliability of RFT determination. At the same time, coagulation parameters other than RFT still demonstrated strong relationships with each other in skimmed bulk milk. In whole bulk milk, the determination of RFT was quite difficult due to adherence of flakes of milk fat to the glass walls during stirring of the milk samples in thin test tubes, which could be confused with the first signs of protein flocculation. Nevertheless, the shape of tryptophan fluorescence pattern was very characteristic and determination of $t_{2\text{min}}$ in whole milk was as simple as in skimmed bulk milk samples. Hence, only the determination of RFT, rather than fluorescence measurements in whole milk should be considered unsuccessful in this case. Further research is still needed for proving the capability of the fluorescence method to assess coagulation time characteristics in whole bulk milk.

4.2. Stages of renneting monitored by changes in tryptophan fluorescence

Intrinsic tryptophan residues in proteins are sensitive fluorophores, whose fluorescence depends on the polarity of the environment and existence of quenching molecules in the vicinity of tryptophan. Changes in microenvironment, induced for example by ligand binding or conformational changes in proteins may result either in enhancement or quenching of fluorescence and in shifts in the spectrum to shorter or longer wavelengths (Ladokhin, 2000).

338 During rennet coagulation and subsequent formation and ageing of gel network, the
339 tryptophan fluorescence intensity profile changes continuously (Fig. 1) and can be tentatively
340 divided into four stages, as described below.

341 1) After the addition of rennet, enzyme binds to casein micelles and a decrease of
342 tryptophan fluorescence could be seen in case of skimmed milk (Fig. 1a, b).

343 Polar amino acid side chains (Asp, Glu, Lys, Arg and protonated His), amide groups of
344 Gln and Asn, phenol group of Tyr, as well as disulfide (Cys-Cys) bridges or single Cys
345 can effectively quench tryptophan fluorescence (Chen & Barkley, 1998; Ladokhin, 2000).
346 The primary structure of chymosin consists of 323 amino acid residues, its polypeptide
347 chain is cross-linked by three Cys-Cys bridges, and two Asp are incorporated into the
348 active site of the enzyme (Gilliland, Winborne, Nachman, & Wlodawer 1990). During
349 renneting, there is about one enzyme molecule per 30 casein micelles (Walstra, Wouters,
350 & Geurts, 2006a). After the addition to milk, the enzyme approaches the micelles
351 sequentially by means of diffusion, part of the diffusion path being in the hairy layer of
352 the micelle, which increases the diffusion time (Walstra at al., 2006a). Hence, the enzyme
353 molecules bind gradually onto the surface of casein micelles – Cys-Cys bridges and Asp
354 residues from the active site get closer to the tryptophans of κ-caseins (κ-CN) located on
355 the surface and can reduce their fluorescence. Similarly to our results, a decrease in
356 tryptophan fluorescence was reported previously in reconstituted skimmed milk during the
357 first minutes of renneting (Lopez & Dufour, 2001).

358 In whole milk, the tryptophan fluorescence signal seems to increase from the very onset of
359 the measurements (Fig. 1c). The creaming effect in non-homogenized whole milk,
360 originating from differences in density between plasma and fat globules (Walstra,
361 Wouters, & Geurts, 2006b), causes a decrease in the concentration of fat globules at the
362 bottom of the optical cell, i.e. within the depth of the sample from there fluorescence

363 emission is measured in the front-face mode. Photons emitted by tryptophan can be
364 absorbed by fat-globule vitamin A in the course of resonance energy transfer (Lopez &
365 Dufour, 2001; Peterson & Rask, 1971) explaining the depression of tryptophan
366 fluorescence in milk containing fat. In whole milk during the first 2-3 minutes of
367 measurement, the increase in tryptophan fluorescence was observed due to the gradual
368 depletion of vitamin A content at the bottom of optical cell with time, either milk was
369 being renneted or not (data not shown); hence, a decrease in tryptophan fluorescence
370 caused by successive binding of coagulating enzyme could not be seen in whole milk
371 because of counteracting and overbalancing effect of creaming.
372 2) Some minutes after rennet addition, initial decrease in tryptophan fluorescence stops
373 and signal starts to increase (Fig. 1).
374 As a result of the hydrolysis of Phe105-Met106 bond in κ-CN by rennet action, the 64
375 amino acid long C-terminal part of κ-CN or glucomacropeptide (GMP) is removed from
376 the surface of the micelles (Fox, Guinee, Cogan, & McSweeny, 2000). GMP does not
377 contain any aromatic amino acids but includes twelve polar (Asp, Glu and Lys) and five
378 amide side chain groups (Asn and Gln) – potential quenchers of tryptophan fluorescence.
379 In contrast, the N-terminal part of κ-CN (para-κ-CN), containing one Trp, nine Tyr and
380 four Phe residues, remains attached with the micelle. Thus, we expect that the release of
381 GMP results in an increase of fluorescence of aromatic amino acid residues retained in the
382 structure of para-κ-CN. In addition, the dissociation of the enzyme – substrate complex
383 after the reaction should also compensate an initial decrease in tryptophan fluorescence.
384 Eventually, during enzymatic phase of renneting, there are two counteracting effects
385 influencing fluorescent signal that occur simultaneously: absorption of chymosin onto the
386 surface of the micelles causing a decrease, and removal of GMP and release of enzyme
387 resulting in an increase of tryptophan emission. In skimmed milk, the increase in

388 tryptophan fluorescence becomes noticeable when the enhancing effect caused by
389 cleavage overcomes the depression induced by enzyme binding.
390 3) At some specific point of time, the fluorescence signal starts to decline with higher or
391 slower rate in RSM (Fig. 1a), or the slope of the ascending fluorescence curve
392 decreases pronouncedly as in bulk milk (Fig. 1b, c).
393 When the majority of κ-CN is cleaved, micelles lose their stability and the second phase
394 of renneting can proceed: para-κ-CN micelles approach to each other and start to form
395 small chain-like aggregates (Fox et al., 2000). The fluorescence of tryptophan residues
396 located near the surface of micelles can be suppressed by the appearance of additional
397 protein-protein interactions between aggregating particles. Enrichment with $CaCl_2$ causes
398 the reduction of the negative charge of micellar caseins, what allows faster aggregation of
399 the micelles (Famelart et al., 1999; Udabage, McKinnon, & Augustin, 2001). Steeper
400 decrease of fluorescence and shorter duration of this stage in RSM with higher level of
401 added $CaCl_2$ were observed in the present study, which confirms increased rate of
402 aggregation and shorter gelation time reported before (Sandra et al., 2012).
403 During aggregation phase, the fluorescence signal showed clear decrease in case of RSM
404 enriched with $CaCl_2$ and only a reduction of the slope of the ascending signal in case of
405 bulk milk, which suggests more intensive aggregation of micelles in RSM. The RSM
406 samples with 0.01 or 0.005 M $CaCl_2$ contained more calcium than raw bulk milk normally
407 has, which explains differences in the behaviour of tryptophan fluorescence patterns in
408 coagulating milk during this phase.
409 4) After reaching the maximum fluorescence signal starts to decrease permanently (in
410 case of bulk milk), or the decline of fluorescence initiated at the previous stage
411 continues with a different slope (in RSM).

As a result of aggregation, a three-dimensional gel is formed, which is dynamic in its nature. After the establishment of gel at t_g, further changes in protein matrix – incorporation of additional caseins, fusion and rearrangements of particles, result in the thickening and hardening of the gel (Mellema, Walstra, van Opheusden, & van Vliet, 2002) and are accompanied with lasting decrease in fluorescence, which supports a dynamic character of the gel.

After gel formation and throughout aging, again the descending fluorescence signal displays more prominent slope in RSM containing $CaCl_2$ compared with bulk milk samples. Addition of $CaCl_2$ allows the formation of supplementary bonds between the micelles during the development of gel structure (Sandra et al., 2012); hence, this can be accompanied with larger changes in protein matrix. We suggest that measurement of tryptophan fluorescence during gel aging may provide the measure of microsyneresis processes occurring in the gel.

The considerations presented are hypothetical based on many cases on the circumstantial evidences. However, the elucidation of the mechanisms of milk coagulation and accompanying structural changes reflected in fluorescence spectra is a very serious challenge.

5. Conclusions

The specific feature of front-face fluorescence spectroscopy is that the technique reflects changes taking place with proteins at the molecular level during renneting, apart from the other optical methods that rely on the changes in particle size. Hence, we suggest that the response of measured tryptophan fluorescence during milk coagulation should be interpreted completely differently from e.g. the response of near-infrared absorbance (Klandar et al., 2007) or light backscatter profile (Fagan et al., 2007b).

An advantage of fluorescence method is its ability to trace all stages of milk coagulation starting with first interactions of enzyme with casein micelles and ending with gel aging, which could find potential implementation first of all by investigators at the laboratory scale research. On the other hand, the present study clearly showed the ability of fluorescence method to estimate coagulation properties of different milk substrates: RSM with wide range of coagulation kinetics as well as skimmed bulk milk samples. During coagulation of whole bulk milk, a characteristic shape of fluorescence pattern was also obtained. The time to the minimum of the second derivative of tryptophan fluorescence profile (t_{2min}) was proposed to be used as a coagulation parameter. We assume that our findings can be a basis for implementation of front-face fluorescence spectroscopy as a non-destructive method in cheese making for monitoring milk coagulation in-line; otherwise, the method could be applied off-line for evaluation on milk suitability for cheese production at everyday milk delivery.

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Table 1

Pearson's correlation matrix of milk coagulability estimating parameters, obtained by different methods, measured in reconstituted skimmed milk (RSM), skimmed or whole raw bulk milk samples.[a]

Milk	Variable	t_{2min}	RFT	$t_{G''}$	t_g
RSM					
(n = 7)	t_{2min}	1			
	RFT	0.98***	1		
	$t_{G''}$	0.98***	0.95**	1	
	t_g	0.94**	0.92**	0.99***	1
Skimmed raw bulk milk					
(n = 9)	t_{2min}	1			
	RFT	0.77*	1		
	$t_{G''}$	0.98***	0.78*	1	
	t_g	0.96***	0.73*	0.94***	1
Whole raw bulk milk					
(n = 9)	t_{2min}	1			
	RFT	0.12	1		
	$t_{G''}$	-	-	-	
	t_g	-	-	-	-

[a] Parameters in the table are defined as follows:

t_{2min} – time to the minimum of the second derivative of tryptophan fluorescence intensity signal,

RFT – visually determined rennet flocculation time,

$t_{G''}$ – time to the initial increase of G'' derived from dynamic rheological measurements,

t_g – time to the crossover of G' and G'' derived from dynamic rheological measurements.

Asterisks indicate the level of correlation: * significant at the 0.05 level; ** significant at 0.01 level; *** significant at 0.001 level.

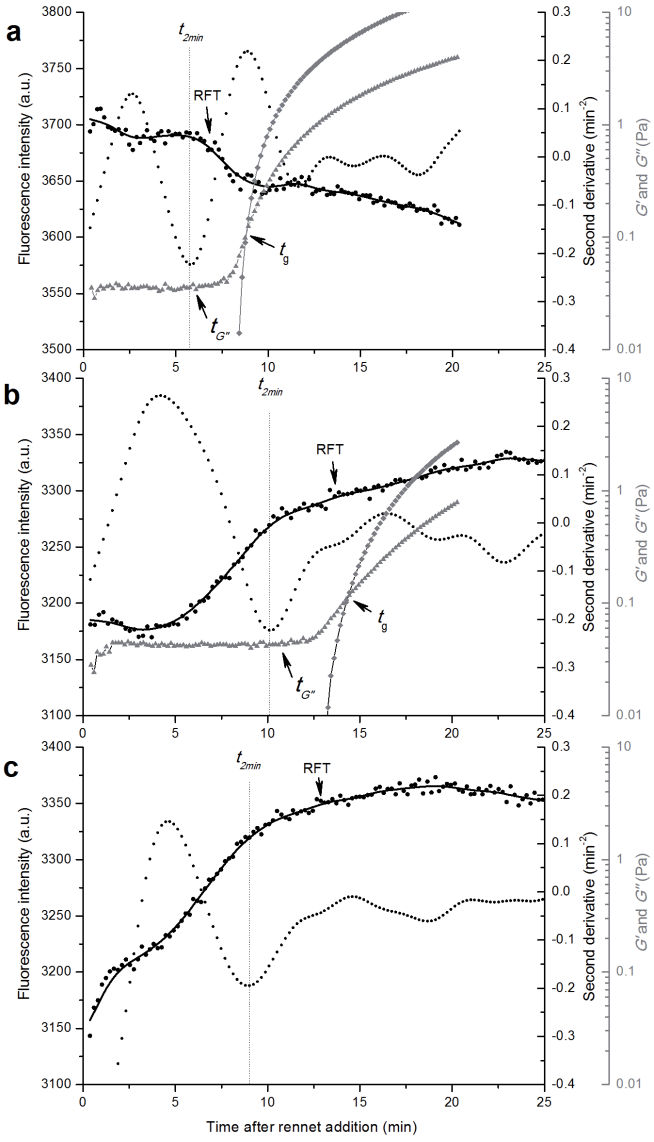

Fig. 1. Examples of tryptophan fluorescence data (black dots) obtained during rennet coagulation of milk and their corresponding second order derivatives: **a)** reconstituted skimmed milk (RSM) enriched with 0.01 M $CaCl_2$, **b)** skimmed or **c)** whole raw bulk milk. Renneting was induced by addition of 5 **(a)** or 10 **(b, c)** mg mL^{-1} solution of chymosin at 1% (v/v). Representative patterns are presented. Smoothed curves and their second derivatives are indicated with black solid or dotted lines, respectively. The times to the minimum of the second order derivative (t_{2min}) are indicated with vertical dashed lines. Evolution of elastic (G') (grey diamonds) and viscous (G'') (grey triangles) moduli during rennet coagulation RSM and skimmed milk are also presented. Time corresponding to initial increase in G'' is defined as $t_{G''}$, time to crossover point of G' and G'' is defined as gelation time (t_g). Approximate time of visually determined rennet flocculation time (RFT) is also indicated.

PUBLICATION IV

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Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder

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ABSTRACT

The growth of *Streptococcus thermophilus* ST12 (ST12) in milk reconstituted from non-irradiated and irradiated at 10 kGy low-heat skim milk powders (RSM and irrRSM, respectively) at 40 °C was monitored by microcalorimetry. Statistically significant differences of the growth patterns of ST12 in RSM and irrRSM were found. Distinctively diauxic growth curves in RSM were replaced by one-stage growth curves in irrRSM. The final pH in RSM was 5.56 while in irrRSM samples it was 4.41. The time of initiation of gel formation was about 36 min shorter; however, the gels were considerably weaker in irrRSM than in RSM. The front-face fluorescence spectra were also used to characterise the differences in acidification processes. The microcalorimetric data together with the concentrations of metabolites determined during fermentation, rheological and fluorescence measurements indicated the substantial changes in the growth of ST12 in irrRSM in comparison with RSM.

1. Introduction

Starter cultures containing lactic acid bacteria (LAB) have an important role in the manufacture of cheese and fermented dairy products. The starters are selected for rapid lactic acid formation and for their ability to contribute to the development of the aroma, flavour and texture of the products – these features being determined by a diverse range of metabolic peculiarities (Leroy & De Vuyst, 2003). *Streptococcus thermophilus* is the second most important dairy starter after *Lactococcus lactis* (Hols et al., 2005; Thomas & Crow, 1984). It is a thermophilic LAB and has traditionally been used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus* for the manufacture of yoghurt and so-called hard 'cooked' cheeses (e.g., Emmental, Gruyere, Grana), respectively (Delorme, 2008). *S. thermophilus* is also used alone or in combination with lactobacilli for the production of mozzarella cheese (Mills, O'Sullivan, Hill, Fitzgerald, & Ross, 2010). The use of *S. thermophilus* in combination with the mesophilic bacteria *L. lactis* subsp. *cremoris* or subsp. *lactis* as starters in experimental Cheddar cheese has also been reported in the literature (Champagne, Gagnon, St-Gelais, & Vuillemard, 2009; Michel & Martley, 2001).

Skim milk powder is among the most abundantly produced dairy products. It is used in a multitude of food applications, many of which require the powder to be reconstituted (Martin, Williams, & Dunstan, 2007). Milk reconstituted from powder is frequently used for investigations of cheese-making processes in pilot-plant conditions (Mohamed, Morris, & Breene, 1982; Peters, 1960; Wang et al., 2012; White & Ryan, 1983) as well as in laboratory practice for studying of activity of starter bacteria (Christopherson & Zottola, 1989) and propagation of mother starter cultures (Horrall, Elliker, & Kensler, 1950). In the early days of the dairy industry raw milk was used as a medium for growing starter cultures at dairies. Considering that raw milk quality variations can affect the uniformity of starter activity, raw milk has been replaced by milk reconstituted from skim milk powder of selected producers for improving product quality and for better control of the production processes (Whitehead, Ayres, & Sandine, 1993). Skim milk powder, which is specially screened to ensure that it is free of antibiotics, is used as the standard medium for growing starter bacteria and for monitoring the acidification activity of LAB. The powder is reconstituted to milk containing 12% solids and heated at 85–95 °C for 30–60 min (Ranken, Kill, & Baker, 1997).

Irradiation has been found to be a prospective technology to ensure food safety and sterility with minimum influence on the functional, nutritional, and sensory properties of some products (Chauhan, Kumar, Nadanasabapathy, & Bawa, 2008; Farkas, 2006; Grolichová, Dvořák, & Musilová, 2004; Żegota & Małolepszy, 2008).

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Gamma rays, emitted by the radioisotopes ^{60}Co and ^{137}Cs, X-rays and electron beams are usually used for the irradiation of foods (Chauhan et al., 2008; Farkas, 2006). It has been shown specifically that the irradiation can prevent spoiling of dairy products (Bandekar, Kamat, & Thomas, 1998; Ham, Jeong, Lee, Han, Jang, et al., 2009; Konteles, Sinanoglou, Batrinou, & Sflomos, 2009). A dose of 5 kGy has shown to be sufficient to decrease the total microflora in milk powder to an acceptable level (Żegota & Małolepszy, 2008). On the other hand, it has been shown that irradiation treatment caused physico-chemical changes of the foods (Cieśla, Salmieri, Lacroix, & Le Tien, 2004). It has led to the conformational changes of proteins (De la Hoz & Netto, 2008; Ham, Jeong, Lee, Han, Chae, et al., 2009), modifications of amino acids (Bhattacharya, Saha, & Mandal, 2000), and breakdown of the protein backbone (Davies, 2012), and has influenced the nutritional value and sensory properties of some irradiated foods (Grolichová et al., 2004; Żegota & Małolepszy, 2008).

Information on irradiated milk, or reconstituted milk from irradiated milk powder, used as growth media for LAB is scarce and somewhat inconsistent. It was claimed by Chamba and Prost (1989) that the growth of thermophilic lactic acid starters was similar in milk reconstituted from milk powder irradiated at 20 kGy and non-irradiated milk powder. However, although it was shown by Favrot and Maubois (1994) that the growth rate of *L. lactis* at an inoculation rate 10^6 cfu mL^{-1} was similar in irradiated and reference milk samples, at a lower inoculation rate (10^4 cfu mL^{-1}) the growth rate of the bacteria was significantly lower in milk reconstituted from skim milk powder irradiated at 20 kGy. In addition, the irradiation of skim milk powder inhibited the acidification rate of milk at both inoculation level (10^6 and 10^4 cfu mL^{-1}) studied (Favrot & Maubois, 1994).

Acidification activity of milk is a very important quality of dairy starters. Methods used to quantify the acidification activity of starter bacteria are usually based on measuring pH changes or on determination of the accumulation of lactic acid (Zanatta & Basso, 1992). In the majority of the studies reported a more detailed analysis of acidification process has not been carried out.

Calorimetry, in particular isothermal microcalorimetry, is ideally suited for the detailed study of acidification processes of milk as it offers many unique advantages: (i) the measured parameter is heat flow, which can be considered as a universal indicator of change – whether of microbial growth, or of physical and chemical processes; (ii) no specific sample treatment or preparation is needed, the samples are housed within an ampoule and monitored in situ; (iii) the technique does not require optical transparency of samples and is invariant irrespective of their physical form (Gaisford, O'Neill, & Beezer, 2009). Modern isothermal microcalorimeters allow measuring heat flows of intensity less than a microwatt. As few as about 10^5 active bacterial cells in a culture are sufficient to produce reliably monitored heat flows (Braissant, Wirz, Göpfert, & Daniels, 2010). It has been shown that isothermal microcalorimetry in combination with other analytical methods is one of the most promising techniques for the characterisation of growth of starter bacteria in opaque media (Kriščiunaite, Stulova, Kabanova, Laht, & Vilu, 2011; Mihhalevski, Sarand, Viiard, Salumets, & Paalme, 2011; Riva, Franzetti, Galli, & Schiraldi, 1997).

The aims of the present study were to characterise and compare the growth parameters of *S. thermophilus* ST12 (ST12) in milk reconstituted from non-irradiated skim milk powder and from skim milk powder irradiated at 10 kGy (RSM and irrRSM, respectively) applying microcalorimetry, and additionally to investigate the effect of irradiation on skim milk powder on the course of coagulation of reconstituted milk samples, using front-face fluorescence and dynamic rheological measurements. The applied combination of the novel methods, which made it possible to study in unprecedented detail the quantitative growth of the LAB in irrRSM in comparison with RSM, and the consequences of irradiation on the properties of the milk powder should be considered as the main justification for the research carried out. The data obtained should be considered as a practical valuable example for similar studies with other foodstuffs.

2. Materials and methods

2.1. Irradiation of milk powder

Low heat skim milk powder (LHSMP) was obtained from Valio Ltd. (Helsinki, Finland). A part of LHSMP was irradiated by gamma rays produced by cobalt-60 in sealed 250 g plastic bags at dose of 10 kGy using dosimetric system GEX WinDose (Centennial, CO, USA).

2.2. Preparation of reconstituted skim milk

LHSMP or irradiated LHSMP (irrLHSMP) were reconstituted in distilled water to yield a final concentration of 10% (w/v) milk solids, mixed thoroughly for 1 h, heated at 90 °C for 30 min and cooled to 40 °C immediately before experiments to make a regular reconstituted skim milk (RSM) or RSM prepared from irrLHSMP (irrRSM).

2.3. Bacterial cultures and preparation of inocula

The strain ST12 was kindly provided by Chr. Hansen (Hørsholm, Denmark). Frozen cultures of ST12 were thawed and pregrown on M17 agar (LAB M, Lancashire, UK) for 24 h at 40 °C. One colony from the M17 agar was inoculated into 10 mL of RSM and left at 40 °C until coagulation. One per cent of this culture was used for inoculation of 10 mL RSM, left until coagulation and further used for inoculation of milk samples. The number of bacteria in the inoculum was determined by plating on M17 agar followed by incubation for 3 days at 40 °C. The final average number of ST12 in the inocula was $(1.15 \pm 0.19) \times 10^9$ cfu mL^{-1}.

2.4. Controlled inoculation of reconstituted skim milk with ST12

RSM and irrRSM as growth media were prepared in 50 mL Erlenmeyer flasks. Milk samples (see Section 2.2) were inoculated with 1% (v/v) of inoculum prepared as described in Section 2.3 and intensively stirred. The calculated concentrations of inocula in milk samples studied varied from 10^6 to 10^1 cfu mL^{-1} with the 10-fold increment. 2 mL from each flask with different inoculum concentrations were transferred into the autoclaved calorimetric ampoules. At least two ampoules were aseptically filled per sample and milk without starter was used as a reference. Microcalorimetric experiments were run at 40 °C.

The remaining inoculated milk in the Erlenmeyer flasks was divided into 1 mL aliquots, placed at 40 °C and used for determination of the concentrations of carbohydrates, lactic acid and amino acids and pH measurements at appropriate time points, determined in accordance with the power–time curves from the microcalorimetric measurements run simultaneously.

2.5. Calorimetric equipment and handling of calorimetric ampoules

A 24-channel isothermal batch microcalorimeter TAM III (Thermal Activity Monitor, TA Instruments, New Castle, DE, USA) was used for the monitoring of the growth of the thermophilic starter ST12. The calorimetric sensitivity was ±0.2 μW and

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detection limit −0.5 μW. Heat generated or absorbed was continuously measured in air-tightly sealed 3 mL calorimetric ampoules.

The filled ampoules were kept in the thermal equilibration position for 15 min in the calorimeter before moving them into measurement position. Data were not collected over ~1 h at the beginning of the experiments to let the thermal equilibration processes to be completed. Heat flow was measured at 10 s intervals. Data acquisition and analysis was carried out using TAM Assistant Program (v 0.9.1012.40, SciTech Software AB, Thermometric AB, Järfälla, Sweden).

Further analysis of calorimetric data was done according to Kabanova, Kazarjan, Stulova, and Vilu (2009) and Kabanova, Stulova, and Vilu (2012). Bacterial growth was also expressed as the change in the number of viable cells in time using the equation:

$$\Delta N = \Delta Q / Y_Q \quad (1)$$

where ΔN (cfu mL^{-1} h^{-1}) is the number of bacteria grown during the selected time interval, ΔQ (J mL^{-1} h^{-1}) is the heat produced during the same time interval, Y_Q (J cfu^{-1}) is the heat yield coefficient, which was calculated on the basis of experimental results (data not shown) and was determined to be $(4.45 \pm 0.15) \times 10^{-9}$ J cfu^{-1}. The Y_Q is effectively the heat evolved during the multiplication of one bacterium.

2.6. Measurement of pH

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

2.7. Determination of carbohydrates and lactic acid

Milk samples were mixed 1:1 with isopropanol for sedimentation of proteins in the samples. The precipitate was removed by centrifugation at 14,000 × *g* for 10 min. The supernatant was filtered through a 13 mm diameter and 0.2 μm pore size regenerated cellulose (RC) membrane filter (Whatman, Maidstone, UK) and diluted with water before analysis. Concentrations of lactose, glucose, galactose and lactic acid were measured with a high-performance liquid chromatography (HPLC) system (Alliance 2695 system, Waters Corp., Milford, MA, USA), using a BioRad HPX-87H column (Hercules, CA, USA) with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} at 35 °C. A Waters 2414 refractive index detector was used for detection and quantification of substances.

2.8. Determination of free amino acids

Analysis of free amino acids was performed on an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp.), including a binary solvent manager, a sample manager and a photodiode array detector (PDA), 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. Prior to injection, free amino acids were derivatised with AccQ•Fluor Reagent (Waters Corp.) according to the manufacturer's procedure. The injection volume was 1.0 μL, the amino acids were eluted at a flow rate of 0.7 mL min^{-1}, and absorbance was recorded at 260 nm. The running time was 12 min. Empower software (Waters Corp.) was used for data processing.

2.9. Dynamic rheological measurements during fermentation

Rheological measurements during fermentation of RSM or irrRSM with ST12 at inoculation rate 10^5 cfu mL^{-1} were carried out using a Physica MCR301 rheometer (Anton Paar GmbH, Graz, Austria) equipped with C-PTD200 Peltier temperature control unit and CC27 coaxial cylinder measuring system (outer and inner diameters 28.92 and 26.66 mm, respectively) as described by Kriščiunaite et al. (2011).

2.10. Front-face fluorescence spectroscopy

An Instant Screener Compact fluorescence spectrophotometer (LDI Ltd., Tallinn, Estonia) equipped with pulsed xenon lamp was used for the measurement of front-face spectral fluorescence signatures (SFS) from the milk powders or inoculated milk samples. Samples were placed in a 10 mL optical cell with quartz bottom window. The SFS spectra were recorded as a matrix of fluorescent intensities depending on excitation and emission wavelengths (λ_{ex} and λ_{em}, respectively) resulting in 3D records: the λ_{ex} was changed in the range 230–400 nm, and λ_{em} in the range 250–615 nm during the measurements. Excitation and emission slits were set at 5 nm.

The SFS spectra were recorded every 15 min throughout the fermentation of RSM and irrRSM samples with ST12 at inoculation rate 10^5 cfu mL^{-1}. SFS spectra recorded were normalised dividing the intensities of the fluorescence by the volume of the spectra and analysed by principle component analysis (PCA).

2.11. Statistical analysis of the data

All microcalorimetric experiments were repeated twice and measurements were carried out with two or three parallel samples. Other analyses were carried out in triplicate. All values of the parallel experimental points were averaged and reported along with the standard deviation (SD). The experimental data were submitted to single-factor analysis of variance (ANOVA), and the differences of the means were evaluated by Fisher's least significant difference (LSD) test. The difference of the mean values was accepted at the significance level $p < 0.05$.

3. Results and discussion

3.1. Effect of irradiation on spectral and physico-chemical properties of skim milk powder

The irrLHSMP was yellowish in colour and had a pronounced off-flavour compared with LHSMP. These odour changes have been reported as typical for irradiated dairy foods (Day, Fross, & Patton, 1957). The SFS spectra of LHSMP and irrLHSMP are presented in Fig. 1. The fluorescence intensity corresponding to tryptophan emission maximum was threefold lower in irrLHSMP than in LHSMP and the coordinates of the maxima $\lambda_{ex}/\lambda_{em}$ were 285/330 and 285/325 nm respectively. After reconstitution, a twofold difference in the fluorescence intensity of tryptophan remained between spectra of irrRSM and RSM (data not shown). The decreased tryptophan fluorescence in irrLHSMP may be associated with the oxidation of aromatic amino acids or production of other oxidation products (Stadtman & Levine, 2003), which can quench the

Fig. 1. Spectral fluorescence signatures of (A) low-heat skim milk powder (LHSMP) and (B) irradiated LHSMP; the black dot corresponds to the fluorescence intensity maximum.

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fluorescence, or with conformational changes of proteins caused by irradiation (Cieśla et al., 2004). A distinctive additional fluorescence signal with a maximum at $\lambda_{ex}/\lambda_{em}$ of 350/410–420 nm, which corresponds most probably to oxidation or Maillard reaction products (Becker, Christensen, Frederiksen, & Haugaard, 2003; Liu & Metzger, 2007), could be observed on the SFS spectra of irrLHSMP but not of LHSMP.

It is known that ionising radiation can lead to the fragmentation, aggregation, conformational and net electrical charge changes, oxidation of amino acids and rupture of covalent bonds (Cho, Song, & Yamda, 2010; Davies, 1987, 2012; Day et al., 1957). According to Arena (1971), ionising radiation also causes water molecules to lose electrons, producing H_2O^+ and "free" solvated electrons. These products react with other water molecules to produce a number of reactive compounds like superoxide anion radicals (O_2^-) and hydroxyl radicals ($OH^•$) (Thibessard, Leblond-Bourget, Fernandez, & Decaris, 2001) and non-radical hydrogen peroxide (H_2O_2) (Davies, 1987). OH^- can also be generated from the reaction of O_2^- with H_2O_2 (Fridovich, 1983). The reactive oxygen formed during exposure to gamma-irradiation can lead to the cleavage of peptide bonds (Stadtman & Levine, 2003). It has been shown by Ham, Jeong, Lee, Han, Chae, et al. (2009) that α_{S1}-casein and β_{A1}-casein were degraded, whereas α_{S0}-, β_B-, and β_{A3}-caseins were formed by gamma-irradiation.

It can be suggested that irradiation treatment can affect also the buffering capacity of milk, which mainly depends on the composition and distribution of minerals and proteins between aqueous and solid phases (Salaün, Mietton, & Gaucheron, 2005). As already in the beginning of fermentation the pH (6.48 ± 0.01 and 6.43 ± 0.01 in RSM and irrRSM, respectively) and total free amino acids (Tables 2 and 3) were different in RSM and irrRSM, this could indicate that initially the samples were differing also in terms of buffering capacity.

All these mechanisms could be involved in changing the spectral characteristics observed. However, a more detailed study of the structural changes of skim milk powder caused by gamma-irradiation is awaiting future research efforts.

3.2. Calorimetric experiments

The growth of ST12 in RSM and irrRSM was monitored by microcalorimetry at 40 °C. Typical calorimetric power-time curves of the growth of ST12, acidification curves and number of bacteria calculated from power–time curves (N; log cfu mL^{-1}) in RSM and irrRSM samples at inoculation rate 10^5 cfu mL^{-1} are presented in Fig. 2. The power–time curves recorded were processed as described by Kabanova et al. (2012) and the numerical results are presented in Table 1.

3.2.1. Typical power–time growth curves of ST12 in RSM and irrRSM

The power–time curves were remarkably different for the growth of ST12 in RSM and irrRSM (Fig. 2). Power–time curves in RSM showed two peaks corresponding to two exponential (fast) growth phases: the first exponential growth phase (a smaller peak, a shoulder) and the second exponential growth phase (a major peak). This shape of the power time curve was in agreement with the results reported in literature for *L. lactis* (Juillard et al., 1995; Letort, Nardi, Garault, Monnet, & Juillard, 2002; Niven, Knight, & Mulholland, 1998) and can be explained by the fact that oligopeptides and presumably also free amino acids were the main sources of nitrogen during the first exponential growth phase, whereas peptides of caseins released by cell wall located proteinases were used as the source of amino acids during the second exponential growth phase. As mentioned above, *L. lactis* and not *S. thermophilus* was studied in the cited papers; however, Juillard et al. (1995) reported that the transport of oligopeptides in these two bacteria is similar.

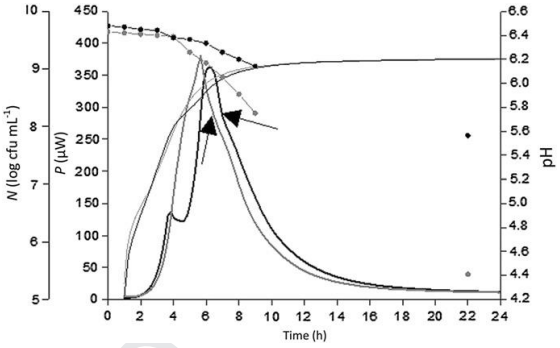

Fig. 2. Calorimetric power–time (P) curves (bold lines) of the growth of *Streptococcus thermophilus* ST12, number of bacteria (N) calculated from the power–time curves (thin lines), and pH changes (circles) in reconstituted skim milk powder (RSM; black) and in irradiated RSM (grey) at an inoculation rate of 10^5 cfu mL^{-1}. Arrows mark the mean gelation times determined rheologically.

According to our data (Fig. 2), the specific feature of the growth of ST12 in irrRSM was the absence of a shoulder (the first smaller peak) on the power–time curve. The maximum specific growth rates (μ_{max}) of bacteria in the single exponential growth phase in irrRSM were close to the values of the first fast exponential growth phase in RSM, still significantly different according to ANOVA (Table 1). Moreover, higher total amounts of free amino acids were observed in the beginning of fermentation (0 h) in irrRSM in comparison with RSM (Tables 2 and 3). This justifies the hypothesis that peptides and amino acids were facilitating the fast growth of bacteria in reconstituted milk. Broad specificity of peptide transport system of *S. thermophilus* and its capacity to use large peptides up to 23 amino acids (Juille, Le Bars, & Juillard, 2005) support the assumption that the growth of the bacteria in irrRSM during the first 7 h of fermentation largely depended on the utilisation of oligopeptides. Peptide transport into the cells has been reported to limit (determine) the growth rate of the bacteria in the media containing casein-derived peptides (Letort et al., 2002). However, our data supported specifically the notion that the presence of peptides in the growth medium may have accelerated the growth of the bacteria in milk, and hydrolysis of caseins was presumably the rate limiting step in the second slower exponential growth phase in RSM.

As seen in Fig. 2, the remarkable differences were observed in the acidification profiles of the reconstituted milk samples studied. The pH of irrRSM just after inoculation (6.43 ± 0.01) was slightly lower than that of RSM (6.48 ± 0.01), which was in agreement with Day et al. (1957), and Ham et al. (2005). The pH of the RSM and irrRSM milk samples decreased slowly up to 4 h of fermentations and then more rapidly in the irrRSM than in the RSM samples. However, pH was higher than 6 during the whole exponential growth phase, which was a clear indication that acidification as such could not be the reason for the termination of the exponential growth. A remarkable difference in the final pH (after 22 h incubation) of the RSM (5.56 ± 0.03) and irrRSM (4.41 ± 0.02) samples was observed. However, also these values were clearly higher than the pH 4.0–4.2 usually observed at the end of the growth of the *S. thermophilus* (Zanatta & Basso, 1992).

To study quantitative peculiarities of the growth of ST12 in the RSM and irrRSM in more detail, different inoculation rates were investigated.

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Table 1
Streptococcus thermophilus ST12 growth parameters in reconstituted skim milk (RSM) and in irradiated reconstituted skim milk (irrRSM)at 40 °C.[a]

Milk	Inoculation rate (cfu mL^{-1})	μ_{max} (h^{-1})		Q_{exp} (J mL^{-1})	Q_{tot} (J mL^{-1})	$N_{exp.} \times 10^8$ (cfu mL^{-1})	P_{max} (μW)	t_{Pmax} (h)	λ (h)
		1st exponential phase	2nd exponential phase						
RSM	10^6	1.88 ± 0.03^{aA}	1.05 ± 0.01^{aA}	2.00 ± 0.05^{aA}	7.12 ± 0.01^{aA}	4.50 ± 0.11^{aA}	362.95 ± 3.80^{aA}	5.50 ± 0.00^{aA}	0.02 ± 0.00^{aA}
	10^5	1.85 ± 0.05^{aA}	1.18 ± 0.00^{bA}	2.10 ± 0.00^{bA}	7.21 ± 0.03^{aA}	4.73 ± 0.00^{bA}	362.77 ± 4.03^{aA}	6.17 ± 0.00^{bA}	0.98 ± 0.05^{bA}
	10^4	1.91 ± 0.03^{aA}	1.17 ± 0.02^{bA}	2.11 ± 0.01^{bA}	7.12 ± 0.01^{aA}	4.74 ± 0.03^{bA}	363.10 ± 0.47^{aA}	7.33 ± 0.00^{cA}	2.08 ± 0.02^{cA}
	10^3	1.85 ± 0.04^{aA}	1.14 ± 0.01^{bA}	1.97 ± 0.02^{aA}	7.24 ± 0.02^{aA}	4.42 ± 0.05^{aA}	359.57 ± 2.30^{aA}	8.67 ± 0.00^{dA}	3.05 ± 0.05^{dA}
	10^2	1.83 ± 0.10^{aA}	1.13 ± 0.02^{bA}	2.00 ± 0.07^{aA}	7.23 ± 0.07^{aA}	4.50 ± 0.15^{aA}	345.52 ± 8.05^{aA}	9.92 ± 0.12^{eA}	4.07 ± 0.19^{eA}
	10^1	1.61 ± 0.04^{bA}	1.01 ± 0.03^{cA}	2.26 ± 0.02^{cA}	7.36 ± 0.13^{aA}	5.08 ± 0.03^{cA}	353.31 ± 12.09^{aA}	10.83 ± 0.00^{fA}	4.98 ± 0.01^{fA}
irrRSM	10^6	1.72 ± 0.05^{abB}	–	1.82 ± 0.01^{aB}	6.83 ± 0.22^{aA}	4.09 ± 0.11^{aB}	407.94 ± 4.57^{aB}	4.33 ± 0.00^{aB}	0.13 ± 0.09^{aA}
	10^5	1.68 ± 0.01^{abB}	–	2.09 ± 0.05^{bcA}	6.94 ± 0.14^{aA}	4.71 ± 0.10^{bcA}	380.02 ± 8.60^{bB}	5.67 ± 0.00^{bB}	1.00 ± 0.01^{bA}
	10^4	1.78 ± 0.05^{aB}	–	2.10 ± 0.03^{bcA}	6.96 ± 0.06^{aB}	4.71 ± 0.10^{bcA}	370.93 ± 1.42^{bcB}	6.92 ± 0.12^{cB}	2.29 ± 0.06^{cB}
	10^3	1.72 ± 0.03^{abB}	–	2.05 ± 0.03^{cA}	6.98 ± 0.02^{aB}	4.60 ± 0.06^{bA}	375.19 ± 2.32^{bB}	8.00 ± 0.00^{dB}	3.38 ± 0.06^{dB}
	10^2	1.66 ± 0.00^{bB}	–	2.17 ± 0.06^{bdB}	7.00 ± 0.10^{aA}	4.88 ± 0.13^{cB}	360.28 ± 3.67^{cA}	9.33 ± 0.00^{eB}	4.43 ± 0.01^{eA}
	10^1	1.46 ± 0.07^{cAB}	–	2.25 ± 0.06^{dA}	7.14 ± 0.00^{aA}	5.05 ± 0.14^{cA}	371.63 ± 6.36^{bcA}	10.67 ± 0.00^{fA}	5.47 ± 0.15^{fB}

[a] Data are means ± SD of maximum specific growth rate (μ_{max}) in the first and second exponential growth phase, the heat evolved during the exponential phase (Q_{exp}), the number of bacteria at the end of exponential growth phase (N_{exp}), the total heat produced during the whole fermentation (Q_{tot}), the maximum heat flow (P_{max}), time of the maximum heat production rate (t_{Pmax}), and lag phase duration (λ) obtained from microcalorimetric power-time curves. Different superscript lowercase letters (a–f) mark significant differences at $p < 0.05$ (ANOVA followed by Fischer's LSD test) between the data of different inoculation rates; different superscript capital letters (A, B) denote significant differences between RSM and irrRSM samples at the same inoculation rates.

3.2.2. *Effect of inoculation rate on power-time curves of ST12 in RSM and irrRSM*

Calorimetric power–time curves and acidification profiles of ST12 were monitored in RSM and irrRSM at different inoculation rates in the range of 10^6–10^1 cfu mL^{-1} (Fig. 3). The diauxic growth of ST12 at inoculation rate 10^5 cfu mL^{-1} observed in RSM and disappearance of diauxy in irrRSM (Fig. 2) were seen at all inoculation rates studied (Fig. 3). It is clearly seen that the power–time curves are strikingly similar in case of all inoculation rates studied. This was not the case in our earlier studies with *L. lactis* in transparent media (Kabanova et al., 2012), and it cannot be considered a trivial phenomenon.

As expected from the similarity of the power-time curves, and seen from the data presented in Table 1 the maximum growth rates μ_{max} of the first exponential phase were practically the same at different inoculation rates ranging from 10^6 to 10^2 cfu mL^{-1}, but differing in the two media, with slightly lower values (about 8%) in irrRSM than in RSM.

Heat effects produced during the exponential growth phase(s) (Q_{exp}) and during the whole studied growth period (Q_{tot}) were calculated in accordance with the scheme proposed in Kabanova et al. (2012), and the results are presented in Table 1. The amount of heat produced during the exponential phases (Q_{exp}) at inoculation rates from 10^6 to 10^2 cfu mL^{-1} was roughly the same in RSM and irrRSM, which in fact means that the numbers of bacteria grown during the fast growth phases should be rather similar in both media studied, however, these occurred statistically different for the inoculation rates 10^6 and 10^2 cfu mL^{-1} according to the data in Table 1. The Q_{exp} at inoculation rate 10^1 cfu mL^{-1} had the highest value compared with other inoculation rates and was the same in RSM and irrRSM. The amount of heat produced during the whole studied growth period of the bacteria was independent of inoculation rate and was almost the same in RSM and irrRSM, except for the inoculation rates $10^4$and 10^3 cfu mL^{-1}. The average Q_{tot} was 7.21 ± 0.09 J mL^{-1} and 6.98 ± 0.10 J mL^{-1}, in RSM and irrRSM, respectively. Using the value $Y_Q = (4.45 \pm 0.15) \times 10^{-9}$ J cfu^{-1} the

Table 2
Changes in the concentrations of free amino acids during fermentation of reconstituted skim milk (RSM) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}.

Amino acid	Concentration of free amino acids (μmol L^{-1}) at various times (hours) after inoculation								
	0	3	4	5	6	7	8	9	22
Histidine	10.4 ± 0.16	13.7 ± 0.0	17.7 ± 0.9	21.8 ± 3.4	28.7 ± 1.2	30.3 ± 1.0	29.0 ± 0.0	33.5 ± 0.7	18.8 ± 1.1
Asparagine	69.0 ± 1.9	40.3 ± 6.0	14.0 ± 1.8	3.6 ± 1.3	0.0	0.0	0.0	0.0	68.0 ± 2.8
Glutamine	14.5 ± 0.8	16.2 ± 0.6	14.3 ± 0.6	18.7 ± 0.6	21.3 ± 0.1	20.6 ± 0.6	20.5 ± 0.7	20.0 ± 0.0	22.2 ± 1.8
Arginine	14.4 ± 0.7	54.0 ± 0.6	65.1 ± 3.7	73.6 ± 1.6	82.0 ± 2.0	82.9 ± 3.0	85.5 ± 3.5	89.0 ± 2.8	94.5 ± 4.9
Glycine	55.5 ± 1.6	58.6 ± 2.7	49.9 ± 1.5	28.1 ± 0.4	13.9 ± 1.5	13.7 ± 0.5	13.5 ± 0.7	13.0 ± 0.0	29.5 ± 2.2
Aspartic acid	26.1 ± 0.7	35.7 ± 1.4	34.9 ± 1.8	33.6 ± 1.1	49.8 ± 1.6	60.6 ± 0.9	74.5 ± 6.4	86.0 ± 1.4	136.6 ± 9.3
Glutamic acid	269.9 ± 7.2	281.9 ± 8.9	279.2 ± 9.7	259.9 ± 8.9	261.8 ± 5.7	267.3 ± 3.3	273.5 ± 14.8	287.5 ± 3.5	374.1 ± 21.3
Threonine	10.1 ± 0.1	10.8 ± 0.5	9.5 ± 0.4	6.8 ± 0.7	4.6 ± 0.3	2.6 ± 0.5	4.0 ± 0.0	5.0 ± 0.0	26.8 ± 1.1
Alanine	33.5 ± 1.1	34.8 ± 0.4	34.4 ± 1.8	32.9 ± 0.5	32.4 ± 1.2	31.9 ± 0.1	33.0 ± 1.4	34.5 ± 2.1	76.6 ± 8.0
Proline	20.5 ± 0.6	27.4 ± 0.5	32.4 ± 0.9	37.7 ± 1.2	50.9 ± 2.0	60.6 ± 0.9	71.0 ± 2.8	81.0 ± 1.4	201.3 ± 14.6
Ornithine	3.2 ± 0.5	3.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.4	3.5 ± 0.4	3.6 ± 0.6	3.0 ± 0.0	3.5 ± 0.7	4.9 ± 0.1
Cysteine	2.5 ± 0.5	15.6 ± 1.8	21.1 ± 2.6	23.9 ± 0.3	21.8 ± 1.0	19.1 ± 0.1	19.5 ± 0.7	20.0 ± 1.4	3.9 ± 1.5
Lysine	15.5 ± 0.3	14.0 ± 0.0	12.2 ± 0.3	14.6 ± 1.9	25.2 ± 0.6	35.4 ± 0.5	44.0 ± 1.4	53.0 ± 0.0	159.9 ± 11.1
Tyrosine	2.5 ± 0.0	3.6 ± 0.2	3.8 ± 0.0	5.7 ± 0.3	11.9 ± 0.1	16.4 ± 0.6	20.5 ± 0.7	25.0 ± 0.0	87.3 ± 6.0
Methionine	2.9 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	2.7 ± 0.1	4.8 ± 0.3	6.0 ± 0.0	8.5 ± 0.7	30.5 ± 2.1
Valine	14.8 ± 0.1	13.4 ± 0.4	9.8 ± 0.2	4.3 ± 0.1	5.0 ± 0.3	9.9 ± 0.1	14.5 ± 0.7	20.5 ± 0.7	107.4 ± 7.7
Isoleucine	5.8 ± 0.1	5.8 ± 0.1	2.9 ± 0.1	1.0 ± 0.2	2.2 ± 0.2	3.3 ± 0.4	4.5 ± 0.7	8.0 ± 1.4	61.6 ± 5.2
Leucine	8.0 ± 0.1	6.0 ± 0.0	2.1 ± 0.0	0.5 ± 0.0	2.5 ± 0.3	7.1 ± 0.1	12.0 ± 1.4	19.0 ± 1.4	124.8 ± 9.7
Phenylalanine	2.7 ± 0.2	4.0 ± 0.2	3.4 ± 0.2	4.0 ± 0.1	10.6 ± 3.9	13.0 ± 0.0	17.0 ± 1.4	20.5 ± 0.7	65.1 ± 4.4
Tryptophan	3.1 ± 0.0	3.2 ± 0.1	3.1 ± 0.2	3.7 ± 0.0	5.5 ± 0.0	6.9 ± 0.1	7.5 ± 0.7	8.0 ± 0.0	19.3 ± 1.8
Total	584.9 ± 5.9	644.1 ± 5.9	614.5 ± 26.5	579.1 ± 7.6	636.3 ± 11.8	689.9 ± 0.1	753.0 ± 36.8	835.5 ± 16.3	1713.2 ± 107.7

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Table 3
Changes in the concentrations of free amino acids during fermentation of irradiated reconstituted skim milk (irrRSM) with *Streptococcus thermophilus* ST12 at inoculation rate 10^5 cfu mL^{-1}.

Amino acid	Concentration of free amino acids (μmol L^{-1}) at various times (hours) after inoculation							
	0	3	5	6	7	8	9	22
Histidine	18.3 ± 2.1	18.0 ± 2.4	16.1 ± 0.0	20.6 ± 0.0	22.8 ± 0.0	21.2 ± 2.6	25.4 ± 4.5	41.5 ± 10.2
Asparagine	40.7 ± 0.6	30.0 ± 1.5	35.7 ± 0.0	43.1 ± 0.0	39.6 ± 4.0	40.8 ± 2.0	61.1 ± 7.4	43.4 ± 0.0
Glutamine	21.2 ± 0.0	15.9 ± 0.7	14.0 ± 0.6	16.8 ± 1.7	20.5 ± 3.2	21.7 ± 3.0	24.4 ± 0.4	17.7 ± 2.2
Arginine	15.1 ± 1.0	25.9 ± 2.3	44.1 ± 5.4	50.6 ± 7.2	64.5 ± 5.5	64.9 ± 8.4	62.6 ± 2.0	36.1 ± 0.0
Glycine	85.3 ± 2.0	75.1 ± 2.9	60.5 ± 3.4	39.8 ± 6.7	0.0	0.0	0.0	0.0
Aspartic acid	43.5 ± 2.1	41.8 ± 1.7	42.5 ± 0.1	36.3 ± 3.5	34.0 ± 1.5	34.1 ± 2.4	28.1 ± 5.3	22.7 ± 0.5
Glutamic acid	305.8 ± 3.9	304.0 ± 4.4	303.7 ± 0.3	254.3 ± 5.4	204.2 ± 7.7	169.4 ± 0.6	117.00 ± 3.1	42.2 ± 6.7
Threonine	10.1 ± 0.2	9.7 ± 0.2	8.6 ± 0.0	5.8 ± 0.7	0.0	0.9 ± 0.3	1.3 ± 0.9	22.8 ± 0.1
Alanine	38.9 ± 0.2	37.7 ± 0.2	36.3 ± 0.4	32.4 ± 0.6	27.4 ± 1.6	28.7 ± 1.9	38.7 ± 5.2	155.5 ± 1.5
Proline	22.0 ± 0.4	22.9 ± 0.5	26.0 ± 1.8	29.3 ± 0.9	47.9 ± 1.6	72.3 ± 0.7	104.0 ± 0.0	215.1 ± 2.5
Ornithine	2.4 ± 0.2	2.4 ± 0.1	2.1 ± 0.0	2.3 ± 0.2	2.9 ± 0.0	3.5 ± 2.1	3.5 ± 0.0	4.6 ± 1.4
Cysteine	0.4 ± 0.0	0.0	1.6 ± 0.3	2.0 ± 0.1	6.3 ± 0.0	5.7 ± 0.2	4.6 ± 0.0	0.0
Lysine	15.0 ± 0.3	14.5 ± 0.1	13.2 ± 0.2	10.7 ± 0.4	17.2 ± 1.8	25.4 ± 0.4	32.2 ± 0.5	53.3 ± 1.4
Tyrosine	2.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.2	4.0 ± 1.6	9.4 ± 1.1	17.9 ± 1.2	26.2 ± 0.4	40.2 ± 0.6
Methionine	trace	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ± 0.0
Valine	14.6 ± 0.2	12.7 ± 0.3	11.8 ± 0.3	6.1 ± 0.3	5.9 ± 1.5	7.3 ± 0.5	9.3 ± 0.9	19.4 ± 0.7
Isoleucine	5.5 ± 0.0	5.4 ± 0.2	3.1 ± 0.2	0.0	0.0	0.0	0.0	38.6 ± 2.7
Leucine	10.4 ± 2.3	9.1 ± 0.3	8.9 ± 2.1	4.9 ± 0.3	11.4 ± 0.1	21.4 ± 0.4	25.5 ± 0.9	57.6 ± 4.6
Phenylalanine	3.6 ± 1.9	2.8 ± 0.1	3.5 ± 1.6	1.8 ± 0.5	5.7 ± 1.2	12.2 ± 0.2	17.6 ± 0.3	60.9 ± 0.6
Tryptophan	0.9 ± 0.1	1.5 ± 0.0	1.5 ± 0.3	1.9 ± 0.3	3.9 ± 0.6	6.9 ± 0.0	4.8 ± 0.0	15.6 ± 2.4
Total	656.7 ± 0.6	632.2 ± 0.6	635.9 ± 4.0	563.6 ± 10.1	524.0 ± 4.8	560.7 ± 23.6	593.0 ± 29.5	909.9 ± 36.0

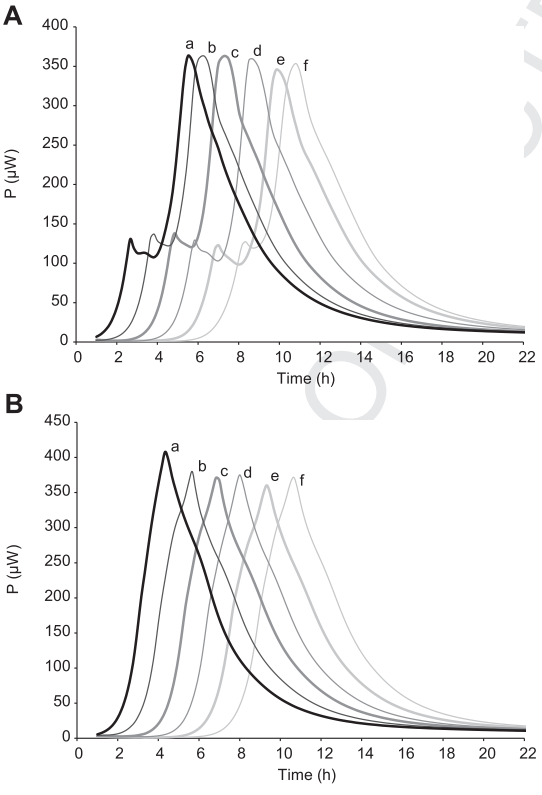

Fig. 3. Calorimetric power–time (P) curves of the growth of *Streptococcus thermophilus* ST12 in (A) reconstituted skim milk powder (RSM) and (B) irradiated RSM at different inoculation rates (cfu mL^{-1}): a, 10^6; b, 10^5; c, 10^4; d, 10^3; e, 10^2; f, 10^1.

total numbers of bacteria (N_{tot}) grown during the whole process were calculated from Q_{tot} and were (1.62 ± 0.02) × 10^9 and (1.57 ± 0.02) × 10^9 cfu mL^{-1} in RSM and irrRSM, respectively.

As mentioned earlier, the noted similarity of the power-time curves for all inoculation rates studied was not observed in the earlier studies (Kabanova et al., 2012). The key to the understanding of these facts is in the comprehension of the growth in the cases with the inocula of different levels (10^1–10^6 cfu mL^{-1}). In the case of an inoculum of 10^6 cfu mL^{-1} the number of bacteria should increase 10^3 times during the growth, and the amounts of growth by-products formed (lactate in the first place), increase from zero to that corresponding to the final density of biomass (10^9 cfu mL^{-1}). In the case of the inoculum of 10^1, the number of bacteria, and the corresponding amounts of growth by-products should increase through the range 10^1–10^9 cfu mL^{-1}, which is remarkably more than in the former case. In full agreement with this, with the "additional" lactate inhibition, μ_{max} observed at lower inoculation rates were previously shown to be lower than those at higher inoculation rates in agar gels (Kabanova et al., 2012). But, as emphasised, this is not the case in the present study in the case of growth on milk: all μ_{max} were practically the same at all inoculation rates studied. This could be explained if we assume that the reconstituted milk has higher buffering capacity in comparison with the solid state matrices of agar, studied by us earlier (Kabanova et al., 2012).

It should be noted, however, that we have no explanation for the fact that the numbers of bacteria grown during the exponential growth phase, and during the total growth processes are also practically the same in the RSM and irrRSM samples. To elucidate the mechanisms terminating the exponential growth and the growth of the cultures, additional measurements of carbohydrates, organic acids, and amino acids were carried out, and the results of these measurements are presented in the Section 3.3.

Looking carefully at power–time curves of both, RSM and irrRSM samples, it can be seen that the curves obtained for tenfold decreasing inoculation rates are shifted with practically regular intervals from each other – the exponential phases are of equal length, the numbers of the bacteria grown during the exponential phase and during the growth of the cultures are the same, but the lengths of the lag phase (λ) are changing with regular interval on

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changing the inoculation rates (Fig. 3A and B, and Table 1). To understand this quite important peculiarity it should be noticed that the sensitivity of the TAMIII is 0.5 μW – this heat flow corresponds to the growing of about $>10^5$ bacteria. The instrument is capable of measuring the growth of the bacteria at a level exceeding this threshold value. The similarity of the curves observed showed that the growth of the population in the range 10^5–10^9bacteria (see Table 1) was taking place very similarly at different inoculation rates. However, at lower inoculation rates bacterial growth is recorded only after the number of bacteria had reached 10^5 cfu mL^{-1}, and it takes time leading effectively to the increase of λ.

The measured λ is, as expected, the shortest in the case of the highest inoculation rates. Taking into account that there also was a lag of 1 h in the starting of the measurements, it could be concluded that 1.0–1.15 h was the shortest lag time observed. Lowering of the inoculation rate by 10 times should lead to the increase of the lag phase by 3.33 doubling times (t_d). Indeed, this was practically the case. The value of t_d in both RSM and irrRSM was approximately 0.38 h, which means that the expected shift of the curves theoretically should have been 1.3 h. As seen from Table 1, the shifts in λ were about 20% less (1 h) than calculated above; however, taking into account that the accuracy of inoculation cannot be too precise and that the value of μ_{max} was reflecting a multi-phased growth pattern the fit obtained should be considered satisfactory.

It should be noted that the heat effect (Q_{exp}) and numbers of grown bacteria (N_{exp}) were 10–20% higher at inoculation rate 10^1 than those at the other inoculation rates. 10–15% lower values of μ_{max} at the lowest inoculation rate 10^1 cfu mL^{-1} in comparison with higher inoculation rates were also observed. The most important difference between the conditions of the lowest inoculation rate and higher rates was the amount of the lactic acid and other possible growth by-products in the samples. Lactic acid concentration in the cultures in the beginning of the measurable power-time curves was highest at the lowest inoculation rate used (10^1 cfu mL^{-1}), and it decreased with the increase of the inocula – 10^2 cfu mL^{-1} and higher. Taking into account the high similarity of the power-time curves at all inoculation rates studied, the conclusion can be made that only the highest lactic acid concentrations in the case of the lowest inoculation rate used (10^1 cfu mL^{-1}) was influencing the growth characteristics of the bacteria in the range 10^5–10^9 bacteria per mL.

These low inoculation rate effects could be explained assuming that diffusion processes of substrates, and bacteria in milk are most probably not as intensive as in other liquids studied (solutions of gelatine and agar), and the descendants of the seed bacteria (inocula) may form loose conglomerates (not real colonies) in which growth retarding factors (lactic acid, etc.) could accumulate, and we could even assume that milk was locally coagulated in the case of the largest agglomerates observed. The same reasoning could be used to explain the lower μ_{max} values in irrRSM than in RSM. The effects of irradiation described in Section 3.1 could lead to the somewhat slower diffusion processes in the irrRSM in comparison with RSM.

3.3. Carbohydrate utilisation and lactic acid production

As seen from Fig. 4, practically all the lactose was hydrolysed by the end of the exponential phases in RSM, except for inoculation rate 10^1.

Glucose and galactose are formed from lactose during the fermentation, glucose is consumed and lactate is synthesised simultaneously by the bacteria. As seen from Fig. 4, this balance was not maintained – there were less amounts of galactose present than expected. The error bars in Fig. 4 are rather small, indicating that deviations from the expected amounts of galactose are not caused by measurement errors. The noticed imbalance could be explained by the fact that not all the possible products involved were measured – polysaccharides in the first place, and also not all growth by-products were determined. About 10–20 mmol L^{-1} of glucose was utilised and about 20 mmol L^{-1} of lactic acid was formed by the end of the exponential phases for the inoculation rates 10^3–10^6 cfu mL^{-1} in RSM.

As seen in Fig. 4 the patterns of hydrolysis of lactose, utilisation of glucose and formation of lactic acid in irrRSM were very different from those in RSM. Less than 30 mmol L^{-1} of lactose was hydrolysed during the active growth phase of the bacteria in irrRSM, less than 15 mmol L^{-1} of glucose as well as of galactose was utilised by the bacteria, and about 10 mmol L^{-1} of lactic acid was formed. These data indicated that irradiation of the skim milk powder led to the deterioration of lactose hydrolysis processes by the bacteria. As the bacteria were obviously not irradiated, the deterioration may be explained by the changes of the state of lactose (Adachi, 1962) in irrRSM.

The pH was practically the same at the end of the exponential phases in RSM and irrRSM (6.31 ± 0.01 and 6.24 ± 0.01, respectively). Taking into account that practically the same amounts of heat (numbers of bacteria) were produced during the exponential growth phase, equality of the pH at the end of the exponential growth phase could be considered as an indication that pH was the factor terminating the fast growth. However, the high values of pH observed cannot support this conclusion.

3.4. Change of free amino acids during fermentation

The changes of the concentration of the individual free amino acids (FAA) and the content of total free amino acids (TFAA) during fermentation of RSM and irrRSM with ST12 at an inoculation rate of 10^5 cfu mL^{-1} are summarised in Table 2 and Table 3, respectively.

The total concentration of the amino acids in irrRSM was about 10% higher than in RSM (657 and 585 μmol L^{-1}, respectively) in the beginning of fermentations, indicating that irradiation has led to hydrolysis of proteins in milk powder. This is supporting the idea that increased content of peptides and amino acids in the irrRSM was leading to the disappearance of the diauxic growth.

Glutamic acid was the dominant amino acid (46% of the total) initially in both types of milk samples; however, its concentration was remarkably higher in irrRSM – 306 μmol L^{-1} in comparison with 270 μmol L^{-1} in RSM. Higher content of other amino acids (histidine, glutamine, glycine, aspartic acid, alanine, leucine and phenylalanine) in irrRSM compared with RSM was also observed. In contrast, asparagine, ornithine, cysteine, methionine and tryptophane concentrations were lower in irrRSM than in RSM.

A period from 3 h to 5 h of incubation in RSM and from 5 to 7 h in irrRSM was characterised by the decrease of TFAA. The largest decrease was observed in the concentration of asparagine, glycine, valine and leucine in RSM and glutamic acid in irrRSM, which decreased continuously during the fermentation. Glycine was fully depleted at the 7th h of fermentation in irrRSM.

During the next period (approximately from 5–7 h–22 h) the intensive liberation of the majority of amino acids was observed and the content of TFAA increased significantly at 22 h of fermentation to 1713 μmol L^{-1} and 910 μmol L^{-1} in RSM and in irrRSM, respectively. The quantitatively dominating amino acids in the RSM at the end of the fermentations were glutamic acid (374 μmol L^{-1}), proline (201 μmol L^{-1}) and lysine (160 μmol L^{-1}); whereas proline (215 μmol L^{-1}) and alanine (156 μmol L^{-1}) were dominating in irrRSM. High aspartic acid, leucine and valine concentrations were also measured at 22 h of fermentation in RSM (137, 125 and 107 μmol L^{-1}, respectively), in contrast to irrRSM, where concentrations of these FAA were much lower (23, 58,

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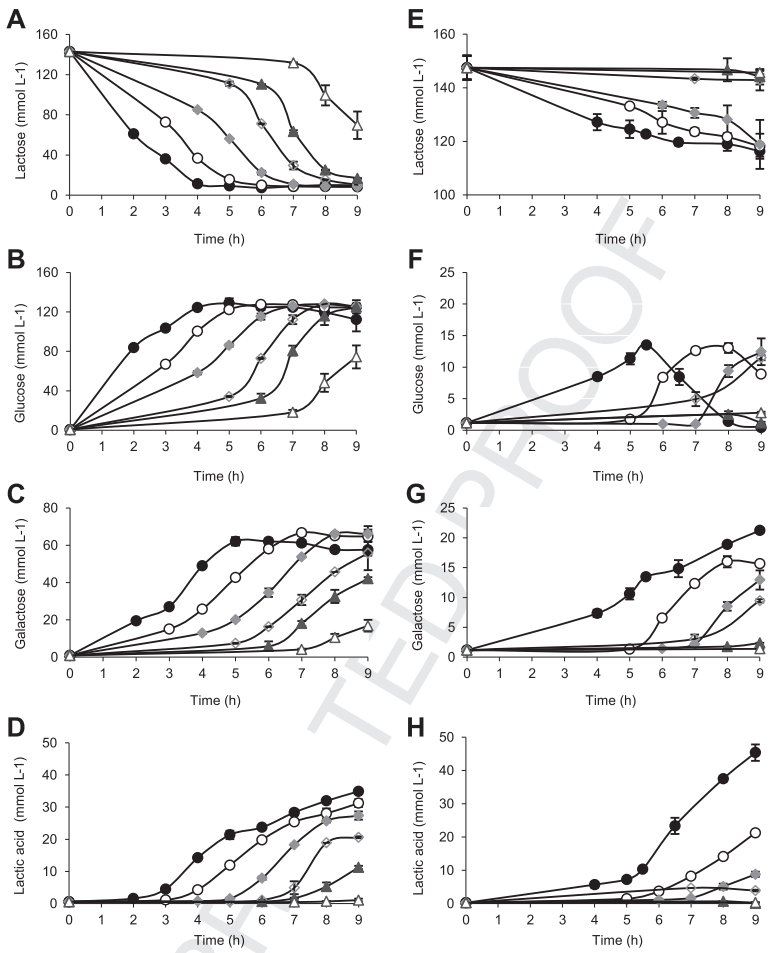

Fig. 4. Comparison of lactose (A, E), glucose (B, F), galactose (C, G) and lactic acid (D, H) profiles during growth of *Streptococcus thermophilus* ST12 in (A, B, C, D) reconstituted skim milk powder (RSM) and (E, F, G, H) irradiated RSM at different inoculation rates (cfu mL^{-1}): ●, 10^6; ○, 10^5; □, 10^4; ◇, 10^3; ▲, 10^2; △, 10^1.

19 μmol L^{-1}, respectively). Ornithine was found at very low concentrations, and despite the fact that the average concentration of this amino acid increased continuously throughout the fermentations, it represented less than 1% of the TFAA content in both growth media.

Summarising, if the pH (5.56 ± 0.03 and 4.41 ± 0.02), the concentration of lactic acid (69 ± 4 and 86 ± 4 mmol L^{-1}), and the total concentration of free amino acids (1713 ± 108 and 910 ± 36 μmol L^{-1}) at the end of the growth in RSM and irrRSM, respectively, are compared for RSM and irrRSM, they were all different, and it was not possible to find a "common denominator" determining the end of the growth of the culture.

As mentioned above irradiation led to the increase of concentration of free amino acids in irrRSM, whereas the amount of TFAA at the end of fermentation (after 22 h incubation) was higher in RSM. This observation is not in accordance with the data found in the literature – no significant differences in the amino acid fraction of the reconstituted milk samples were noted by Favrot and Maubois (1994) in case of 20 kGy irradiation experiment.

3.5. Dynamic rheological measurements

Growth of the bacteria in the RSM and irrRSM samples led to coagulation. The development of storage (G′) and loss (G″) moduli during fermentation in both media with ST12 at inoculation rate 10^5 cfu mL^{-1} is shown in Fig. 5.

Samples of RSM gelled after somewhat longer time (6.9 ± 0.5 h) than irrRSM (6.3 ± 0.4 h), but with a higher gel firming rate (63.4 ± 6.2 Pa h^{-1} and 59.4 ± 4.6 Pa h^{-1} in RSM and irrRSM, respectively), and resulted in firmer gel (186 ± 9 Pa and 177 ± 4 Pa in RSM and irrRSM, respectively).

Gelation pH was near 6.3 and 6.1 respectively for RSM and irrRSM samples inoculated with ST12 at 10^5 cfu mL^{-1}. It can be seen from the data obtained, that gelation is taking place after the exponential growth (Fig. 2).

The data obtained indicated that the gelation as the results of fermentation was presumably not caused by a 'simple' acidification but possibly also by enzymatic processes with participation of bacterial proteases.

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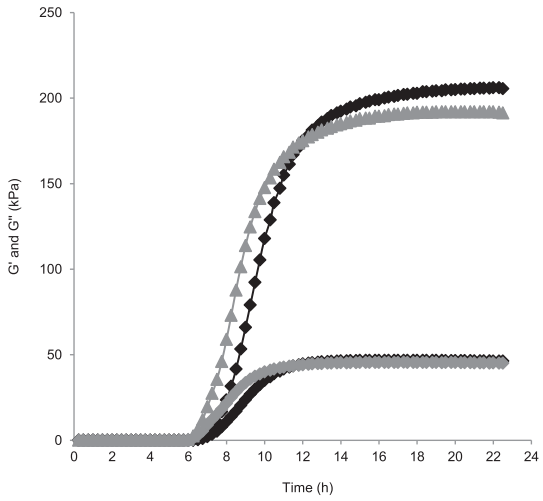

Fig. 5. Development of storage (G′; diamonds) and loss (G″; triangles) moduli during fermentation of reconstituted skim milk powder (RSM; black symbols) and irradiated RSM (grey symbols) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}.

3.6. Monitoring of fermentation by front-face fluorescence spectroscopy

The development of tryptophan fluorescence intensity at maximum emission (285/330 nm) recorded continuously with 15 min interval during fermentation of RSM and irrRSM with ST12 at inoculation rate 10^5 cfu mL^{-1} is shown in Fig. 6. Changes in tryptophan fluorescence intensity profiles of RSM and irrRSM milk during fermentation were very similar, except for the fact that changes in fluorescence intensity occurred much faster in irrRSM, which is in line with the faster drop of pH in irrRSM sample. Changes in tryptophan fluorescence intensity at maximum emission corresponded presumably to the modifications of the environment of the tryptophan residues caused by pH decrease during acidification; whereas changes in protein network associated with gelation phenomenon were not clearly distinguishable. The gelation points of RSM and irrRSM determined by rheological measurements (marked in Fig. 6) did not coincide with the same specific point on tryptophan fluorescence intensity change profile, but were different for the two milk samples. Change of tryptophan fluorescence intensity during fermentation with ST12 was similar to that shown in the case of milk acidified by glucono-δ-lactone (Lopez & Dufour, 2001).

The SFS spectral data recorded at 15 min intervals during fermentation of RSM and irrRSM with ST12 at an inoculation rate of 10^5 cfu mL^{-1} were pooled in one matrix and analysed by PCA. The similarity map (Fig. 7) defined by the principal components 1 and 2 (PC1, PC2) fully discriminated two systems along PC1 axis. Changes of SFS in time were observed along PC1 and PC2 axes. Two phases with turning point at pH 5.6–5.7 and pH 6.1 in the case of RSM and irrRSM, respectively, were observed on the similarity map. However, the layout of the scores on both axes during the time span of fermentations of RSM and irrRSM samples followed rather different profiles. The data show that the turning point, separating two phases on the similarity map, did not necessarily correspond to the gelation point. Two-phase change of tryptophan emission spectra during lowering pH until 4.5 during acidification induced by glucono-δ-lactone or yoghurt starter has previously been observed by Herbert, Riaublan, Bouchet, Gallant, and Dufour (1999) and Laligant, Famelart, Paquet, and Brulé (2003).

However, in contrast to the above cited studies, no shift in tryptophan emission was observed in the results presented here, possibly due to lower resolution spectra used in the present study (5 nm). On the contrary, the discrimination of the SFS spectra during fermentation was associated with PC1 and PC2 due to specific λ_{ex} of tryptophan fluorescence peak at maximum λ_{em} of near 330 nm. Considering PC1, fluorescence intensity increased after excitation at 295–300 nm during the first phase of fermentation, and after the

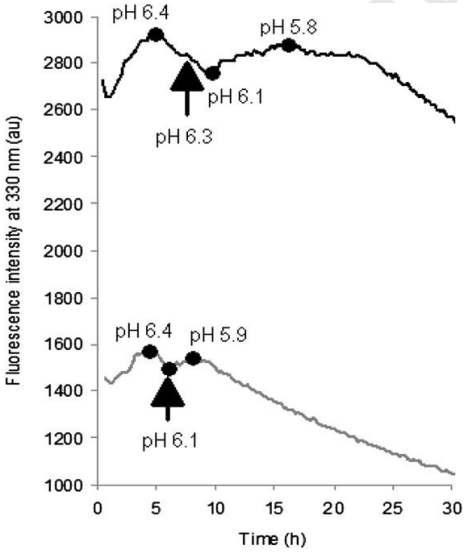

Fig. 6. Development of fluorescence intensity at 285/330 nm during fermentation of reconstituted skim milk powder (RSM; black line) and irradiated RSM (grey line) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}. Arrows mark the mean gelation times determined rheologically.

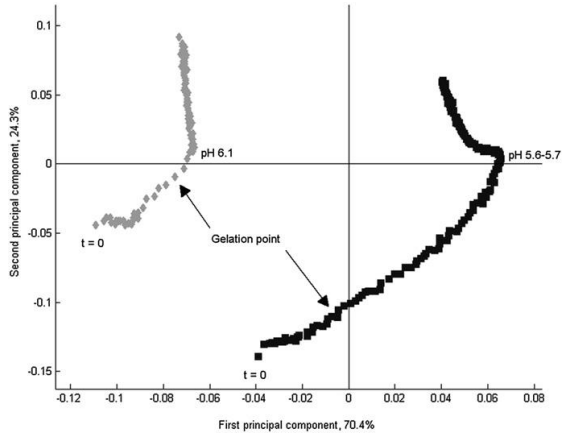

Fig. 7. Principal component analysis map defined by two first principal components for spectral fluorescence signatures data recorded during fermentation of reconstituted skim milk (RSM; black squares) and irradiated RSM (grey diamonds) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}. Arrows mark the mean gelation times determined rheologically.

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turning point a change in the opposite direction was observed. Considering PC2, fluorescence intensity mainly decreased after excitation near 275 nm during the time course of fermentation.

Previously, tryptophan fluorescence signal measured in processed cheese samples was reported to be composed of two tryptophan components with $\lambda_{ex}/\lambda_{em}$ of 300/347 and 280/339 nm, which were assumed to the fluorophores in different protein structures (Christensen, Povlsen, & Sørensen, 2003). Similar to what was found by Christensen et al. (2003), two types of tryptophan residues could be differentiated in milk during fermentation based on PC1 and PC2 loadings. From the data obtained it can be concluded that depending on milk sample used as a substrate for ST12 growth the comparable reduction of pH can lead to different changes in protein structures depending on the history of the samples.

4. Conclusions

The present work is, to our knowledge, the first extensive study of the influence of irradiation treatment on the skim milk powder. Processing of low-heat skim milk powder with gamma irradiation resulted in addition to the visible modification in colour and odour also differences in pH, free amino acids content, SFS spectra as well as substantial differences in fermentation patterns of irradiated reconstituted milk fermented by *S. thermophilus* ST12 and in dynamic viscoelastic properties of gels. To the best of our knowledge, the study carried out was also the first systematic microcalorimetric study of the growth of a LAB in reconstituted milk. The data obtained showed that use of microcalorimetry together with the determination of metabolites is a powerful combination for the detailed study of growth of anaerobic bacteria and of bacterial acidification processes in milk. The results of the study also clearly indicated that further detailed studies would be needed for the elucidation of the mechanisms of acidification and coagulation processes, for the careful assessment of the consequences of the use of sterilising gamma irradiation of skim milk powder.

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2000-2004 Tallinn University of Technology, bio- and food technology, BSc
1988-2000 Tallinn High School of Humanities

4. Language skills

Russian	mother tongue
Estonian	fluent
English	fluent
German	basic skills
Lithuanian	basic skills

5. Special Courses

19-24.08.2007 PhD course „Ripening of Northern European Cheeses", University of Copenhagen, Denmark
21-25.08.2006 PhD course „From Milk to Cheese", Norwegian University of Life Sciences, Norway
28.03-8.04.2004 Socrates/Erasmus Intensive Course "Food Safety", Larenstein University of Professional Education, the Netherlands

6. Professional Employment

2005-… Competence Centre of Food and Fermentation Technologies, researcher

7. Defended theses

Master thesis: Development of Front-Face Fluorescence Spectroscopy for Studying Milk Renneting – Monitoring Influence of Strong Oxidants
Bachelor thesis: Influence of Protein Composition and pH on Coagulation Properties of Milk

ELULOOKIRJELDUS

1. Isikuandmed

Ees- ja perekonnanimi	Tiina Kriščiunaite
Sünniaeg	13.08.1982
Sünnikoht	Tallinn
Kodakondsus	Eesti

2. Kontaktandmed

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3. Hariduskäik

2006-…	Tallinna Tehnikaülikool, keemia ja geenitehnoloogia doktoriõpe
2004-2006	Tallinna Tehnikaülikool, bio- ja toiduainetehnoloogia magistriõpe
2000-2004	Tallinna Tehnikaülikool, bio- ja toiduainetehnoloogia bakalaureuseõpe
1988-2000	Tallinna Humanitaargümnaasium

4. Keelteoskus

Vene keel	emakeel
Eesti keel	kõrgtase
Inglise keel	kõrgtase
Saksa keel	algtase
Leedu keel	algtase

5. Täiendusõpe

19-24.08.2007 PhD kursus „Põhja-Euroopa juustude valmimine”, Kopenhaageni Ülikool, Taani

21-25.08.2006 PhD kursus „Piimast juustuni”, Norra Maaülikool, Norra

28.03-8.04.2004 Socrates/Erasmus intensiivkursus "Toiduohutus", Larenstein Ülikool, Holland

6. Teenistuskäik

2005-… Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, teadur

7. Kaitstud lõputööd

Magistritöö: Uue front-face fluorestsentsmeetodi väljatöötamine piima kalgendatavuse hindamiseks – tugevate oksüdeerijate mõju uurimine

Bakalaureusetöö: Piima kalgendumis- ja hapnemisomaduste sõltuvus valgulisest koostisest ja pH-st

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

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