THESIS ON NATURAL AND EXACT SCIENCES B160

The Effects of Milk Composition and Treatment on the Growth of Lactic Acid Bacteria

IRINA STULOVA

TUT PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

This dissertation was accepted for the defence of the degree of Doctor of Philosophy (in Chemistry) on 19 June 2013

Supervisor: Professor Raivo Vilu
Chair of Biotechnology
Tallinn University of Technology

Reviewed by: Professor Toomas Paalme
Department of Food Processing
Tallinn University of Technology

Opponents: Dr. Olivier Braissant
Senior scientist
Biomechanics & Biocalorimetry Center Basel
University of Basel, Switzerland

Dr. Inga Ciprovič a
Professor, Faculty of Food Technology
Latvia University of Agriculture, Latvia

Defence of the thesis: 25 September 2013

Declaration:

Hereby I declare that this doctoral thesis, submitted for the doctoral degree at TUT, is my original investigation and achievement and has not been submitted for the defence of any other academic degree elsewhere.

Irina Stulova

ISSN 1406-4723
ISBN 978-9949-23-543-8 (publication)
ISBN 978-9949-23-544-5 (PDF)

LOODUS- JA TÄPPISTEADUSED B160

Piima koostise ja töötlemise mõju piimhappebakterite kasvule

IRINA STULOVA

TTÜ
KIRJASTUS

CONTENTS

LIST OF PUBLICATIONS

The dissertation is based on the following publications, referred to in the text by the Roman numbers I – IV and listed in the appendices with permission from the publishers.

I. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Laht, T.-M., Vilu, R. 2011. The effect of milk heat treatment on the growth characteristics of lactic acid bacteria. *Agronomy Research*, 9, 473–478

II. Kriščiunaite, T., **Stulova, I.**, Kabanova, N., Laht, T.-M., Vilu, R. 2011. 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

III. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Laht, T.-M., Vilu, R. 2013. Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder. *International Dairy Journal,* 31(2), 139–149

IV. **Stulova, I.**, Adamberg, S., Kriščiunaite, T., Kampura, M., Blank, L., Laht, T.-M. 2010. Microbiological quality of raw milk produced in Estonia. *Letters in Applied Microbiology*, 51 (6), 683–690

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

In **Publication I**, the author planned and performed the experimental work, interpreted the data, and wrote the manuscript.

In **Publication II**, the author participated in the experimental work and was a co-author of the publication.

In **Publication III**, the author planned and performed the experimental work, calculated and interpreted the data, and wrote the manuscript.

In **Publication IV**, the author participated in planning the study, was responsible for the experimental work (microbiological evaluation of milk quality), interpreted the data, and wrote the manuscript.

OTHER PUBLICATIONS IN RELATED FIELD

V. Kabanova, N., **Stulova, I.**, Vilu, R. 2013. Microcalorimetric study of growth of *Lactococus lactis* IL1403 at low glucose concentration in liquids and solid agar gels. *Thermochimica Acta*, 559, 69–75

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

VII. Kabanova, N., **Stulova, I.**, Vilu, R. 2012. Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels. *Food Microbiology*, 29 (1), 67–79

VIII. Kabanova, N., Kazarjan, A., **Stulova, I.**, Vilu, R. 2009. Microcalorimetric study of the growth of *Lactococcus lactis* IL1403 in broth. *Thermochimica Acta*, 496, 87–92

LIST OF CONFERENCE PRESENTATIONS

1. **Stulova, I.**, Laht T.-M. Isolation and preliminary characterization of psychrotrophic and spore-forming bacteria from Estonian farm milk. *NordOst I workshop: Potential of Lactobacillus helveticus and Lactobacillus paracasei group in Northen European cheeses. Indigenous bioactive compounds, Denmark, Copenhagen, 6–7 November 2005 (oral presentation)*

2. **Stulova, I.**, Siegumfeldt, H., Ardö, Y. Microscopical interactions between *Lactobacillus paracasei* and *Lactococcus lactis. NordOst II workshop: Potential of Lactobacillus in Northern European cheeses. Bioactive compound, Finland, Viiki, 19–20 June 2006 (oral presentation)*

3. **Stulova, I.**, Kazarjan, A., Vilu, R. Microcalorimetric investigation on the submerged bacterial growth. *XXVIII National Conference on Calorimetry, Thermal Analysis and Chemical Thermodynamics, Italy, Milan, 11–15 December 2006 (poster presentation)*

4. **Stulova, I.**, Kazarjan, A., Kriščiunaite, T., Laht, T.-M., Vilu, R. Milk thermal treatment: influence on lactic starters growth. *Symposium „Potential of Lactobacillus in Northern European cheeses", Estonia, Tallinn, 23–25 April 2007 (oral presentation)*

5. **Stulova, I.**, Kampura, M., Blank, L, Laht., T.-M. Microbial characterization of Finnish and Estonian open texture cheeses: manufacturing and ripening. *NordOst Symposium „Health Aspects of Cheese", Norway, Drøbak, 6–8 October 2009 (oral presentation)*

6. **Stulova, I.**, Taivosalo, A., Blank, L., Laht, T-M. Characterisation of the dynamics of the lactic acid bacterial population of Finnish and Estonian open texture cheeses. *22nd International ICFMH Symposium FoodMicro 2010, Denmark, Copenhagen, 30 August–02 September 2010 (poster presentation)*

7. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Taivosalo, A., Laht, T.-M, Vilu, R. A microcalorimetric study of the growth of *Streptococcus thermophilus* ST12 in irradiated milk and rennet curd. *10th Symposium on Lactic Acid Bacteria, Holland, Egmond aan Zee, 28 August–01 September 2011 (poster presentation)*

8. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Laht, T.-M., Vilu, R. The effect of milk heat treatment on the growth characteristics of lactic acid bacteria. *Conference Food/Feed Quality, Safety and Risks in Agriculture, Tallinn, Estonia, 25–28 October, 2011 (poster presentation)*

9. **Stulova, I.** Application of isothermal microcalorimetry in dairy microbiology. *International seminar „New methods in assessment of food/feed quality and safety", Saku, Estonia, 17–18 January, 2012 (oral presentation)*

10. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Taivosalo, A., Laht, T.-M, Vilu, R. Growth of thermophilic starter bacteria in non-irradiated and irradiated reconstituted milk. *23rd International ICFMH Symposium FoodMicro 2012, Global Issues in Food Microbiology, Istanbul, Turkey, 03–07 September 2012 (poster presentation)*

11. **Stulova, I.**, Kabanova, N., Kriščiunaite, T., Laht, T.-M, Vilu, R. Characterization of growth of lactic acid bacteria in milk: microcalorimetric approach. *1st Congress of Baltic Microbiologists, Riga, Latvia, 31 October–04 November 2012 (oral presentation)*

OTHER PUBLICATIONS & CONFERENCE PRESENTATIONS

Orav, A., Haab, K., **Stulova, I.** The effect of drying method and grade of grinding to the composition of the essential oil from black, green and white pepper (*Piper nigrum L.*) *27th Estonian Chemistry Days*, *Estonia, Tallinn, 15–16 November 2001*

Stulova, I., Orav, A. Effect of storage of spices on the essential oil composition. *28th Estonian Chemistry Days, Estonia, Tallinn, 20–22 November 2002*

Orav, A., **Stulova, I.**, Kailas T., Müürisepp, M. (2004) Essential oil composition of *Piper nigrum* L. fruits of different ripening states. *Journal of Agricultural and Food Chemistry*, 52 (9), 2582–2586

Laht, T.-M., **Stulova, I.** Possible mechanisms of liberation of bioactive peptides during digestion of caseins in vivo and in ripening cheese by indigenous milk proteinases. *Conference „Milk products and components in health and disease", Island, Blue Lagoon, 20–23 August 2005*

Menert A., Kazarjan A., **Stulova I.**, Lee C.C., Vilu R. Microcalorimetric monitoring of microbial growth in solid-state fermentations. *XIVth International Society for Biological Calorimetry Conference, Poland, Sopot, 2–6 June 2006*

Kriščiunaite, T., Laht, T.-M., **Stulova, I.** Cold storage and mechanical treatment affects renneting properties of milk. *2nd Baltic Conference on Food Science and Technology: FoodBalt-2007, Lithuania, Kaunas, 13–14 June 2007*

Kriščiunaite, T., **Stulova, I.**, Laht, T.-M. The effect of hydrogen peroxide on the growth characteristics of thermophilic lactic starters and rheological properties of acid milk gels. *Symposium „Potential of Lactobacillus in Northern European cheeses", Estonia, Tallinn, 23–25 April 2007*

Kampura, M., **Stulova, I.**, Laht, T-M. *Pseudomonas* spp in Estonian farm milk. *The 21st International ICFMH Symposium „Evolving microbial food quality and safety", FoodMicro 2008, Scotland, Aberdeen, 1–4 September 2008*

Siegumfeldt, H., **Stulova, I.**, Ryssel, M., Ardö, Y. A Microscope Chamber System to Study Microbial Interactions and Cell Death in Cheese. *5th IDF Symposium on cheese ripening, Swizerland, Bern, 9–13 March 2008*

Kabanova, N., **Stulova I.**, Kriščiunaite T., Vilu R. Microcalorimetry allows to elucidate effectively quantitative peculiarities of growth of bacteria in colonies of solid state matrices – study and optimize solid-state fermentation processes. *Recent Advances in Fermentation Technology (RAFT VIII*), *San Diego, CA, USA, November 2009*

Kabanova, N., **Stulova, I.**, Kriščiunaite, T., Vilu, R. Microcalorimetry allows studying and optimizing solid-state fermentation processes and explaining the quantitative peculiarities of growth of bacteria in colonies of solid state matrices. *22nd International ICFMH Symposium FoodMicro 2010, Denmark, Copenhagen, 30 August–02 September 2010*

Kabanova, N., **Stulova, I.**, Vilu, R. Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in solid matrices. *10th Symposium on Lactic Acid Bacteria, Holland, Egmond aan Zee, 28 August–01 September 2011*

Blank, L., Panke, M., **Stulova, I.**, Adamberg, S., Laht, T.-M. Dynamics of lactic acid bacteria throughout the ripening of semi-hard open structure cheese. *10th Symposium on Lactic Acid Bacteria, Holland, Egmond aan Zee, 28 August–01 September 2011*

Kabanova, N., **Stulova, I.**, Kriščiunaite, T., Taivosalo, A., Vilu, R. Microcalorimetric study of the growth of *Streptococcus thermophilus* ST12 in liquid and solid media. *1st Central and Eastern European Conference on Thermal Analysis and Calorimetry, CEEC-TAC, Craiova, Romania, 7–10 September, 2011*

Blank, L., Panke, M., **Stulova, I.**, Saareleht, J., Laht, T.-M. Dynamics of lactic acid bacteria and flavour formation during ripening of hard cheese, *6th IDF Cheese Ripening &Technology Symposium*, *Madison, USA, 21–24 May, 2012*

Kabanova, N., **Stulova, I.**, Vilu, R. Microcalorimetric study of the growth of bacterial colonies of *Lactococus lactis* IL1403 in gelatine matrices. *23rd International ICFMH Symposium FoodMicro 2012, Global Issues in Food Microbiology, Istanbul, Turkey, 03–07 September 2012*

ABBREVIATIONS

ANOVA	analysis of variance
ASFBC	aerobic spore-forming bacteria count
B.	*Bifidobacterium*
CCP	colloidal calcium phosphate
CFU	colony forming unit
CEP	cell wall-bound extracellular proteinase
Fischer´s LSD	*Fischer´s* least significant difference test
G´	elastic or storage modulus
G´´	viscous or loss modulus
GRAS	Generally Regarded as Safe
EPS	exopolysaccharides
HPLC	high-performance liquid chromatography
irrLHSMP	irradiated LHSMP
irrRSM	RSM prepared from irrLHSMP
IC	isothermal calorimeter
IMC	isothermal microcalorimetry
kGy	kiloGrey
L.	*Lactococcus*
LAB	lactic acid bacteria
LABC	lactic acid bacteria count
Lb.	*Lactobacillus*
LHSMP	low-heat skim milk powder
Leuc.	*Leuconostoc*
MRS	agar growth media that favours the growth of *Lactobacilli*, developed in 1960 by de Man, Rogosa and Sharpe
N	number of bacteria
N_{exp}	number of bacteria produced during the exponential phase
N_{tot}	number of bacteria produced at the end of growth
P_{max}	maximum heat flow
PBC	psychrotrophic bacteria count
Q_{exp}	amount of heat produced during the exponential growth phase
Q_{tot}	amount of heat produced during the whole cultivation period
RSM	reconstituted skim milk
r-RSM	RSM with rennet addition
r-irrRSM	irrRSM with rennet addition
SCC	somatic cell count
SD	standard deviation

SFS	spectral fluorescence signatures
St.	*Streptococcus*
subsp.	subspecies
TAMIII	Thermal Activity Monitor III
TBC	total bacterial count
t_d	doubling time
t_{Pmax}	time of the maximum heat production rate
UHT	ultra-high treated
UPLC	ultra-performance liquid chromatography
var.	varietas
Y_Q	yield coefficient
α-La	*alpha*-lactalbumin
β-Lg	*beta*-lactoglobulin
κ-casein	*kappa*-casein
μ_{max}	maximum specific growth rate
μ_{max1}	maximum specific growth rate of the first exponential phase
μ_{max2}	maximum specific growth rate of the second exponential phase
λ	lag phase

INTRODUCTION

Carefully selected strains of different species of lactic acid bacteria (LAB) – starter cultures – are added to milk to initiate the processes of manufacturing most fermented dairy products. The major function of starter bacteria is to produce lactic acid and, in some cases, flavour compounds. It is generally accepted that three main factors determine the properties of fermented dairy foods: the quality of raw milk, the pretreatment of the milk, and the fermentation process by the starter bacteria.

The microbiological quality of raw milk is assessed, to a large extent, by total bacterial count and somatic cell count, and these parameters are routinely measured in milk grading. Very little attention has been paid to the control of predominant bacterial groups, such as lactic acid bacteria, psychrotrophic bacteria and spore-forming bacteria, in milk at the farm level. High hygienic standards coupled with rapid cooling applied to raw milk on modern farms gives growth advantages to psychrotrophic bacteria, including the most common spoilage species *Pseudomonas* spp., and suppresses indigenous lactic acid bacteria. The presence of even a small number of proteolytic psychrophilic bacteria can lead to the significant proteolysis of caseins, and therefore to changes in the growth conditions of bacteria in milk.

Most industrial dairy products are produced from milk that has been processed prior to starter addition, or at least heat-treated. Therefore, it is crucial to understand the influence of different treatments on the composition and properties of milk and, as a result, on the growth of starter bacteria.

There is a growing demand for comprehensive/systematic studies of the metabolic activities of LAB. Industrial-level food fermentations should be fully standardised and easy to control. To optimize bacterial growth resulting in high quality stable products, the influence of environmental factors on microbial physiology can be elucidated using a systems biology approach.

The growth of thermophilic starter bacteria in milk was studied in this thesis using a precise and sensitive method: isothermal microcalorimetry. The effects of heat treatment, milk composition, irradiation and rennet coagulation on the growth characteristics of *St. thermophilus* were analysed. The data obtained provide valuable information about the metabolic capacities of lactic acid bacteria, and can be used for the optimisation of dairy fermentations.

1. LITERATURE REVIEW

1.1 Lactic acid bacteria

The term Lactic Acid Bacteria (LAB) was gradually accepted at the beginning of the 20th century and was first differentiated in the pioneering systematization of Orla-Jensen (published in 1919). The classification of genera of LAB has been based on morphology, mode of glucose fermentation, growth at certain temperatures, and range of sugar utilization (Khalid, 2011). There are currently 12 genera of LAB, of which four – *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus* – are commonly used as dairy starter cultures (Leroy & De Vuyst, 2004). These cultures are called starters because they initiate the production of acid in dairy products. The types of starters used for different dairy products are listed in Table 1.

Table 1. Dairy products and lactic acid bacteria used for their production (Leroy & De Vuyst, 2004)

Type of fermented product	**Lactic acid bacteria**[a]
Hard cheeses without eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*
Cheeses with small eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
Swiss- and Italian-type cheeses	*Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *St. thermophilus*
Butter and buttermilk	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus*, *St. thermophilus*
Fermented, probiotic milk	*Lb. casei*, *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. johnsonii*, *B. lactis*, *B. bifidum*, *B. breve*
Kefir	*Lb. kefir*, *Lb. kefiranofacies*, *Lb. brevis*

[a] *B. = Bifidobacterium*; *L. = Lactococcus*; *Lb. = Lactobacillus*; *Leuc. = Leuconostoc*; *St. = Streptococcus*

All LAB used in starter cultures are Gram-positive, catalase-negative, non-motile, acid-tolerant, non-sporulating and facultative anaerobic microorganisms (Savijoki et al., 2006). LAB include both homofermentative, producing mainly lactic acid, and heterofermentative species, which, apart from lactic acid, yield a large variety of other fermentation products, such as acetic acid, ethanol, carbon dioxide and formic acid (McDonald et al., 1987). Additionally, LAB used as dairy starter cultures perform a number of other important functions, such as the production of flavour compounds, enzymes and other products that have profound effects on the texture and body characteristics of cheese and cultured milk (Leroy & De Vuyst, 2004; Routray & Mishra, 2011).

These bacteria also differ in their optimal temperatures for growth: 20–30°C as the optimal temperature for mesophilic bacteria and 35–45°C as the optimal temperature for thermophilic bacteria (Table 2). Mesophilic starters are used in the production of many cheese varieties, fermented milk products, and ripened cream butter (Table 1). Thermophilic starters are used for yoghurt and cheese varieties with high cooking temperatures, such as Emmental, Gruyère, Comte, and such Italian cheeses as the blue-veined Gorgonzola and the hard cheese Grana (Iyer et al., 2010).

Table 2. Characteristics of lactic acid bacteria used in fermented dairy products (Walstra et al., 1999)

	Morphology	Lactose fermentation	Temperature (C)			Primary metabolic products
			Min	Opt	Max	
L. lactis subsp. *lactis*	cocci	homo-fermentative	8–10	28–32	40	lactate
L. lactis subsp. *cremoris*	cocci	homo-fermentative	8–10	22	37–39	lactate
St.thermophilus	cocci	homo-fermentative	20	40	50	lactate, acetaldehyde
Leuconostoc mesenteroides subsp. *cremoris*	cocci	hetero-fermentative	4–10	20–25	~37	lactate, diacetyl, carbon dioxide
Lb. delbrueckii subsp. *bulgaricus*	rods	homo-fermentative	22	40–45	52	lactate, acetaldehyde
Lb. helveticus	rods	homo-fermentative	20–22	42	54	lactate
Lb.delbrueckii ssp.*lactis*	rods	homo-fermentative	18	40	50	lactate
Lb.acidophilus	rods	homo-fermentative	20–22	37	45–48	

1.1.1 Yoghurt starter cultures

Thermophilic lactic starters generally contain *St.thermophilus* and one or several lactobacilli (*Lb. bulgaricus*, *Lb. helveticus*).

St. thermophilus is a dairy bacterium that has been consumed for centuries, is in the European Qualified Presumption of Safety list of food bacteria and is generally recognized as a safe species (GRAS status) (Iyer et al., 2010; Goh et al., 2011). Morphologically, *St. thermophilus* cells are spherical to ovoid, 0.7–0.9 μm in diameter, and grow in pairs to long chains (10-20 cells). *St. thermophilus* has a high optimum growth temperature: it grows best at about 42–45°C. It can be isolated from milk. Because *St. thermophilus* can survive moderate thermal processes (60°C for 30 min), it is often found in milk pasteurization equipment and in pasteurized dairy products.

The genus *Lactobacillus* consists of a genetically and physiologically diverse group of rod-shaped lactic acid bacteria. The genus can be divided into three groups based on fermentation end-products. Obligately homofermentative lactobacilli (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. helveticus*) commonly found in starter cultures grow at higher temperatures (>45°C) than other lactobacilli and are thermoduric. Facultatively heterofermentative lactobacilli, such as *Lb. casei*, carry out secondary fermentation during cheese ripening. Obligately heterofermentative lactobacilli (*Lb. brevis, Lb. buchneri, Lb. fermentum* and *Lb. reuteri*) are not important as dairy starter cultures, except for kefir production (Beresford et al., 2001).

Lactobacilli are the most acid tolerant of the lactic acid bacteria, preferring to start growing at acidic pH (5.5–6.2) and lowering the pH of milk to below 4.0 (Hassan & Frank, 2001). Lactobacilli grow slowly in milk in pure cultures and they are generally used in combination with *St. thermophilus*. Several explanations have been proposed to account for this "symbiotic growth behaviour" (Zourari et al., 1992). The lactobacilli are generally more proteolytic than *St. thermophilus*; thus in dairy fermentations their extracellular proteinases hydrolase casein and other milk proteins and thereby provide *St. thermophilus* with necessary amino acids. In contrast, *St. thermophilus* produce small amounts of formic acid that may stimulate *Lb. delbruecki* subsp. *bulgaricus*. The production of CO_2 from urea by *St. thermophilus* may also enhance the growth of lactobacilli (Pernoud et al., 2004). Finally, the excretion of galactose by most strains of *St. thermophilus* may provide galactose-fermenting lactobacilli with a source of fermentable carbohydrate when lactose is not available (Zourari et al., 1992).

The role of thermophilic lactic starters in milk fermentations is two-fold. Firstly, they transform lactose to lactic acid, thus lowering the pH of milk or cheese curd. This step is essential for yoghurt production in which the final pH is below 4, as it prevents the development of spoilage microorganisms and possible pathogens. In the case of cooked cheeses, acidification contributes to syneresis.

After pressing, the cheese has a low water content and a pH sufficiently low to ensure and support the long ripening period. Secondly, they contribute to the organoleptic qualities of the final product. In the case of yoghurt, this role is particularly important, as the consistency and flavour of the product depend on the metabolism of the lactic starters. Many *St. thermophilus* strains synthesize exopolysaccharides (EPS) that contribute to the desirable viscous texture and rheological properties of fermented milk products (Vaningelgem et al., 2004; Nordmark et al., 2005). EPS yields achieved in controlled fermentations (45–600 mg L^{-1}) depend on strain and fermentation parameters. For "real commodities", such as yoghurt, traditionally manufactured with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, the EPS content is 13–170 mg L^{-1} (Mende et al., 2013).

1.1.2 LAB as indigenous microflora of raw milk

LAB are widely distributed in nature and are generally considered to be indigenous microflora of raw milk (Singh & Sharma, 2009). They are found in the dairy farm environment and in decomposing vegetation, including silage. Some species colonize animal digestive organs, including the mouth and intestine. They are also part of the microflora of the streak canal of the mammary gland (Hayes & Boor, 2011).

LAB are present in all raw milk samples with a mean value of $5 \times 10^2 – 8 \times 10^2$ CFU mL^{-1} (Desmasures et al., 1997a; Espeche et al., 2012). Representatives of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* are usually found (Lafarge et al., 2004; Giannino et al., 2009). *Lactococcus lactis* has been confirmed to be a major raw milk species (Lafarge et al., 2004). As was shown by Desmasures (1997b), average counts of *Lactococcus* spp. are 3.4×10^2 CFU mL^{-1} in milk, 2.6×10^3 CFU mL^{-1} per total area on teats and 7.7×10^2 per 25 cm^2 on udders.

Populations of LAB in raw milk have decreased over the last 20 years (Champagne et al., 1994). The decrease in the number of LAB, together with other contaminating bacteria in raw milk, is probably the most significant change in milk quality caused by technological progress (Guessas & Kihal 2004).

1.2 Milk as a growth medium for LAB

The production of fermented foods is based on the use of LAB (added to milk as "starter" cultures), which initiate rapid acidification of milk. To perform this function, LAB must grow to high numbers, from $\sim 1 \times 10^6$ CFU mL^{-1} in inoculated milk to $\sim 1 \times 10^9$ CFU mL^{-1} in end-products (yoghurt and cheese).

Milk is a suitable growth medium for LAB because of the variety of substrates available for fermentation (carbohydrates and proteins), as well as due to the presence of such growth stimulants as vitamins and minerals (Mäyrä-

Mäkinen & Birget, 2004). Nitrogen in milk occurs in forms which are readily available for microbial metabolism. These include free amino acids (0.4%), urea (1.2%) and ammonia (0.6%), which together represent 2.0% of the total nitrogen in milk (Frank & Marth, 1999). The amount of these compounds is sufficient for the initiation and support of only 25% of the maximum final cell densities of LAB in the case of optimal nutrition (Fox et al., 2000).

1.2.1 Main components of milk

Milk is a complex biological fluid consisting of seven main components: water, sugar (lactose), protein, fat, minerals, vitamins and enzymes. The most abundant component of milk is water, which comprises about 87% of its content. The following is a short overview of the main components of cow's milk.

Carbohydrates. The principal fermentable substrate, lactose, is the only carbohydrate existing in sufficient quantities in milk to support microbial growth. Milk contains only trace amounts of other sugars, including glucose (50 mg L^{-1}), fructose, glucosamine, galactosamine, neuraminic acid and neutral acidic oligosaccharides (Fox et al., 2000). Bovine milk contains about 4.8% lactose (from 45 to 50 g L^{-1}), which is a disaccharide (β-D-galactopyranosyl-(1→4)-D-glucose) composed of one glucose and one galactose residue, linked by a β1-4 glycosidic bond. Citric acid is also an important fermentable compound in milk. Citrate fermentation by LAB leads to the production of 4-carbon compounds, mainly diacetyl, acetoin and butanediol, which are aroma substances (Quintans et al., 2008).

Proteins. The proteins in milk are classified into two families: structural caseins and soluble whey proteins. Caseins form the most important group, constituting over 80% of milk proteins. Four caseins, α_{s1}-, α_{s2}-, β- and κ-, represent approximately 38%, 10%, 36% and 15%, respectively, of the casein fraction, while β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) represent approximately 50% and 20%, respectively, of total whey proteins (O'Farrell, 2004). Milk also contains several minor whey proteins, including bovine serum albumin (BSA) and immunoglobulins (Ig), which are transferred from the blood, each representing about 10% of the whey proteins in mature bovine milk. The remaining 10% is mainly non-protein nitrogen and trace amounts of several proteins, including approximately 60 indigenous enzymes, which represent a minor but very important part of the milk protein fraction (Santos et al., 2012). In raw milk, 95% of the total casein is associated with calcium phosphate ($Ca_3(PO_4)_2$) and exists as coarse colloidal particles, called micelles, with an average diameter of 150 nm (varying from 80 to 300 nm) (O'Farrell, 2004). The casein molecules which form micelles have an open, largely random structure, which makes them readily susceptible to proteolysis. In contrast, the principal whey proteins (β-Lg and α-La) are globular molecules with a high degree of secondary and tertiary structure. Steric factors make whey proteins in native form remarkably resistant to proteolysis (Thomas & Pritchard, 1987).

Lipids. The lipid fraction of milk is mainly comprised of triglycerides (98%), with 1% phospholipids and small amounts of diglycerides, monoglycerides, cholesterol, cholesteryl esters and traces of fat-soluble vitamins and other lipids. The lipids occur as fat globules, 0.1-20 μm in diameter, surrounded by the milk fat globule membrane, which serves as a protective layer against chemical deterioration and as an emulsifier (Dewettinck et al., 2008). Compounds derived from the breakdown of glyceride esters of milk fat, such as free fatty acids and short chain esters, are recognised as playing an important role in the flavour of fermented foods (McSweeney, 2004). However, LAB are generally non-lipolytic.

Vitamins and minerals. Milk is a rich source of vitamins and other organic substances that stimulate microbial growth. The presence of an ample quantity of B-complex vitamins makes milk an excellent growth medium for LAB. Lactococci are naturally auxotrophic for several vitamins and require niacin (B3), pantothenic acid (B5), pyridoxine (B6) and biotin (H) for their growth. Thermophilic streptococci require, in addition to the above-mentioned thiamine (B1) and riboflavin (B2), vitamins B5, B3 and B2 for the growth of lactobacilli (Mäyrä-Mäkinen & Birget, 2004). Such minerals as calcium, magnesium, manganese, zinc, iron, potassium, cobalt, copper and molybdenum, important in microbial fermentations (Lante et al., 2006), are also present in milk in sufficient concentrations.

1.3 Metabolism of LAB in milk

1.3.1 Lactose transport and metabolism

Carbohydrates are the primary carbon and energy sources for LAB, and lactose is the major substrate for microbial fermentation in milk (Martinussen, et al., 2013).

Before lactose can be fermented, it must be transported into the cell. Starter LAB use two different systems to transport lactose, the phosphotransferase (PTS) and permease, both of which require energy.

In the *phosphoenol pyruvate phosphotransferase system* (PEP-PTS) – the group translocation system – lactose is phosphorylated during passage across the cytoplasmatic membrane. The initial source of phosphate is the energy-rich glycolysis intermediate phosphoenol pyruvate (PEP). The lactose-phoshate is hydrolyzed to glucose and galactose-6-phoshate by a phospho-β-galactosidase (P-β-gal). Glucose is converted to glucose-6-phosphate, and both sugar phosphates formed are metabolised further.

The second lactose transport system of LAB involves cytoplasmic proteins (*permeases*), which translocate the sugar into the cell without chemical modification. Located in the cytoplasmic membrane, the lactose permease translocates lactose along with protons into the cytoplasm. This is an active transport system, and the energy is provided in the form of a proton motive force

developed by the transmembrane ATPase at the expense of ATP hydrolysis. The lactose is cleaved into glucose and galactose by an intracellular enzyme, β-galactosidase (β-gal). Lactococci use the PTS system to transport lactose, while all of the other starter LAB use the permease system.

Depending on the mode of transport, lactose molecules enter the cell either as free sugars or as sugar phosphates. In the former case, free disaccharides are split by specific hydrolases into monosaccharides, which then enter the major pathways: the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) or the phosphoketolase pathway (PKP). In general, homofermentative LAB (*Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and some species of *Lactobacillus*) use the glycolytic pathway for glucose fermentation, whereas heterofermentative LAB (*Leuconostoc*, *Oenococcus* and certain *Lactobacillus* species) use the PKP pathway (Axelsson, 2004).

Homolactic fermentation of glucose results in a maximum of two mol of lactic acid and a net gain of 2 ATP per mole of glucose consumed. The heterolactic fermentation of glucose through the PKP pathway ends with the formation of one mol each of lactic acid, ethanol and CO_2, and one mol of ATP/mol glucose. In practice, these theoretical values are seldom obtained (Neves et al., 2005).

1.3.2 Lactose uptake by *St. thermophilus*

Lactose metabolism in *St. thermophilus* is different from that of most other LAB. This organism lacks a lactose PEP-PTS system and instead accumulates and hydrolyzes lactose via an ATP-energized proton motive force (PMF) mediated system and β-galactosidase (Hutkins et al., 1987). Another unusual feature of lactose metabolism by *St. thermophilus* is that most strains ferment only the glucose moiety of lactose and excrete galactose equal to the amount of lactose initially transported.

Three models of the counterflow-like galactose-lactose exchange system were described by Hutkins & Ponne (1991) (Figure 1):

in the first model (Figure 1A), galactose efflux and lactose uptake are indirectly coupled via a proton circuit. Galactose efflux-generated PMF is used to drive a separate PMF-mediated uptake system for lactose. Lactose uptake occurs via proton symport by lactose permease (Poolman et al., 1989);

in the second model (Figure 1B), a lactose-galactose antiporter serves directly as the means of heterologous exchange. In this system, downhill galactose efflux drives the uptake of lactose. This model implies that galactose and lactose uptake systems function via a common carrier (Poolman et al., 1989);

in the third model (Figure 1C), galactose-lactose exchange occurs via a common galactose antiporter, but both sugar uptake and efflux may occur in symport with protons.

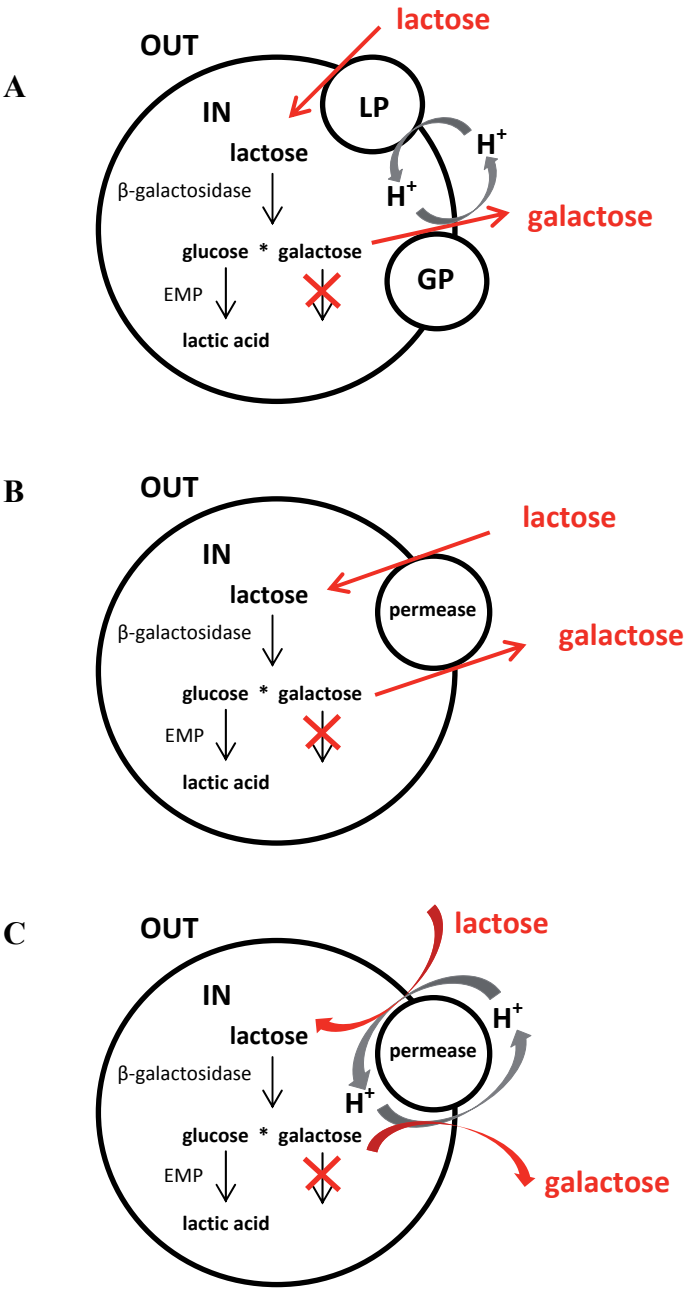

Figure 1. Proposed models for galactose-driven lactose uptake in galactose nonfermenting (Gal⁻) St.thermophilus. Adapted from Hutkins & Ponne, 1991

1.3.3 Protein and peptide metabolism

LAB are nutritionally fastidious and are unable to synthesize many amino acids, vitamins and nucleic acids (Pritchard & Coolbear, 1993). Depending on the specific strain, their auxotrophy can vary from four to 14 different amino acids out of a total of 20 amino acids required to build cellular proteins (Kunji et al., 1996).

One of the main functions of the extracellular proteolytic systems of LAB is to make the amino acids present in casein molecules available for growth. This is accomplished in a three-step process: (1) the hydrolysing of proteins by proteinases into oligopeptides, (2) translocation of the breakdown products across the cytoplasm membrane, and (3) degradation of peptides by peptidases into free amino acids (Figure 2) (Mayo et al., 2010). These amino acids are further degraded by strain-dependent metabolic pathways to generate the actual volatile compounds responsible for the aroma profiles of fermented products (Liu et al., 2008).

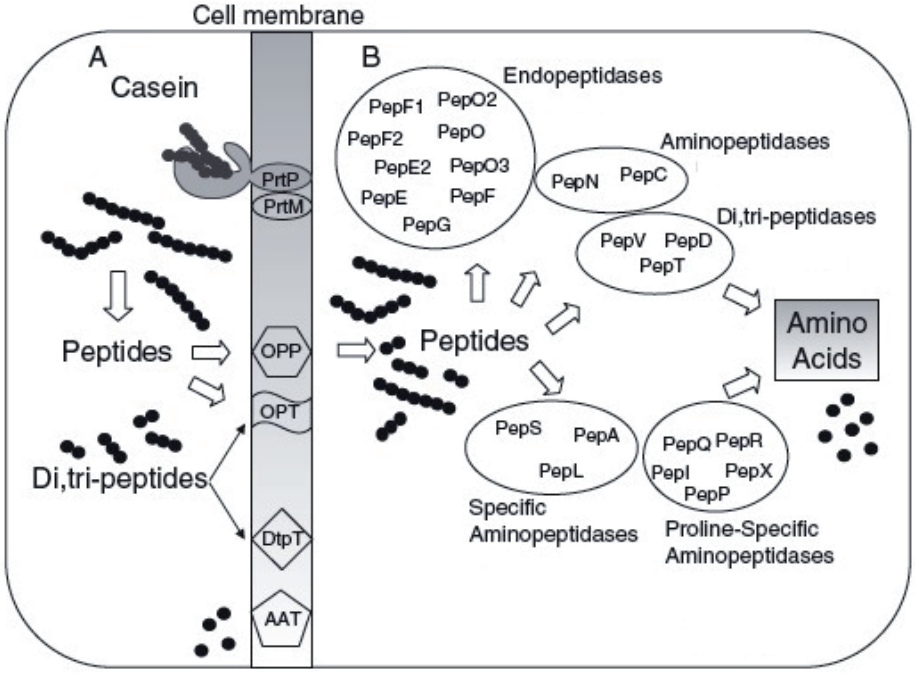

Figure. 2. Diagram of the proteolytic systems of lactic acid bacteria. The pentagon at the bottom of the cell membrane marked as AAT refers to the different types of amino acid transporters. Adapted from Mayo et al., 2010

Proteinases. The key enzyme – cell envelope proteinase (CEP) - is generally considered to be responsible for the initial breakdown of caseins by dairy LAB (Smid et al., 1991). This enzyme is a serine protease belonging to the subtilisin family. Five different types of this enzyme have been characterized from LAB: PrtP from *L. lactis* and *Lb. paracasei*, PrtH from *Lb. helveticus*, PrtR from *Lb. rhamnosus*, PrtS from *St. thermophilus* (Fernandez-Espla et al., 2000), and PrtB from *Lb. delbrueckii* subsp. *bulgaricus*. All enzymes are synthesized as pre–pro–proteins of approximately 2000 residues, consisting of several functional domains. A membrane–bound lipoprotein, PrtM, has been found to be essential for the autocatalytic activation of the proteinases in *L. lactis* and *Lb. paracasei* (Fernandez-Espla et al., 2000).

Most strains of *St. thermophilus* express a very low level of CEP. A screening of *St. thermophilus* strains revealed that only three out of 97 possessed a level of proteinase activities close to those of the proteinase-positive lactococci strains (Shahbal et al., 1991). The presence or absence of PrtS divides the *St. thermophilus* strains into two phenotypic groups: slow and fast acidifying strains (Dandoy et al., 2011). Since *St. thermophilus* is generally found in mixed cultures, the growth of PrtS-deficient strains relies on the use of oligopeptides released by other LAB, such as *L. delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *L. lactis* (Courtin et al., 2002). However, during the last 10 years this important metabolic trait has been drastically selected as a result of the dairy practice, resulting in a strong increase in PrtS+ strains in industrial dairy products (Rasmussen, 2008; Galia et al., 2009)

Peptide and amino acid uptake systems. The second step in protein degradation and utilization is the transport of di -, tri -, and oligopeptides into cells by the action of different peptide uptake systems. Three functional peptide transport systems have been described in *L. lactis*: Opp, DtpT and DtpP (Kunji et al., 1996).

The oligopeptide transporter (Opp) is solely responsible for the uptake of oligopeptides (4–35 amino acid residues) (Doeven et al., 2005). The Opp system is a part of the ABC (ATP binding casette) family of transporters and is composed of five proteins: an oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC) and two ATP binding proteins (OppD and OppF) (Doeven et al., 2005). Up to now, oligopeptide uptake has been thought to be due only to the ABC transporter Opp. Nevertheless, the analysis of several Opp-deficient *L. lactis* strains has revealed the presence of a second oligopeptide ABC transporter, the Opt system, in cells (Lamarque et al., 2004). The proton-motive-force-driven DtpT has a more general specificity, with preferences for hydrophilic and charged peptides, whereas DtpP has a preference for hydrophobic peptides, especially those containing branched chain amino acids, and is dependent on ATP or another energy-rich phosphorylated compound (Doeven et al., 2005, Foucaud et al., 1995).

The oligopeptide transport system in *St. thermophilus* is different from that of *Lactococcus*. It has been called Ami since it shows the highest identity with the Ami system of *St. pneumoniae* (Garault et al., 2002). However, the specificity of the *St. thermophilus* oligopeptide uptake system is broader than those of streptococci and similar to that specified by OppA from *L. lactis* (Garault et al., 2002). The Ami transporter is composed of three oligopeptide-binding proteins (AmiA1, AmiA2 and AmiA3), two membrane proteins (AmiC and AmiD), and two ATPases, which provide energy to the system (AmiE and AmiF).

Peptidases. The third step of protein catabolism involves the peptidolytic cleavage of peptides. The peptides are degraded intracellularly by the concerted action of an array of peptidases, with distinct but overlapping specificities (Kunji et al. 1996). Peptidases can be divided into endopeptidases, which hydrolyse internal peptide bonds, and exopeptidases, which remove amino acids from the ends of the peptide chains. The majority of the exopeptidases in LAB are aminopeptidases, and their specificity depends on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al., 1996). Thirteen homologous peptidases were identified in species of *L. lactis* and *St. thermophilus,* and in addition an oligopeptidase and an aminopeptidase (PepS) were discovered in *St. thermophilus* (Rul & Monnet, 1997).

1.4 Changes in milk components during processing

Milk is a very flexible raw material for the production of many types of dairy products in a great diversity of flavours and forms around the world. The processability and functionality of milk and milk products are determined by the properties and concentrations of its principal constituents (proteins, sugars, fats and salts). Milk is usually subjected to heat treatment to ensure the microbiological safety of dairy products.

1.4.1 Heat-treatment of milk

The main reason for the heat treatment of milk is to guarantee safe food and to improve its storage quality by decreasing the number of spoiling microorganisms and to achieve desirable properties in the final product (Vasbinder et al., 2003). The following are the customary processes of heat treatment:

- thermization: heating for 20 sec at 60 to 69°C;
- low temperature pasteurization: heating for 30 min at 63°C (LTLT), or for 15 sec at 72°C (HTST – high temperature short time);
- high temperature pasteurization: heating for 20 sec at 85°C
- sterilization: heating for 30 min at 110°C in-bottle sterilization or 30 sec at 130°C, or 1 sec at 145°C in line (UHT – ultra high temperature treatment).

1.4.1.1 Heat-induced changes in milk

Several structural changes and chemical reactions take place during the heat treatment of milk. The biological activities, properties, interactions and structures of natural milk components are influenced. The extent of change is proportional to the temperatures used: more pronounced (irreversible) changes occur at higher temperatures.

Changes in proteins. Upon the heat treatment of milk above 60°C, several changes take place, of which the denaturation of whey proteins is the most obvious (Raikos, 2010). The most abundant whey protein is *β*-Lg, in which a heat-induced conformational change exposes a reactive thiol group. This thiol group can form disulfide bonds with other cysteine-containing proteins, such as *β*-Lg and bovine serum albumin, or with proteins having disulfide bridges, such as *α*-La, *κ*- and α_{s2}-casein (Jang & Ewaisgood, 1990). Interactions of *β*-Lg with *κ*-casein present at the exterior of the casein micelles, like a hairy brush, lead to the coating of the casein micelles with *β*-Lg (Vasbinder et al., 2003).

Not all the denaturated whey proteins form complexes with the casein micelles; some remain in serum, where they may form aggregates with other whey proteins. The extent of the association of denaturated whey proteins with casein micelles is markedly dependent on the pH of the milk prior to heating. Heating at pH values below 6.7 results in a greater quantity of denaturated whey proteins associating with casein micelles, whereas at higher pH values whey protein-*κ*-casein complexes dissociate from the micelle surface, apparently due to the dissociation of *κ*-casein (Kethireddipalli et al., 2010; Blecker et al., 2012).

Although caseins are extremely heat-stable, primarily due to their lack of tertiary structure, casein micelles are susceptible to heat-induced changes. Heating at temperatures below 70°C causes some reversible changes in the associations of micellar caseins and may result in the interaction of denaturated whey proteins with the casein micelles. The heat-treatment of milk at >70°C leads to increased levels of non-micellar casein in milk, suggesting the heat-induced dissociation of caseins. Furthermore, heating at 110-150°C causes extensive dephosphorylation of caseins, which may affect the structure of micelles. The treatment of milk at temperatures <100°C may also lead to the hydrolysis of caseins, leading to the formation of peptides (Tamime, 2009).

Changes in lactose. Heat-induced changes in lactose include isomerisation, degradation and its participation in Maillard reactions. If the heating of milk is not intense, lactose is decomposed, primarily via isomerisation reactions, whereas only a small proportion will be degraded via Maillard reactions; at intense heating, however, the degradation of lactose is primarily via the Maillard reaction (O´Brien, 1995). This reaction involves the condensation of lactose with the free amino groups of protein, with subsequent rearrangements and degradations, leading to a variety of brown-coloured compounds. Products (consequences) of Maillard reactions which are important in milk processing include brown colour substances, the formation of off-flavour, the loss of

available lysine, lowered nutritional value, reduced digestibility, and reduced solubility. In addition, the heat-induced isomerisation of lactose leads to the formation of lactulose. If the temperature of heating is higher than 100°C, lactose can be converted to acids, especially formic acid, thereby decreasing the pH of the milk (Robinson, 2002).

Changes in milk fat and fat globules. The thermal degradation of lipids in milk is generally not observed, because the temperature required for the non-oxidative decomposition of fatty acids (>200°C) is well outside the range used for the heating of milk products. However, the heat treatment of milk can alter the properties of milk fat globule membrane (MFGM). The denaturation of MFGM proteins occurs at temperatures >70°C, leading to the exposure of reactive groups. A noticeable effect of the heating is the release of sulphydryl compounds, most notably H_2S. Heating at 80°C for 3-18 min leads to the incorporation of whey proteins, especially β-Lg, into MFGM, thus increasing the protein content of the membrane and decreasing the proportion of lipids. Heating also decreases the number of free sulphydryl (SH) groups in the MFGM and increases the number of disulphide (SS) groups, suggesting that the incorporation of β-Lg might be due to association with membrane proteins via disulphide bonds (Morin et al., 2008).

Changes in milk salts. Milk salts exist in an equilibrium between the soluble and colloidal phases of milk, and this distribution is affected by the heat treatment of the milk. Heating milk reduces soluble and ionic calcium and phosphate concentrations by converting the soluble calcium phosphate to a colloidal state. On subsequent cooling, some of the indigenous or heat-precipitated calcium phosphate molecules may redissolve, especially if the heating temperatures remain below 85°C. However, after heating at higher temperatures, the precipitated material does not resolubilize. The decrease in soluble calcium, phosphate and citrate during heating in the temperature range 70-90°C involves two steps (Pouliot et al., 1989). The majority of the decrease takes place during the first minutes of heating, after which a small decrease occurs over an extended period of time (up to 120 min).

1.4.1.2 Influence of heating milk on the growth of starter cultures

From the practical point of view, the effect of heating milk on starter development is important in the manufacture of cheese and cultured milk products. Most dairy plants employ a heat treatment before starter inoculation to insure optimal development of desirable bacteria, as well as the uniformity of the final product.

In general, heating raw milk improves its ability to support the growth of starters, whereas “overheating” does not result in a consistent and uniform improvement in starter growth. It was shown by Greene & Jezeski (1957) that

treatments ranging from 62°C for 30 minutes to 120°C for 60 minutes resulted in a series of related changes in starter activity.

A primary zone of stimulation was observed from 62°C for 30 minutes to 72°C for 40 minutes, and was followed by a zone of inhibition, which began at 72°C for 45 minutes and lasted through 82°C for 10 to 120 minutes and 90°C for one to 45 minutes. A second zone of stimulation began at 90°C for 60 to 180 minutes and autoclaving at 120°C for 15 to 30 minutes. Final inhibition occurred by autoclaving for more than 30 minutes (Greene & Jezeski, 1957).

In practical dairy plant operations, the first three phases of this cycle (with treatments below 100°C) are usually the most important.

1.4.2 Irradiation of milk

Ionizing radiation (radiation sterilization or "cold sterilization") is a decontamination technology that upgrades food safety and extends the shelf-life of food. 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 irradiation can also prevent the spoilage of dairy products (Bandekar et al., 1998; Ham et al., 2009b; Konteles et al., 2009). However, only a limited number of countries have approved irradiation of milk products for sanitation purposes. The disinfection and microbial control of dried milk products by irradiation has been approved in Croatia, and the irradiation of casein or caseinates has been approved as a microbial control method in Belgium (Ham et al., 2009a). A dose of 5 kGy has been shown to be sufficient to decrease the total microflora in milk powder to the acceptable level (Żegota & Małolepszy, 2008). The irradiation of pasteurized milk at 0.25 kGy, at room temperature, was found to double the shelf-life at 4°C (Sadoun et al., 1991), while the irradiation of raw milk at 0.5 kGy did not increase shelf-life significantly.

1.4.2.1 Changes in milk induced by γ-irradiation

Besides hygienization, however, it has been shown that irradiation treatment causes physico-chemical changes in foods and particularly in milk (Cieśla et al., 2004).

Off-flavour. Milk and dairy products are very susceptible to radiation-induced flavour changes (Diehl, 1983). The threshold value for off-flavour development in milk depends on its composition. A threshold of 0.3 kGy or lower has been reported for whole liquid milk; skim milk and dried milk powders are less sensitive (Diehl, 1983). The off-flavours and off-odours which characterize irradiated dairy products (malty and caramel-like) originate from the casein and appear to arise from a group of potent, disagreeable-smelling sulphur compounds, predominantly methyl mercaptan, methyl sulphide and metyl disulphide (Day et al., 1957).

Formation of free radicals. It is known that ionizing radiation can lead to fragmentation, aggregation, conformational and net electrical charge changes, oxidation of amino acids and the rupture of covalent bonds (Davies, 1987; Davies, 2012; Day et al., 1957). According to Arena (1971), ionizing 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, such as superoxide anion radicals (O_2^-), hydroxyl radicals ($OH^\bullet$) (Thibessard et al., 2001) and non-radical hydrogen peroxide (H_2O_2) (Davies, 1987). $OH^\bullet$ can also be generated in 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).

Effect on proteins. Gamma irradiation of proteins can induce structural changes and alter their functional properties (de la Hoz & Netto, 2008). It was shown by Ham et al. (2009a) that α_{s1}-casein and β_{A1}-casein were degraded, whereas α_{s0}-, β_B-, and β_{A3}-caseins were formed by gamma irradiation at 10 kGy. The effect of doses up to 10 kGy on the tertiary and secondary structure of β-Lg irradiated in solid state was not significant, and slight changes were observed at 50 kGy. The changes observed on β-Lg irradiated in solution, related to secondary and mainly to tertiary structure, explain, at least partially, the protein aggregation. However, the observed secondary and tertiary structural changes were less pronounced than those occurring by heat treatment (de la Hoz & Netto, 2008).

Effects on milk fats. Free radicals have been related to milk lipid oxidation, especially of unsaturated fats (de Oliveira Silva et al., 2011). Irradiation-induced changes in milk fat are caused as a result of hydroperoxide production, carotenoid destruction, and oxidized flavour production (Day & Papaioannou, 1963). The irradiation-specific odour of milk fat has been described as tallow, chalky, metallic and candle-like by various investigators (Wertheim et al., 1956, Hoff et al., 1959; Day & Papaioannou, 1963). It was shown by de Oliveira Silva (2011) that gamma radiation, even in small doses (1-2 kGy), was able to produce alterations in the lipids in raw whole milk, with the liberation of malonaldehyde. At a 3 kGy dose, the alteration was intense, demonstrating total degradation of the lipids with this treatment.

Effect on lactose. Wertheim et al. (1956) have reported that lactose plays an important role in radiation-induced browning and that carbonyl compounds, the browning precursors of milk, are formed from lactose. The degradation of lactose in aqueous solutions by ionizing radiation at 20 kGy was studied by Adachi (1962) and it was evident that lactose had been degraded into at least ten fragmentation products. It was suggested that the amounts of galactose and glucose may be equimolar in the irradiated lactose and that galactonic acid is formed mainly from the galactose portion of lactose by oxidative degradation.

1.4.2.2 Growth of starter cultures in irradiated milk

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 of 10^6 CFU mL^{-1} was similar in irradiated and reference (non-irradiated) milk samples, the growth rate of the bacteria at a lower inoculation rate (10^4 CFU mL^{-1}) 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 inoculation levels of 10^6 and 10^4 CFU mL^{-1} as reported by Favrot & Maubois, 1994.

1.4.3 Coagulation of milk

Milk coagulation is the primary step in the production of a number of dairy products, such as fermented milks and cheeses (Herbert et al., 1999). Coagulation starts with the induction of gel formation, i.e. destabilization of the colloidal system of dispersed casein micelles, either as a result of slow acidification by lactic acid bacteria (yoghurt), or as a result of the enzymatic action of chymosin (cheese), or a combination of both factors, e.g. quark and cottage cheese with chymosin levels considerably lower than in cheese-making (Aichinger et al., 2003). The development and properties of these milk gels are remarkably different.

Acid-induced coagulation. The fermentation of lactose to lactic acid by thermophilic starter bacteria gradually causes the pH of milk to drop from about 6.7 to below 5.0. The neutralization of negative charges on the surfaces of casein micelles leads to the collapse of the κ-casein hairy layer, destabilizes the micelles and induces clotting as the isoelectric point is approached (pH ~4.6) (Phadungath, 2005). As the pH is reduced, the colloidal calcium phosphate (CCP) is dissolved and this alters/weakens the internal structure of micelles.

The acidification of milk takes place in three pH regions from pH 6.7 to 4.6 (Phadungath, 2005). A decrease in the net negative charge on the micelle surface and reduced electrostatic repulsion takes place as pH is lowered from 6.7 to 6.0. CCP and micelle integrity is preserved. A further decrease of pH from 6.0 to 5.0 neutralizes the charge of the surface of the micelles and, with the shrinkage/collapse of κ-casein hairs, micelles are sterically and electrostatically destabilized. CCP is completely dissolved if a pH of around 5.9 is reached and this increases the internal flexibility of the micelles (Donato et al., 2009). Finally, at a pH below 5.0 the destabilized micelles come closer, hydrophobic interactions come into play and gelation occurs at pH 4.9 in unheated milk, but at a higher pH (5.2) in heat-treated milk.

Enzymatic coagulation. There are three important stages in the kinetics of rennet-induced coagulation of milk. The first phase of coagulation is initiated by the cleavage of milk proteins using chymosin (EC 3.4.23.4): the enzyme responsible for the cleavage of the Phe_{105}-Met_{106} bond in the κ-casein molecules in the casein micelles. As a result, hydrophilic glycomacropeptides (residues 106 to 169) are released into the solution and hydrophobic para–κ–caseins (residues 1 to 105) are left in the micelle. When a sufficient proportion of hydrolyzed κ-casein molecules have been generated, the lower net negative charge of the micelles and the greater hydrophobicity of para–κ–casein lead to the aggregation of micelles (secondary phase). This time of incipient aggregation is considered the "clotting time". As coagulation proceeds, fat globules, water and water-soluble materials are trapped in the aggregating protein matrix, first forming linear chains and later a 3D branched structure (third phase) (Lucey, 2009).

1.4.3.1 Growth of LAB in solid matrix

The growth of bacteria in solid media fundamentally differs from that in liquid media (Wilson et al., 2002). In contrast to liquid systems, where microbial growth occurs planktonically, microorganisms in (semi-)solid matrices are immobilised, and constrained to form colonies (Mertens et al., 2012). The growth of starter bacteria occurs during cheese making in changing environmental conditions: starting with liquid, followed by enzymatic gel and then in drying cheese grains (Favrot & Maubois, 1996). Starter bacteria provide the most significant contribution to the microbial biomass in young curd, starting with $1–5 \times 10^6$ CFU mL^{-1} and typically attaining densities of $>10^8$ CFU g^{-1} within one day of manufacture. The mechanisms and time of gel formation and consequently the properties of the final gels are quite different, which can affect the dynamics of growth and biochemical activity of microorganisms (Giraffa et al., 2010).

1.5 Methods of studying the growth of LAB in milk

The monitoring and screening of the growth and activities of starter cultures are of great importance in the starter industry and food technology to guarantee high quality products, such as fermented milks, cheese and butter.

1.5.1 Methods based on measurements of acidity and metabolite analysis

The importance of methods of evaluation of the acidifying capacity of lactic acid cultures has been emphasized in the literature (Corrieu, 1988; Zanatta & Basso, 1992). The quality of process control based on acidity changes depends

on the accuracy of the method used. The acidifying capacity of cultures of lactic acid bacteria has been estimated automatically by measuring pH changes continuously during fermentations (Corrieu 1988). The monitoring of changes in conductance (changes in the electrical properties of media) has also been used to determine the activity of lactic acid cultures (Lanzanova et al., 1993; Mucchetti et al., 1994).

Nowadays, the dynamic CINAC system is highly reliable (Corrieu, 1988). iCINAC is a novel piece of equipment for on-line pH monitoring and for the calculation of acidification rates and feature points (descriptors) which characterize acidification curves and the corresponding kinetics. iCINAC also makes it possible to obtain additional information on temperature and oxidation/reduction potential in parallel with pH.

An alternative approach is to measure the production of organic acids that lower pH. The methods available are based on the determination of lactic acid by titration with NaOH, or by using HPLC (Zanatta & Basso, 1992).

Undoubtedly, the acidification activity of LAB is a very important and relatively easy-to-measure parameter, whereas for more detailed analysis of acidification processes more sophisticated methods are required.

1.5.2 Methods based on heat flow measurement

Nearly all processes (chemical, physical and biological) produce heat. Heat is a form of energy whose exchange always accompanies life processes. There are different types of calorimeters used for the measurements of heat exchange in different types of processes: temperature-scanning calorimeters, isoperibol calorimeters, bomb calorimeters and isothermal (heat conduction) calorimeters (IC) (Kemp, 2001).

In an IC, the thermal power emitted from samples is measured under essentially isothermal conditions. Small volume and sensitive calorimeters are called microcalorimeters, and modern isothermal microcalorimetres (IMC) are capable of measuring heat fluxes in the order of 1 μW and even less. As few as 10,000–100,000 active (growing) bacterial cells in a sample are sufficient to produce a real-time signal dynamically related to the number of cells present and their activity (Braissant et al., 2010). The IMC TAM III was used in the present study for the investigation of the growth of starter bacteria under different conditions (Publications I, II and III).

IMC is unique in its ability to provide rapid detection and real-time, quantitative monitoring of a wide variety of microbiological phenomena (Braissant et al., 2010). IMC offers several unique advantages: a) the measured parameter is heat flow, which can be considered to be a universal indicator of change, b) no specific sample treatment or preparation is needed; the samples are housed within an ampoule and monitored *in situ*, and c) the technique does not require optical transparency of samples and is invariant irrespective of physical form.

Microcalorimetry has been used for the experimental study of bacterial growth during the last 50 years, and over the past 10 years the use of IMC in the biological field has been increasing (Braissant et al., 2013). Calorimetry is especially helpful in studies of growth in opaque media, for example solid media where agar, gelatine, etc. are used as jellifying agents (Mitchell et al., 2004), but it can also be successfully used for studies of spoilage processes and shelf-life determination (Wadsö & Galindo, 2009): anywhere that traditional microbiological measurements (optical density, cell count and metabolic assays), microscopical and other physical methods can not be used due to the properties of non-transparent media (Wilson et al., 2002; Antwi et al., 2006; Braissant et al., 2013).

The main drawback in the use of IC may be the batch nature of the process, i.e. there is no steady state but continuous change in the culture conditions (substrate, product concentrations, growth rate etc.) and difficulty in collecting samples for additional analyses (e.g. content of substrates or products) during a run.

1.5.2.1 Analysis of calorimetric power-time curves

In a batch culture, microbial growth proceeds through four main phases: the lag phase, the exponential (or logarithmic) growth phase, the stationary phase and the decline (or death) phase (Figure 3). A typical calorimetric power-time curve (a curve that are the output of a microcalorimeter) can be devide into three growth phases (Figure 4a).

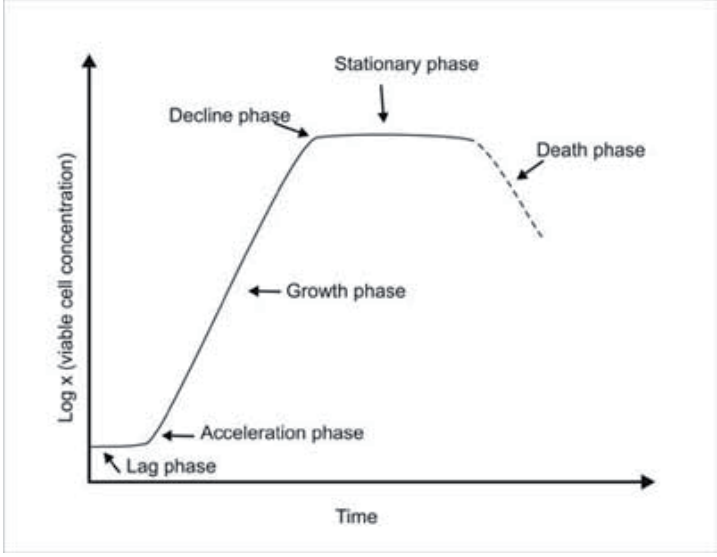

Figure 3. A typical growth curve of bacteria

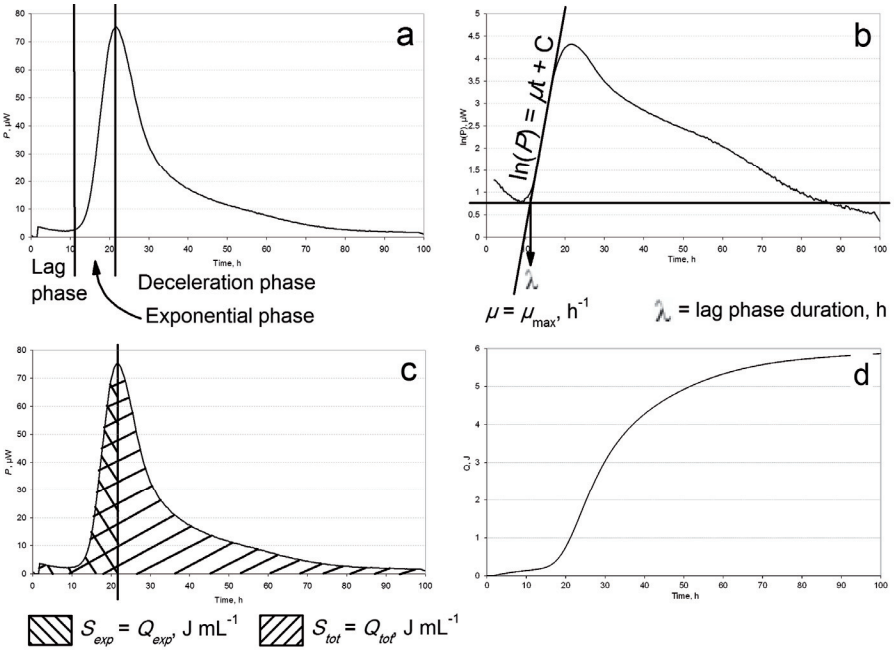

Figure 4. The explanation of the processing of calorimetric power-time curves; (a) the division of calorimetric power-time curves into three phases: lag phase, exponential growth phase, and deceleration phase; (b) determination of the maximum growth rate (μ_{max}, h^{-1}) and the lag phase duration (λ, h); (c) determination of heat amounts during the exponential phase (Q_{exp}, J mL^{-1}) and during the total experiment (Q_{tot}, J mL^{-1}); (d) an integrated power-time curve: accumulated heat change over time (Kabanova et al., 2012)

The length of the lag-phase (λ, h) can be determined as shown in Figure 4b. The calculation procedure is practically the same as in Swinnen et al. (2004). However, it should be emphasized that in calorimetric experiments the length of the lag-phase is defined by the physiology of the cell, and by the level of sensitivity of the microcalorimeter. The sensitivity of a TAM III in the registration of heat is 7×10^{-4} J (0.5 mW), which means that only a certain number of growing (metabolizing) bacteria can produce heat fluxes surpassing the sensitivity threshold of the instrument. This means that the lag-phase duration measured on the basis of calorimetric curves should be calculated by taking into account the time when the heat produced by the growing bacterial population exceeds the level of the sensitivity of the instrument (Kabanova et al., 2012).

The second phase of the power-time curve, following the lag-phase, corresponds to the exponential growth phase (fast growth phase), during which bacteria grow at practically the maximal growth rate μ_{max} possible in the experimental conditions studied.

According to our data (Publications I, II and III), the typical power-time curve of thermophilic starters in milk samples contained two peaks corresponding to two exponential growth phases: first the smaller one (the first exponential growth phase) and after that the main larger peak (the second exponential growth phase).

It is interesting to note that a decline in heat flow does not always mean a decrease in cell number, but may refer to metabolic changes. It is known that if, for example, two different carbon sources are present in a medium, microbes may consume these substrates sequentially, resulting in diauxic growth. The first growth phase occurs when cells use a preferred carbon source, and the second growth phase begins after the preferred carbon source has been depleted. The less-preferred carbon source is used during the second exponential growth phase. The transition from one stage to another often requires the synthesis of new enzymes and during this time the heat flow of the culture tends to decrease (Braissant et al., 2010).

We assume that the occurrence of two peaks on the power-time curves of the growth of *St. thermophilus* are also explained by a switch from growth in free amino acids present in milk to growth in the amino acids and peptides of caseins released by the enzymes synthesized by the bacteria.

The end of the exponential phase of bacterial growth is defined in our data analysis by the peak of the power-time curves. According to the results obtained by plate count data, the end of the exponential growth occurred at some time after the peak of the calorimetric curve, but then judging by the power-time curve, inhibitory processes and decrease in the growth rate had obviously started already. Therefore, the exponential growth phase ending at the peak value of the power-time curves was justified.

The return of the heat flow to its baseline at the end of the experiment does not imply that all of the cells are dead. In most cases, the return to the baseline indicates that the heat producing reactions (i.e., the microbial metabolism) have ceased, although the number of cells in the medium may remain rather constant.

1.5.3 Application of isothermal microcalorimetry in dairy microbiology

Calorimetry was first applied in dairy research for milk grading in the seventies (Berridge et al., 1974). Thermograms of the growth of individual strains of LAB were obtained with a defined medium containing limiting concentrations of several carbohydrates by Fujita in 1978 (Fujita et al., 1978). The first observations of the interactions of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in single and mixed cultures in milk date back to 1979 (Monk, 1979). Several calorimetric studies have been carried out in dairy research: Riva et al. (1997) investigated the shelf life of raw milk using isothermal calorimetry by continuously monitoring the kinetics of microbial growth, Gardea et al. (2002) compared heat evolution by bacteria with

traditional plate counts, assessing the microbiological quality of milk that had undergone different treatments, Litz & Schäffer (2005) used isoperibolic batch calorimetry for the indication of the proliferation of LAB in mixed cultures, and Wadsö et al. (2009) compared the thermal power profiles of the fermentation of milk at two incubation temperatures using two different buttermilk cultures.

In the present work, IMC was used to evaluate the peculiarities of the growth of lactic acid bacteria in differently pretreated milk samples (Publications I-III).

2. AIMS OF THIS DISSERTATION

The main aim of the dissertation research was to study the growth and metabolism of thermophilic lactic acid bacteria in milk with different pretreatments, using isothermal microcalorimetry. In addition, microflora of raw milk were studied as a possible source of biochemical changes prior to milk processing in the dairy, possibly interfering with the growth of LAB.

The specific aims of the study were the following:

- study the effect of milk heat treatment on the growth parameters of thermophilic starter bacteria (Publication I).

- study the influence of O_2 on the growth of thermophilic starter bacteria in UHT milk (Publication II).

- study the growth characteristics of *Streptococcus thermophilus* ST12 in skim milk prepared from irradiated at 10 kGy skim milk powder in comparison with the growth in non-irradiated reconstituted skim milk (Publication III).

- characterize the growth of *Streptococcus thermophilus* ST12 in renneted milk (unpublished data).

- evaluate the microbial quality of farm bulk-tank raw milk produced in Estonia and determine the technologically significant bacterial groups (lactic acid bacteria, psychrotrophic bacteria and aerobic spore forming bacteria) not routinely analysed by dairies that possibly interfere with the growth of LAB (Publication IV).

3. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used are available in Publications I to IV.

3.1 Bacterial cultures

The strain of *Streptococcus thermophilus* (*St. thermophilus* ST12) used in this work was provided by Chr. Hansen (Denmark) (Publications I and III).

Liquid bulk starter containing *St. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chr. Hansen XY-11) was purchased from a small dairy (Nõmmiku Talu Meierei, Estonia) (Publication II).

Strain *Lactobacillus paracasei* S1R1 culture was isolated from Estonian cheese and kept frozen in MRS-glycerol at -80°C (Kask et al., 2003) (Publication I). All experiments were started from a single colony on an agar plate.

3.2 Milk samples

Low-heat skim milk powder (LHSMP) was obtained from Kalev Paide Tootmine AS (Paide, Estonia) (Publication I) and from Valio Ltd. (Helsinki, Finland) (Publications III and IV), and used for the preparation of the laboratory 10% skim milk. A part of the LHSMP (Valio Ltd.) was irradiated at a dose of 10 kGy (Publication III) and used for the preparation of skim milk similarly to conventional RSM.

RSM and irrRSM, with the addition of $CaCl_2$ to yield a 10 mM final concentration, were used for milk gel preparation. Milk gels were prepared by the addition of 100 µl of a 10 gL^{-1} chymosin (CHY-MAX Powder Extra, Hørsholm, Denmark) solution to 10 mL of RSM and irrRSM

Commercial pasteurized milk with 3.5%, 2.5% and 0.05% fat content (Tere AS, Tallinn, Estonia) and commercial UHT milk with 3.5% and 0.05% fat content (Kalev Paide Tootmine AS) were obtained from retail sellers (Publications I and II).

The detailed description of the catalase and nitrogen gas pretreatments of the milk samples prior to the inoculation of the starter is described in Publication II.

Raw milk samples from individual dairy farms were collected from 131 farms at geographically different locations in Estonia by licensed milk haulers and from dairy silos between February 2004 and June 2007 (Publication IV).

3.3 Enumeration of microorganisms

The determined plate counts of spore-forming (ASFBC), psychrotrophic (PBC) and lactic acid bacteria (LABC) were combined with the regular milk quality data of total bacterial count (TBC) and somatic cell count (SCC) kindly provided by the Animal Recording Centre (Tartu, Estonia) (Publication IV).

3.4 Analytical methods

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 bacteria. Heat generated or absorbed was continuously measured in maximally filled hermetically sealed 3-mL calorimetric ampoules. Data was not collected for ~1 h at the beginning of the experiments to let the thermal equilibration processes be completed. Heat flow was measured at 10 sec intervals. Data acquisition and analysis were carried out using the TAM Assistant Program (v 0.9.1012.40, SciTech Software AB, Thermometric AB) (Publications I, II and III).

The pH of the samples was measured using a pH meter S20 Seven Easy Mettler Toledo with an InLab 413 Mettler Toledo electrode (Publications III and IV).

Concentrations of lactose, glucose, galactose and lactate were determined using the HPLC system (Alliance 2695 system, Waters Corp., Milford, MA) with a Refractive Index Detector 2414 and column BioRad HPX-87H 300 x 7.8 mm (Hercules, CA) (mobile phase – 0.005 N H_2SO_4, flow rate 0.6 mL min^{-1} at 35°C) (Publication III).

The analysis of free amino acids was performed by a UPLC system (Acquity UPLC; Waters Corp.), including a binary solvent manager, a sample manager and a photodiode array (PDA) detector connected to 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 (Publications III and IV).

Dynamic rheological measurements were carried out using a Physica MCR301 rheometer (Anton Paar GmbH, Graz, Austria) equipped with a C-PTD200 Peltier temperature control unit and CC27 coaxial cylinder measuring system (outer and inner diameters 28.92 and 26.66 mm, respectively) (Publications II and III).

Measurements of front-face spectral fluorescence signatures (SFS) from the milk powders and inoculated milk samples were performed in The Instant Screener Compact fluorescence spectrophotometer (LDI Ltd., Tallinn, Estonia) equipped with a pulsed xenon lamp (Publication III).

3.5 Parameters describing bacterial growth

The following definitions and formulas were used to express the growth characteristics of the cultures of LAB in IMC:

1) *Maximum growth rate* – μ_{max} (Wh^{-1})

Maximum specific growth rate (μ_{max}) in the first and second exponential growth phases was measured as shown in Figure 5.

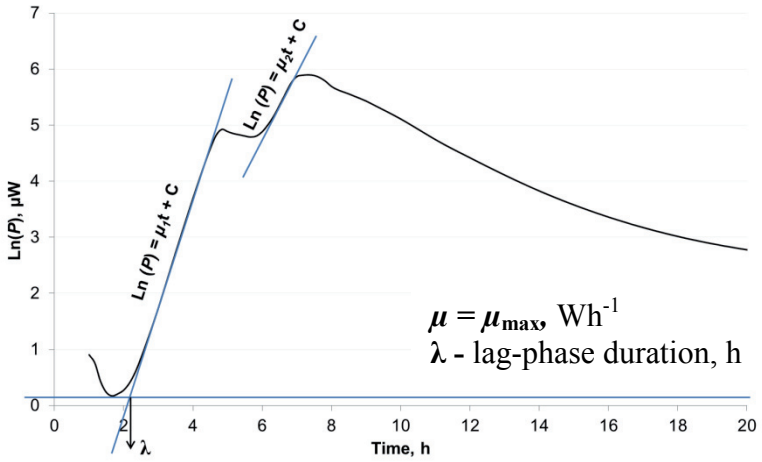

Figure 5. Determination of the maximum growth rate (the slope of the tangent to the first (μ_{max1}) and second exponential phase (μ_{max2}), Wh^{-1}) and lag-phase duration (the crossing point of X-axis and the tangent, λ, h)

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

$$\frac{dX}{dt} = \mu X \qquad (1)$$

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

$$\ln\frac{dQ}{dt_t} = \frac{dQ}{dt_0} + \mu_{max}t \qquad (2)$$

2) *Heat amounts produced during growth – Q* (J mL^{-1})

The heat produced during the exponential growth phase Q_{exp} (area between the power-time curve, the vertical line that goes through the peak, and the baseline) and the total heat produced during the whole process of growth Q_{tot} (area between the power-time curve and baseline) were determined using the TAM Assistant program (v 0.9.1012.40, SciTech Software AB, Thermometric AB, Järfälla, Sweden) and Microsoft Excel.

3) *Heat production rate – P_{max}* (μW mL^{-1})

The value P_{max} is the aggregate rate of heat production of all bacteria present at the time when the maximum occurs. It depends on both the number of active bacteria present at that time, and the rate at which bacteria present are producing heat.

4) *Number of bacteria calculated from power-time curves – N* (CFU mL^{-1})

Bacterial growth was expressed as the change in the number of viable cells over time using the equation:

$$\Delta N = \frac{\Delta Q}{Y_Q} \qquad (3)$$

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, and 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 ± 0.15) × 10^{-9} J CFU^{-1} for the exponential growth phase (a major peak). The Y_Q is effectively the heat evolved during the multiplication of one bacterium.

3.6 Calculation of growth characteristics

The growth characteristics of the bacteria during the growth in milk were calculated on the basis of the heat which was converted to biomass concentration and organic acid concentrations in the culture medium (mol L^{-1}) as follows:

$$\mu = \frac{\ln(N_2 / N_1)}{(t_2 - t_1)} \qquad (4)$$

$$I_{Lactose} = \frac{d(C_{Lactose})}{d(X)} \qquad (5)$$

$$O_{Lactate} = \frac{d(C_{Lactate})}{d(X)} \quad (6)$$

$$O_{PS} = \frac{d(C_{Lactose}) - d(C_{Gal})}{d(X)} \quad (7)$$

$$Y_{Lactate/Lactose} = \frac{d(C_{Lactate})}{d(C_{Lactose})} \quad (8)$$

$$Y_{XS} = \frac{d(X)}{(d(C_{Lactose}) - d(C_{Gal}) - d(Glc)) \times 180/1000} \quad (9)$$

$$I_{AA} = \frac{d(C_{AA})}{d(X)} \quad (10)$$

where μ is the specific growth rate (h^{-1}), t is time (h), N is the cell count, assuming that heat produced per cell formation is constant (4.45×10^{-9} J per cell, unpublished data), X is the dry biomass (gDW L^{-1}), assuming that one cell is 0.2×10^{-12} g (unpublished data), $I_{Lactose}$ is the lactose consumption per biomass produced (mmol gDW^{-1}), $O_{Lactate}$ is the lactate production per biomass produced (mmol gDW^{-1}); Gal is galactose, Glc is glucose, C is the concentration of corresponding compound (mM), O_{PS} is the polysaccharide production per biomass produced, assuming that galactose was not degraded by cells (lactate production per glucose consumed was lower than 2 mol mol^{-1}), $Y_{Lactate/Lactose}$ shows the lactate yield per lactose consumed, Y_{XS} is the biomass yield per hexose consumed (in the case of RSM where PS was consumed after the 6^{th} h calculated based on the lactate production, assuming that 2 mol of lactate is produced per 1 mol of hexose), and I_{AA} is the consumption of corresponding amino acid per biomass produced (mmol gDW^{-1}).

3.7 Statistical analysis of the data

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 $p<0.05$ (Publications I, II, III and IV).

4. RESULTS AND DISCUSSION

4.1 A tool for microcalorimetric data processing

A tool for the quick analysis of microcalorimetric signals, *microCalCurveGUI*, was developed and used for the evaluation of the complex power-time curves (see 4.2.4).

The graphic user interface of *microCalCurveGUI* consisted of three main parts: file I/O, the input data part and derivative analysis parameters (Figure 6 and Table 3). The file I/O part is for selecting the input data file (MS Excel file) and the right worksheet with calorimetric data. The input data part is used for editing input data name and units, and for selecting data for the analysis. The derivative analysis button makes it possible to start the analysis of the microcalorimetry curves and fix the values of the parameters influencing the analysis.

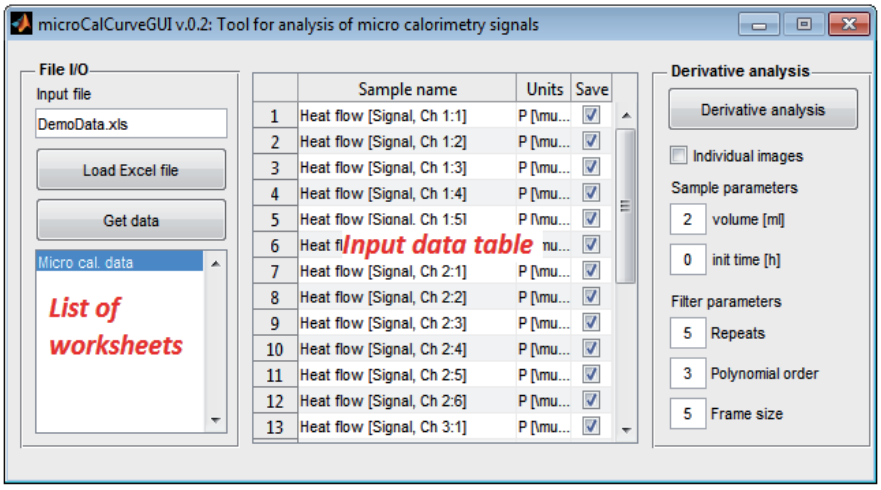

Figure 6. microCalCurve graphical user interface with file I/O on the left, input data in the centre and derivative analysis controls on the right

The *microCalCurveGUI* tool expresses the results of the derivative analysis of microcalorimetry signals in the form of image file(s) and an MS Excel spreadsheet table. Images saved in the form of *.png* files contain the original microcalorimetric curve and exponential growth phases detected by the derivative analysis. The image corresponding to the analysis of one single microcalorimetric is depicted in Figure 7.

Different phases are marked by a light red or blue colour. Additionally, the fitted lines describe the exponential phases, and the numbers next to them express the maximum growth rate μ_{max}, start and end of a particular phase t_{start}

and t_{end}, and Pearson's correlation coefficients between fitted data and an exponential growth file model within the whole growth phase r^2. The same data are written into Excel (the data are not shown). Additionally, in the MS Excel file the length of the lag-phase can be found. The length of the lag phase is calculated as the point where the exponential curve of the first growth phase crosses the y-axis.

Table 3. Description of the files of microCalCurve

File I/O	
Input file	Name of the MS Excel file
Load Excel file	Button that opens new dialogue for selecting input file
Get data	Button to load worksheet from MS Excel input file
List of worksheets	List of all worksheets in the MS Excel input file to choose from
Data	
Input data table	Table with three columns corresponding to sample name and unit name. The third column consists of check-boxes. All checked samples are included in the derivative analysis
Derivative analysis	
Derivative analysis	Button that starts the analysis.
Individual images	If checked results of analysis are saved in separate images. Otherwise all samples are saved in one image file.
Sample parameters	***Volume*** (sample parameter): sample volume; information is used to normalize results to units per ml. Default value is 2 ml.
	Init time (sample parameter): time between the preparation of the sample and starting microcalorimetric experiment. Data is added to the time axis of microcalorimetric curves. This is necessary to obtain correct values for parameters in the time domain: length of lag phase, etc.
Filter parameters	***Filter parameters***: Savitzky-Golay filter is used for a smoothing purpose on the microcalorimetric signal. This is required for successful detection of local extremes, inflection points and other points required for the analysis of microcalorimetric curves. The performance of the filtering can be adjusted using several parameters.
	Repeats (filter parameter): number of times the filter is used. Higher value results in smoother signal. Default value is 5. Value 0 means no filter has been used.
	Polynomial order (filter parameter): polynomial order of Savitzky-Golay filter. Default value is 3.
	Frame size (filter parameter): frame size of Savitzky-Golay filter. Frame size must be an odd number greater than the polynomial order. Default value is 5.

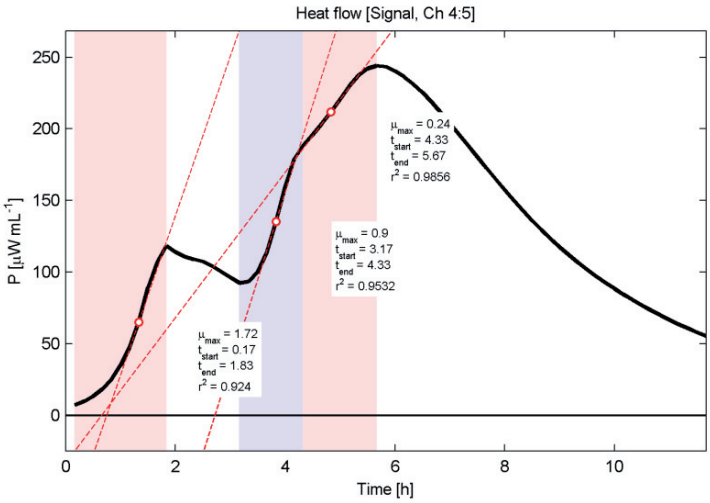

Figure 7. Image of the derivative analysis results of one single microcalorimetric curve by microCalCurveGUI tool

4.1.1 Performance of *microCalCurveGUI*

The derivative analysis of microcalorimetric curves is based on finding local extremes, inflection points and other specific points, i.e. points where the first, second or third derivative is zero. Using these points, the microcalorimetric curve is split into one or more growth phases. In the most primitive situation, the growth phase starts in the bottom of the curve (local minima) and ends at the top of the curve (local maxima), as in the example in Figure 8a. If there is more than one local minima or maxima in the microcalorimetric curve, more than one growth phase has been detected (Figure 8b). The distance between local maxima and minima is disregarded, as there is no growth and the microcalorimetric curve is decreasing. In more complicated situations, exponential phases alternate without any decrease in microcalorimetric signal (Figure 8c), i.e. there is no pause between two phases. Points where the third derivative is zero are used to detect these growth phases.

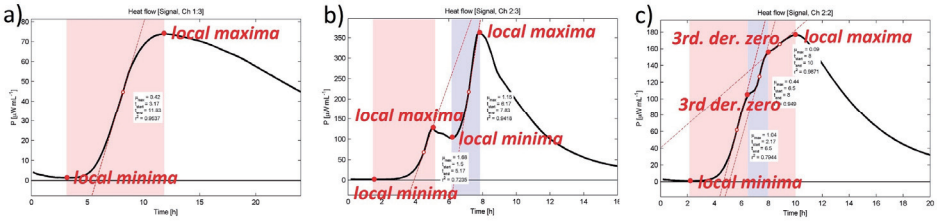

Figure 8. Results of the derivative analysis written into an MS Excel output file

Due to the noise in the signal, it is very likely that algorithm searching for local extremes (or other interesting points) produces more results than are necessary for detecting growth phases in microcalorimetric curves. To reduce the number of non-specific points, microcalorimetric signals have been applied for Savitzky-Golay filtering. Additionally, all detected points where the first, second or third derivatives are zero that are located before the first local minima or after the total maxima have been disregarded.

4.2 Analysis of growth of LAB in milk

4.2.1 Effect of milk heat treatment (Publication I)

The growth of ST12 in milk with different heat treatments and different fat contents was studied. The typical dual-peak power-time curve of the growth of *St. thermophilus* ST12 was registered in reconstituted milk prepared from LHSMP, and in pasteurized and UHT milk, although with different curve shapes (Figure 9). In RSM the heat evolved during the first exponential phase was 6.5% of the total, whereas the contribution of this phase was only 1.1-1.9% in the case of other milk samples. There were no significant differences in Q_{tot} (6.5-6.7 J mL^{-1}) between pasteurized milk with 0.05%, 2.5%, 3.5% fat content and RSM ($p > 0.05$), but the total evolved heat Q_{tot} was the smallest in UHT milk (5.9 J mL^{-1}).

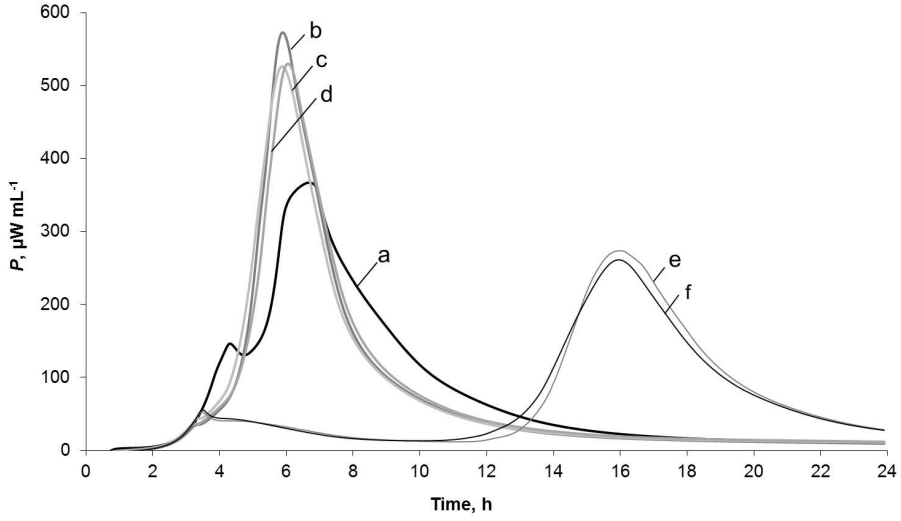

Figure 9. Thermal profiles of differently pretreated milk inoculated with thermophilic lactic starter bacteria Streptococcus thermophilus ST12. Mean power-time curves (n = 3) of milk prepared from low-heat skim milk powder (a), pasteurized milk with 0.05% fat content (b), 2.5% fat content (c), 3.5% fat content (d), and UHT milk with 0.05% fat content (e), 3.5% fat content (f)

The mean times needed to reach the maximum heat production rate (t_{Pmax}) were approximately 6 h for pasteurized milk with 0.05%, 2.5% and 3.5% fat content, and significantly longer (about 16 h) for UHT milk with 0.05% and 3.5% fat content. These data clearly indicated the effect of different heat treatments of milk on the growth patterns of ST12.

Table 4. Parameters describing Streptococcus thermophilus ST12 growth in differently pretreated milk samples: means of maximum specific growth rate (μ_{max}), heat evolved during the exponential growth phase (Q_{exp}), total heat registered (Q_{tot}) and time at maximum heat production rate (t_{Pmax}) obtained from microcalorimetric power-time curves

Sample	μ_{max}, Wh^{-1}		Q_{exp}, J mL^{-1}		Q_{tot}, J mL^{-1}	t_{Pmax}, h
	1st exp. phase	2nd exp. phase	1st exp. phase	2nd exp. phase		end of 2nd exp. phase
RSM	1.72^{a}	1.38^{a}	0.44^{a}	2.43^{a}	6.73^{a}	6.69^{a}
Past 0.05%	2.03^{b}	1.80^{b}	0.07^{b}	1.89^{b}	6.51^{a}	5.88^{b}
Past 2.5%	2.03^{b}	1.55^{c}	0.08^{c}	2.05^{c}	6.51^{a}	5.88^{b}
Past 3.5%	1.94^{c}	1.47^{c}	0.07^{b}	2.01^{bc}	6.58^{a}	6.05^{b}
UHT 0.05%	2.03^{b}	1.22^{d}	0.10^{d}	2.39^{a}	5.98^{b}	15.87^{c}
UHT 3.5%	1.99^{bc}	0.98^{e}	0.11^{e}	2.41^{a}	5.88^{b}	16.02^{c}

For *St. thermophilus* ST12, the calculated μ_{max} in the first exponential growth phase was higher than in the second exponential phase in all milk samples (Table 4). These results are in agreement with the results obtained earlier (Letort et al., 2002). During the first exponential growth, such readily available nitrogen sources as free amino acids and short peptides were preferably metabolised until some amino acids became growth limiting and the culture entered a post-exponential growth phase in which the synthesis of extracellular proteases was needed and initiated for the production of free amino acids. In the second exponential phase, the proteolytic system of ST12 was able to supply sufficient peptides, although with lower maximum growth rates, probably due to the limited capacity of the peptide uptake systems (Letort et al., 2002; Sieuwerts et al., 2008).

It has been reported that the growth of several strains of LAB is identical in whole milk and in skim milk, but the organisms grow much better in milk heated at 115°C for 15 min compared to milk heated at 80 °C for 10 min (Foster, 1952). As seen from our data, *St. thermophilus* ST12 had a slightly higher μ_{max} in the first exponential growth phase in pasteurized skim milk (0.05% fat) in comparison with 3.5% milk. A remarkable decrease in the value of μ_{max} in the second exponential growth phase was also observed in pasteurized and UHT milk with 3.5% fat content compared with low-fat samples: 1.94 ± 0.02 h^{-1} and 1.47 ± 0.01 h^{-1} in the case of 3.5% and low fat samples of pasteurized milk, respectively, and 1.99 ± 0.05 h^{-1} and 0.98 ± 0.05 h^{-1} in the case of 3.5% and low fat UHT milk, respectively (Table 4).

The length of the lag-phase was similar in all milk samples, but the second exponential growth phase was markedly delayed in UHT milk. The poor growth of *St. thermophilus* ST12 during the second exponential phase in UHT milk can be explained by the specific amino acid requirements which could not be met by the proteolytic action of the bacteria, considering that casein micelles in heated milk are coated with denatured whey proteins (Vasbinder et al., 2003).

For *Lb. paracasei* S1R1, the power-time curves corresponded to the two-stage growth pattern in pasteurized milk and multiphase growth in RSM. *Lb. paracasei* S1R1 was characterized by low μ_{max} in the second exponential growth phase in pasteurized milk with 2.5% fat content and three times lower in reconstituted milk (data not shown). No heat production was recorded in UHT milk with 0.05% fat and negligible growth was observed in UHT milk with 3.5% fat (Figure 2 in Publication I).

4.2.2 Effect of oxygen (Publication II)

Characteristic diauxic growth was observed during the growth of the yoghurt starter culture in milk with oxygen present at the beginning of growth, or in milk that was de-aerated by flushing the nitrogen gas before inoculation.

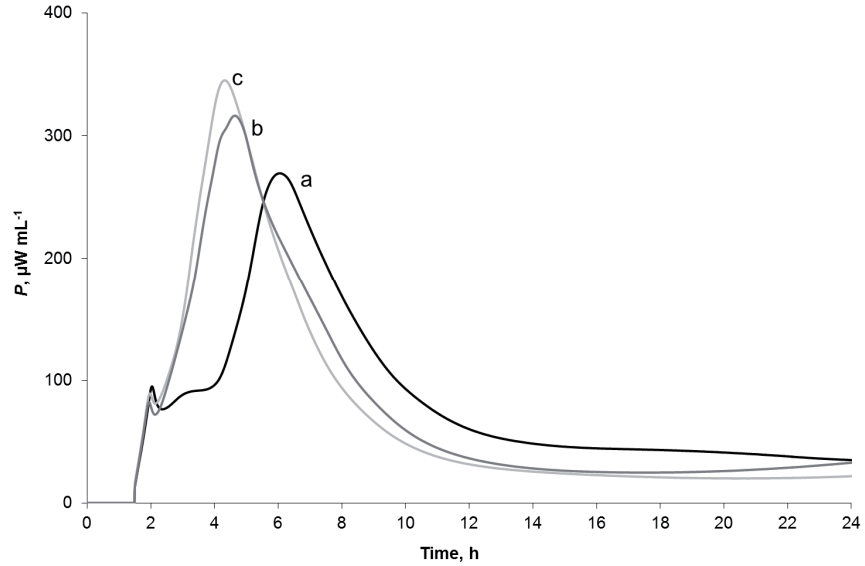

Figure 10. Characteristic thermal profiles of milk inoculated with thermophilic lactic starter. Mean power-time curves (n=3) of a) UHT 3.5% fat milk control samples, b) UHT 3.5% samples treated with catalase and c) UHT 3.5% samples sparged with N_2 before inoculation

The thermal profiles of thermophilic mixed starter growth in UHT milk treated with catalase and samples sparged with N_2 before inoculation were similar to the typical diauxic growth patterns observed with ST12.

The first, smaller peak did not change after the milk treatment with catalase or N_2 (Figure 10b and 10c). However, the lag-phase between the first and the second growth phases disappeared in 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 h^{-1} and 1.06 ± 0.31 h^{-1} in the catalase and N_2-treated milk samples, respectively. The amount of the bacteria formed during fermentation expressed by Q_{tot} did not change upon the aforementioned treatments, according to ANOVA (data not shown). Milk can contain about 10 mg L^{-1} of oxygen introduced during milking and further processing (Siegenthaler & Kosikowski, 1969). Gilliland & Speck (1969) showed 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 environment. 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 way similar to that shown for the glucose oxidase and catalase systems (Parpinello et al., 2002), but indigenous milk enzymes are not likely to sustain the UHT process. On the other hand, some lactobacilli can produce H_2O_2 during their growth (Ito et al., 2003; Juffs & Babel, 1975), which may turn self-inhibitory for them (Reiter & Härnulv, 1984), and thus the removal of the produced H_2O_2 by catalase may be another reason for the stimulated growth of starter bacteria. Based on the aforementioned studies, we assume that the removal of oxygen by N_2 and the elimination of possible H_2O_2 produced by starter lactobacilli by catalase treatment enhanced the growth of thermophilic starters in our experiments.

According to our data, the first exponential growth phase of the thermophilic starter containing *St. thermophilus* and *Lb. bulgaricus* was normally shorter and the calculated μ_{max} was higher than in the second phase (Table 1 in Publication II). It was also shown for *St. 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* has been shown to grow steadily in milk in the presence of *St. thermophilus*, exhibiting no diauxia until the end of fermentation (Courtin et al., 2002).

4.2.3 Effect of γ-irradiation of skim milk powder used for the preparation of reconstituted skim milk (Publication III)

The power-time curves describing the growth of *St. thermophilus* ST12 in milk reconstituted from non-irradiated and irradiated at 10 kGy low-heat skim milk powders (RSM and irrRSM, respectively) were remarkably different. 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, and the stopping of the growth at the end of the exponential phase was abrupt (Figure 11).

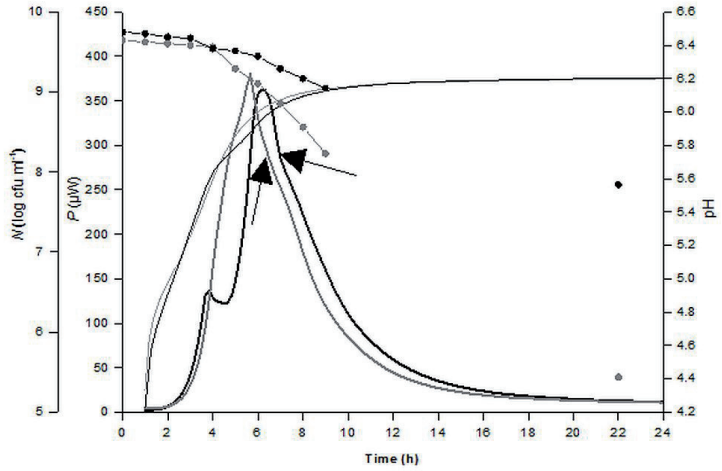

Figure 11. Calorimetric power-time (P) curves (bold lines) of the growth of Streptococcus thermophilus ST12, number of bacteria (N) calculated from the power-time curves (thin lines), and pH changes (circles) in reconstituted skim milk powder (RSM; black) and in irradiated RSM (grey) at an inoculation rate of 10^5 CFU mL^{-1}. Arrows mark the mean gelation times determined rheologically

Remarkable differences were observed in the acidification profiles of these two skim milk cultures, measured in parallel samples kept at the same temperature in an incubator. The initial pH of the sample of irradiated RSM (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 the fermentation and then more rapidly in the irrRSM than in the RSM samples. About one unit 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, both final values were clearly higher than the pH 4.0-4.2 usually observed at the end of the growth of the *St. thermophilus* (Zanatta & Basso, 1992). Differences in the acidification profiles are obviously linked to irradiation-related changes in casein and lactose in irrRSM.

The maximum specific growth rates of starter bacteria in the exponential growth phase (μ_{max}) in irrRSM were close to the values of the first fast exponential growth phase in RSM, although significantly different according to ANOVA (Table 1, Publication III). 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, Publication III). This justifies the hypothesis that peptides and amino acids would facilitate the fast growth of bacteria in reconstituted milk. The broad specificity of the peptide transport system of *St. thermophilus* and its capacity to use large peptides up to 23 amino acids (Juille et al., 2005) support the assumption that the growth of the bacteria in irrRSM during the first seven hours of fermentation would largely depend on the utilization 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 suggest specifically that the presence of short peptides and free amino acids in the growth medium can accelerate the growth of starter bacteria in milk, and the hydrolysis of caseins was presumably the rate-limiting step in the second exponential growth phase in RSM.

4.2.3.1 Growth differences depending on inoculation rate

The diauxic growth of ST12 at inoculation rate 10^5 CFU mL^{-1} observed in RSM and the disappearance of diauxy in irrRSM were seen at all inoculation rates studied (Figure 12A and B). It is also clear that the *power-time curves are strikingly similar* (see P_{max} value) in the case of all inoculation rates studied. This was not the case in our earlier studies with *L. lactis* in agar (Figure 12 C) (Kabanova et al., 2012) and gelatin gels (Kabanova, unpublished data), and it cannot be considered a trivial phenomenon. The key to understanding these facts lies in understanding the peculiarities of growth in cases with 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 to reach the final biomass density, and the amounts of growth by-products formed (primarily lactate) increase from zero to values corresponding to the final density of the biomass (10^9 CFU mL^{-1}). In the case of an inoculum of 10^1, the number of bacteria and the corresponding amounts of growth by-products increase through a range of 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 was not the case in the present study in the growth in milk: all μ_{max} were practically the same at all inoculation rates studied. This can be explained if we assume that the reconstituted milk has a higher buffering capacity in comparison with the solid state matrices of agar and gelatin, studied by us earlier (Kabanova et al., 2012).

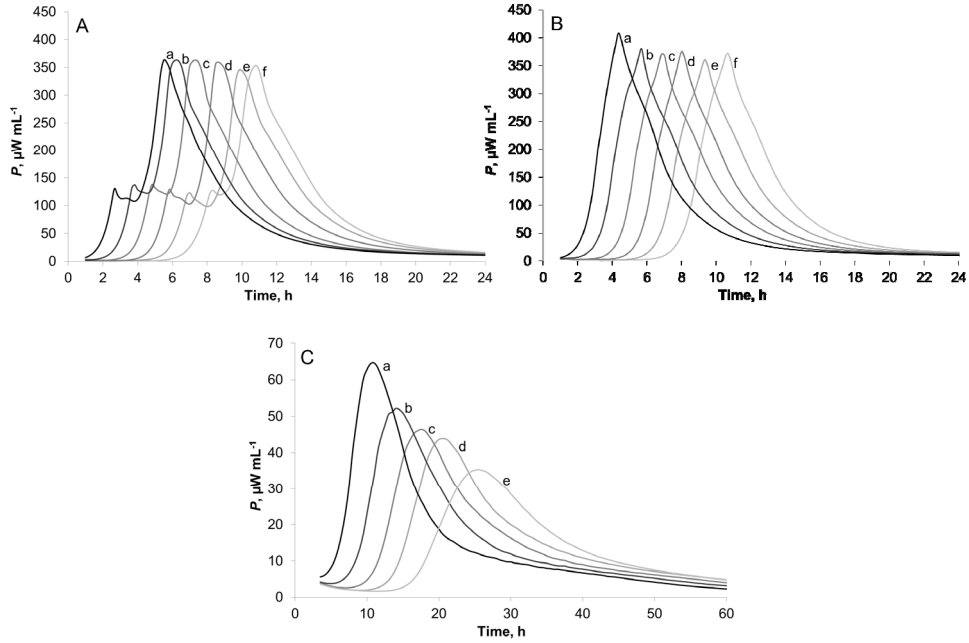

Figure 12. Power-time curves describing the growth of Streptococcus thermophilus ST12 in (A) reconstituted skim milk (RSM) and (B) irradiated RSM, as well as the growth of L.lactis in (C) carbohydrate-restricted medium broth at different inoculation rates (CFU mL^{-1}): a) 10^6; b) 10^5; c) 10^4, d) 10^3; e) 10^2; f) 10^1

As expected from the similarity of the power-time curves, and as seen in the data presented in Table 5, the maximum growth rates of the first exponential phase (μ_{max1}) were practically the same at different inoculation rates ranging from 10^6 to 10^2 CFU mL^{-1}, but differed in the two media, with slightly lower values (about 8%) measured in irrRSM than in RSM.

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 have been rather similar in both media studied; however, these were different with high confidence for the inoculation rates 10^6 and 10^2 CFU mL^{-1} according to the data in Table 1 (Publication III).

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

Table 5. Streptococcus thermophilus ST12 growth parameters in reconstituted skim milk (RSM) and in irradiated reconstituted skim milk (irrRSM) at 40°C. Means ± SD[a] of maximum specific growth rate (μ_{max}) in the first and second exponential growth phases, the heat evolved during the exponential phase (Q_{exp}) and the total heat produced during the whole fermentation (Q_{tot}) obtained from microcalorimetric power-time curves are presented

Milk	Inoculation rate (cfu mL^{-1})	μ_{max} (Wh^{-1})		Q_{exp} ($J\ mL^{-1}$)	Q_{tot} ($J\ mL^{-1}$)
		1st exp. phase	2nd exp. phase		
RSM	10^6	1.88^{aA}	1.05^{aA}	2.00^{aA}	7.12^{aA}
	10^5	1.85^{aA}	1.18^{bA}	2.10^{bA}	7.21^{aA}
	10^4	1.91^{aA}	1.17^{bA}	2.11^{bA}	7.12^{aA}
	10^3	1.85^{aA}	1.14^{bA}	1.97^{aA}	7.24^{aA}
	10^2	1.83^{aA}	1.13^{bA}	2.00^{aA}	7.23^{aA}
	10^1	1.61^{bA}	1.01^{cA}	2.26^{cA}	7.36^{aA}
irrRSM	10^6	1.72^{abB}	-	1.82^{aB}	6.83^{aA}
	10^5	1.68^{abB}	-	2.09^{bcA}	6.94^{aA}
	10^4	1.78^{aB}	-	2.10^{bcA}	6.96^{aB}
	10^3	1.72^{abB}	-	2.05^{cA}	6.98^{aB}
	10^2	1.66^{bB}	-	2.17^{bdB}	7.00^{aA}
	10^1	1.46^{cAB}	-	2.25^{dA}	7.14^{aA}

[a]Different superscript lower-case letters (a, b, c, d) 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

It can also be seen that the curves obtained for tenfold decreasing inoculation rates *are shifted at 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) change at regular intervals on changing the inoculation rates (Figure 12 A and B).

To understand this quite important peculiarity, it should be noted that the sensitivity of the TAMIII is 0.5 mW, and this heat flow corresponds to the growing of $>10^5$ bacteria. The instrument is capable of measuring the growth of bacteria at a level exceeding this threshold value. The similarity of the curves observed showed that the growth of the population in a range of 10^5–10^9 bacteria was taking place very similarly at different inoculation rates. However, at lower inoculation rates bacterial growth was recorded only after the number of bacteria had reached 10^5 CFU mL^{-1}, and it took time to lead effectively to the increase in the lag phase measured. The measured λ was, 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 can be concluded that 1.0–1.15 h was the shortest lag time observed. The lowering of the inoculation rate by a factor of 10 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 in Table 1 (Publication III), the shifts in l 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} reflected a multi-phased growth pattern, the fit obtained can be considered satisfactory.

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 were 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.

4.2.3.2 Differences in the utilization of carbohydrates and production of lactic acid

As seen in Figure 13, practically all of the lactose was hydrolyzed to glucose and galactose by the end of the exponential phases in RSM, except for at an inoculation rate of 10^1.

Glucose was consumed with simultaneous lactate production by the bacteria. About 10-20 mmol L^{-1} of glucose was utilized and about 20 mmol L^{-1} of lactic acid was formed by the end of the exponential phases for the inoculation rates of 10^3–10^6 CFU mL^{-1} in RSM. Galactose was not fermented by *St.thermophilus*. As seen in Figure 13, the amounts of accumulating galactose were lower than expected. This difference can be explained by the fact that not all possible (by-) products, such as polysaccharides, were determined and involved in the calculations.

As seen in Figure 13, the patterns of hydrolysis of lactose, utilization 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 hydrolyzed during the active growth phase of the bacteria in irrRSM, less than 15 mmol L^{-1} of glucose and of galactose was utilized by the bacteria, and about 10 mmol L^{-1} of lactic acid was formed. These data suggest that the lactose state and metabolism by ST12 is affected by irradiation.

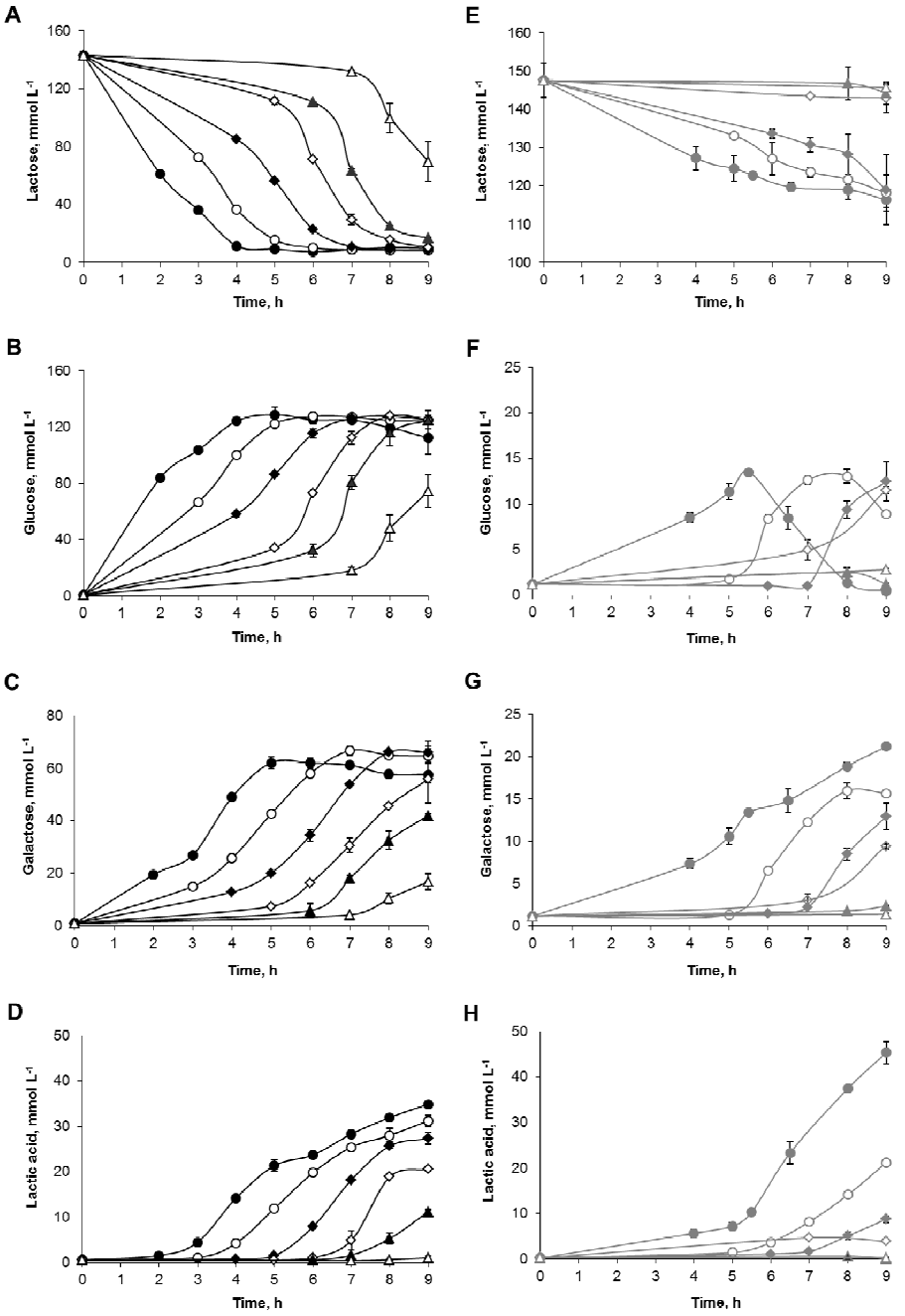

Figure 13. Comparison of lactose (A, E), glucose (B, F), galactose (C, G) and lactic acid (D, H) profiles during growth of Streptococcus thermophilus ST12 in RSM (A, B, C, D) and irrRSM (E, F, G, H) at different inoculation rates (cfu mL^{-1}): 10^6 (●), 10^5 (○), 10^4 (◆), 10^3 (◇), 10^2 (▲), 10^1 (△)

4.2.3.3 Change of free amino acids during fermentation

The changes in the concentrations of the individual free amino acids (FAA) during the fermentation of RSM and irrRSM with ST12 at the inoculation rate of 10^5 CFU mL^{-1} are shown in Figure 14.

At the beginning of the fermentations, the total concentration of FAA in irrRSM was about 10% higher than in RSM: 657 and 585 μmol L^{-1}, respectively (Table 1 and Table 2; Publication III).

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 ± 4 μmol L^{-1} in comparison to 270 ± 7 μmol L^{-1} in RSM. Higher contents of several other amino acids (histidine, glutamine, glycine, aspartic acid, alanine, leucine and phenylalanine) in irrRSM compared to RSM were also observed. In contrast, asparagine, ornithine, cysteine, methionine and tryptophane concentrations were lower in irrRSM than in RSM.

Periods from 3 h to 5 h of incubation in RSM and from 5 h to 7 h in irrRSM were characterized by a decrease in TFAA. The largest decrease was observed in the concentrations 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 7^{th} h of fermentation in irrRSM.

During the next period (approximately from 5-7 to 22 h), the intensive release of the majority of the amino acids from the caseins was observed, and the content of TFAA was significantly higher at 22 h of fermentation than at the beginning of fermentations: 1713 L^{-1} and 910 μmol L^{-1} in RSM and in irrRSM, respectively. The quantitatively dominant amino acids in the RSM at the end of the fermentation 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 dominant 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 and 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.

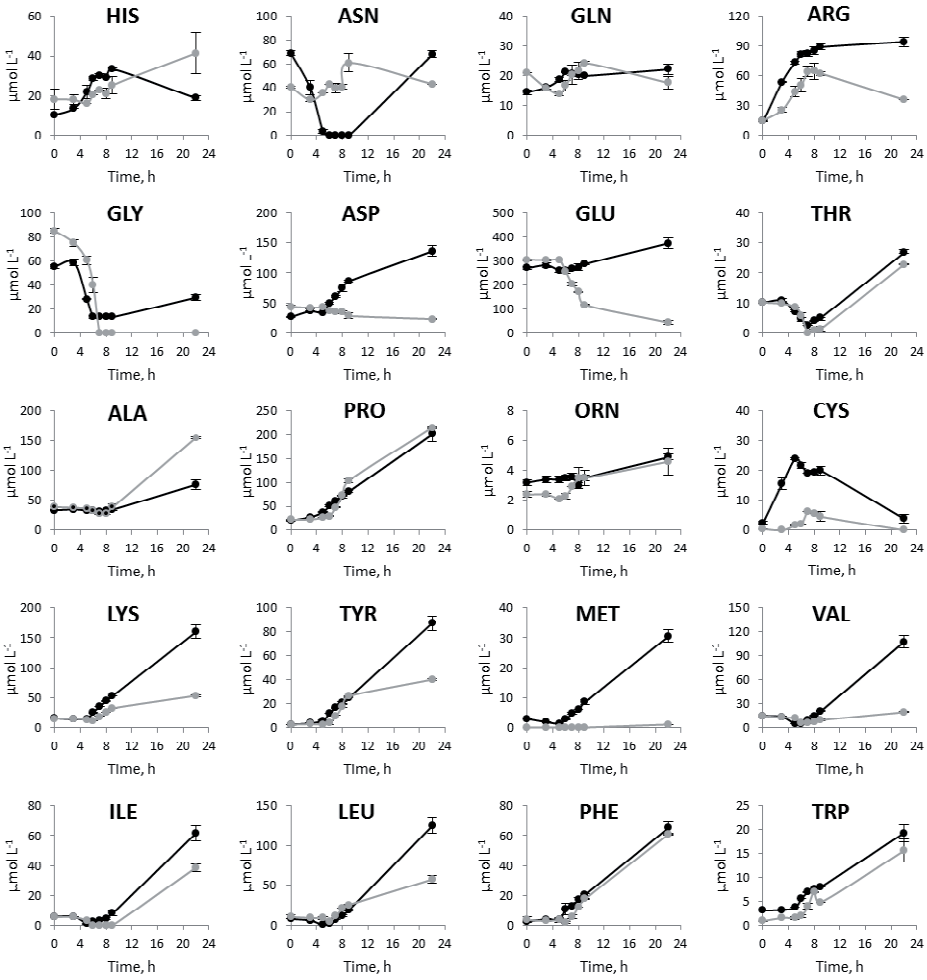

Figure 14. Changes in the concentrations of free amino acids during fermentation of reconstituted skim milk (RSM) (black) and irradiated RSM (grey) with Streptococcus thermophilus ST12 at an inoculation rate of 10^5 CFU mL^{-1}

4.2.3.4 Additional measurements for the characterization of effects of γ-irradiation

The differences in the growth behaviour of ST12 in milk reconstituted from non-irradiated and irradiated at 10 kGy LHSMP were also monitored by front-face fluorescence spectroscopy, and differences in coagulation processes were detected using dynamic rheological measurements.

At the outset, the effect of γ-irradiation on the physico-chemical and spectral properties of skim milk powder and reconstituted skim milk prepared from it was studied. Visually, 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 products (Day et al., 1957). The fluorescence intensity corresponding to the tryptophan emission maximum was threefold lower in irrLHSMP than in LHSMP, and the coordinates of the maxima $\lambda_{ex}/\lambda_{em}$ were 285/330 instead of 285/325 nm (Figure 15).

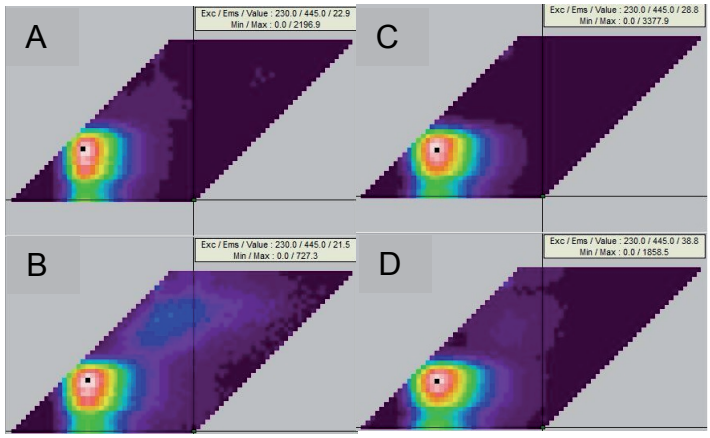

Figure 15. Spectral fluorescence signatures of (A) low-heat skim milk powder (LHSMP), (B) irradiated LHSMP, (C) reconstituted skim milk (RSM), and (D) irradiated RSM; the black dots correspond to the fluorescence intensity maxima

The decreased tryptophan fluorescence in irrLHSMP may be associated with the oxidation of aromatic amino acids or the production of other oxidation products (Stadtman & Levine, 2003) which can quench the fluorescence, or with conformational changes in 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 corresponds most probably to oxidation or Maillard reaction products (Becker et al., 2003; Liu & Metzger, 2007), can be observed on the SFS spectra of irrLHSMP, but not of LHSMP.

The development of tryptophan fluorescence intensity at maximum emission (285/330 nm) recorded continuously at 15 min intervals during the fermentation of RSM and irrRSM with ST12 at an inoculation rate of 10^5 CFU mL^{-1} is shown in Figure 16A. The 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 in pH in the irrRSM sample. Changes in tryptophan fluorescence intensity at maximum emission seemed to correspond to the modifications of the environment of the tryptophan residues caused by pH decrease during acidification, whereas changes in the protein network associated with the gelation phenomenon were not clearly distinguishable. The gelation points of RSM and irrRSM determined by rheological measurements (marked in Figure 16A) did not coincide with the same specific point on the tryptophan

fluorescence intensity change profile, but were different for the two milk samples. The change in tryptophan fluorescence intensity during fermentation with ST12 was similar to that shown in the case of milk acidified by glucono-d-lactone (Lopez & Dufour, 2001).

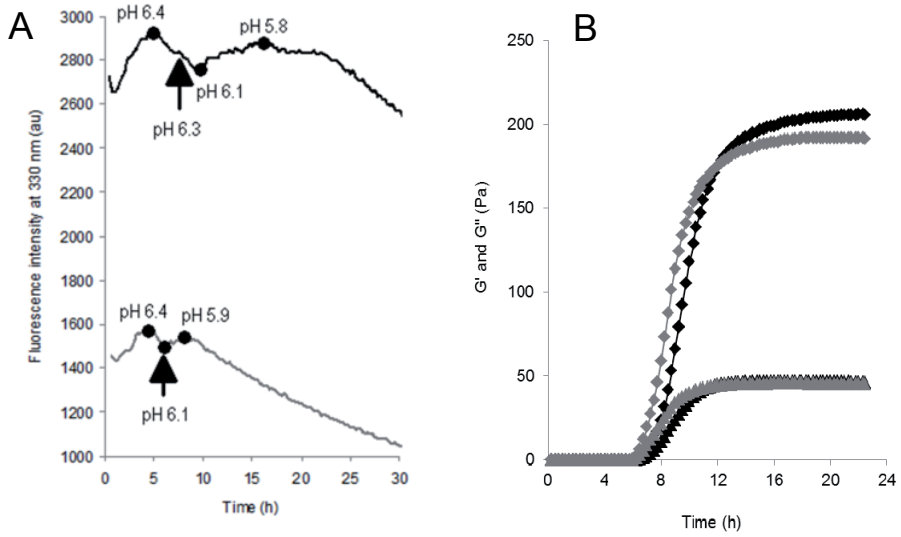

Figure 16. A) Development of fluorescence intensity at 285/330 nm during fermentation of reconstituted skim milk powder (RSM; black line) and irradiated RSM (grey line) with Streptococcus thermophilus ST12 at an inoculation rate of 10^5 mL^{-1}. Arrows mark the mean gelation times determined rheologically. B) 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}

The development of storage (G´) and loss (G´´) moduli during fermentation in both media with ST12 at an inoculation rate of 10^5 CFU mL^{-1} is shown in Figure 16B. Samples of RSM gelled after a 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 a firmer gel (186 ± 9 Pa and 177 ± 4 Pa in RSM and irrRSM, respectively). The 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 took place after the exponential growth (Figure 11). The data obtained indicate that the gelation as a result of fermentation was presumably not caused by a "simple" acidification but possibly also by enzymatic processes.

4.2.4 Evaluation of the growth of LAB in renneted reconstituted skim milk (unpublished data)

The representative calorimetric power-time curves of the growth in *Streptococcus thermophilus* ST 12 in non-irradiated (A) and irradiated (B) reconstituted skim milk (RSM) and in RSM with rennet addition (r-RSM) at different inoculation rates, from 10^6 to 10^1 CFU mL^{-1} with increments of 10-fold, are presented in Figure 17. It can be clearly seen that two distinct types of power-time curves were measured, depending on the relative contribution of bacteriological acidification vs. renneting to gel formation.

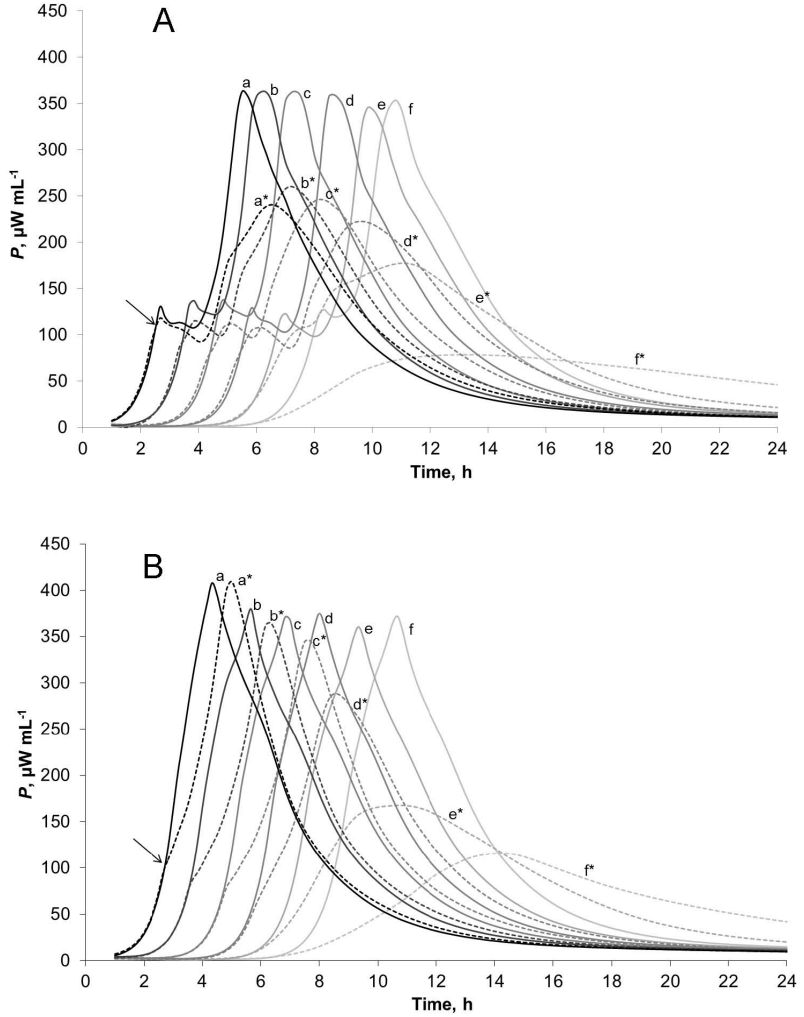

Figure 17. Calorimetric power-time curve of the growth of Streptococcus thermophilus ST12 in non-irradiated (A) and irradiated (B) reconstituted skim milk (bold lines) and in RSM with rennet addition (dashed lines) at different inoculation rates (CFU mL^{-1}): a) 10^6; b) 10^5; c) 10^4; d) 10^3; e) 10^2; f) 10^1. Asterisks (*) mark the renneted samples

According to Figure 17, the growth of ST12 in RSM and r-RSM was quite similar at the beginning, although starting from a certain number of the bacteria in the samples (from 0.63 × 10^7 CFU mL^{-1} at 10^1 CFU mL^{-1} to 6.12 × 10^7 CFU mL^{-1} at 10^6 CFU mL^{-1}), at the power-time curves "deviation moments" (marked by arrows) the maximum specific growth rate (μ_{max}) in r-RSM decreased.

As shown in Figure 18, the calorimetric power-time curves in RSM were divided into five phases (I: lag-phase, II: first exponential growth phase (a smaller peak), III: non-exponential phase, IV: second exponential growth phase (a major peak), and V: deceleration phase). The second exponential growth phase in r-RSM separated into faster (IV´) and slower (IV´´) phases.

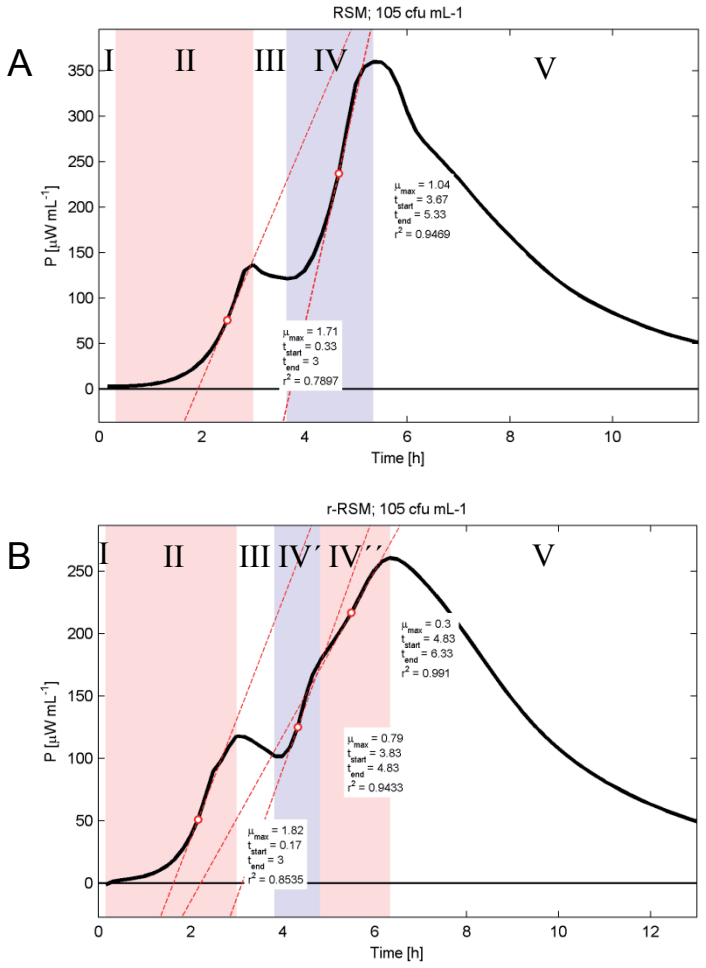

Figure 18. The derivative analysis of the power-time curves of the growth of Streptococcus thermophilus ST12 in RSM (A) and in r-RSM (B) at an inoculation rate of 10^5 CFU mL^{-1} by a microCalCurveGUI tool

Table 6. Streptococcus thermophilus ST12 growth parameters in renneted reconstituted skim milk (r-RSM) and in renneted irradiated reconstituted skim milk (r-irrRSM) at 40°C [a]

Milk	Inoculation rate (cfu mL^{-1})	μ_{max} (Wh^{-1})		Q_{exp} (J mL^{-1})	Q_{tot} (J mL^{-1})	$N_{exp,} \times 10^8$ (cfu mL^{-1})	P_{max} (μW mL^{-1})	t_{Pmax} (h)	λ (h)
		1st exp. phase	2nd exp. phase						
r-RSM	10^6	1.89^{abA}	1.00^{aA}	2.41^{aA}	7.02^{aA}	5.42^{aA}	240.67^{abA}	6.50^{aA}	0.04^{aA}
	10^5	0.26^{aA}	1.06^{aA}	2.36^{aA}	6.92^{aA}	5.30^{aA}	260.32^{aA}	7.17^{aA}	1.15^{bA}
	10^4	1.89^{abA}	0.94^{aA}	2.26^{aA}	6.89^{aA}	5.08^{aA}	247.32^{aA}	8.17^{bA}	1.96^{cA}
	10^3	1.88^{abA}	0.93^{aA}	2.28^{aA}	7.12^{aA}	5.13^{aA}	222.73^{bA}	9.59^{cA}	2.97^{dA}
	10^2	1.67^{bA}	0.49^{bA}	2.45^{aA}	7.35^{aA}	5.51^{aA}	177.90^{cA}	11.00^{dA}	3.87^{eA}
	10^1	1.12^{cA}	-	1.37^{bA}	6.47^{aA}	3.12^{bA}	78.75^{dA}	13.2^{eA}	4.64^{fA}
r-irrRSM	10^6	1.70^{aB}	0.65^{aB}	2.08^{aB}	6.88^{aAB}	4.67^{aB}	409.68^{aB}	5.00^{aB}	0.05^{aA}
	10^5	1.73^{aAB}	0.56^{bcB}	2.07^{aB}	6.76^{bA}	4.66^{aB}	364.52^{bB}	6.25^{bB}	1.06^{bA}
	10^4	1.75^{aB}	0.55^{bB}	2.06^{aA}	6.78^{bA}	4.64^{aA}	345.62^{bB}	7.60^{cB}	2.29^{cB}
	10^3	1.82^{aA}	0.61^{acB}	1.79^{bB}	6.58^{bB}	4.02^{bB}	288.29^{cB}	8.50^{dB}	3.51^{dB}
	10^2	1.42^{bB}	-	1.86^{bA}	6.62^{bA}	4.17^{bA}	167.68^{dB}	10.67^{eA}	4.26^{eB}
	10^1	0.84^{cB}	-	1.59^{cA}	6.06^{bA}	3.57^{cA}	116.02^{eA}	14.33^{fA}	4.69^{fA}

[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 total heat produced during the whole fermentation (Q_{tot}), the number of bacteria at the end of exponential growth phase (N_{exp}), maximum heat flow (P_{max}), time at 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 r-RSM and r-irrRSM samples at the same inoculation rates

Table7. The t-test analysis denoting the differences between liquid and renneted RSM and irrRSM samples

Milk	Inoc. rate (CFU mL^{-1})	μ_{max} 1st exp. phase	μ_{max} 2nd exp. phase	Q_{exp}	Q_{tot}	N_{exp} $\times 10^8$	P_{max}	t_{Pmax}	λ
RSM vs r-RSM	10^6	NS	*	*	*	*	**	***	NS
	10^5	NS	**	*	NS	*	***	***	NS
	10^4	NS	**	NS	NS	*	*	*	*
	10^3	NS	**	**	NS	**	***	**	NS
	10^2	NS	**	*	NS	*	**	*	NS
	10^1	**	-	*	*	*	**	*	NS
irrRSM vs r-irrRSM	10^6	NS	-	NS	NS	NS	NS	***	NS
	10^5	NS	-	NS	NS	NS	NS	*	NS
	10^4	NS	-	NS	NS	NS	NS	*	NS
	10^3	NS	-	**	**	**	**	***	NS
	10^2	NS	-	*	*	*	***	***	NS
	10^1	**	-	*	**	*	**	**	*

***p<0.001, **p<0.01, *p<0.05, NS: not significant

As seen from the data presented in Table 6 and Table 7, the maximum growth rates of the first exponential phase (μ_{max1}) were very similar (p>0.05) in RSM and r-RSM at different inoculation rates, ranging from 10^6 to 10^2 CFU mL^{-1}, except for 10^1 CFU mL^{-1}. The values of maximum growth rates of the second exponential phase (μ_{max2}) in r-RSM were significantly lower (p<0.01).

According to Table 6, the average amounts of heat produced during the exponential phase ($Q_{exp,\ average}$) in r-RSM at inoculation rates ranging 10^6–10^2 CFU mL^{-1} was 2.35 J mL^{-1} (corresponds to 5.30×10^8 CFU mL^{-1}), which was about 13% higher than in RSM. This shows that during the exponential growth phase more bacteria were produced in r-RSM. This result is in agreement with data presented by Favrot & Maubois (1996), where the maximum population level of *L.lactis* was also higher in renneted curd compared with liquid milk.

The amount of heat produced during the total growth (Q_{tot}) was around 7.14 ± 0.09 J mL^{-1}, which corresponds to the number of bacteria 1.62×10^9 CFU mL^{-1} in both r-RSM and RSM at inoculation rates ranging 10^5–10^2 CFU mL^{-1}. This indicates that the numbers of bacteria growing at the end of fermentation (at 22 h) were similar in both media studied. According to the data presented in Table 5, Q_{tot} in RSM and r-RSM at the inoculation rates of 10^6 and 10^1 CFU mL^{-1} were statistically different. The Q_{tot} and N_{tot} values had the lowest value in r-RSM at the inoculation rates of 10^1 CFU mL^{-1}, 6.47 ± 0.06 J mL^{-1} and 1.45×10^9 CFU mL^{-1}, respectively.

P_{max} values in r-RSM were lower compared to RSM (Figure 17A). The most likely explanation for the inhibition of ST12 growth in r-RSM is the colonial

growth of bacteria in a structured matrix. According to statistical analysis (Table 7), no major differences were observed in the lag-phase (λ) durations in the two media studied (liquid *vs* renneted samples). The time it took to reach the maximum heat effect (t_{Pmax}) increased with decreasing amounts of inoculum (Table 6). As seen in Table 6, there were significant differences noted in t_{Pmax} between the r-RSM and RSM samples. Depending on the inoculation rate, 1 to 2.5 h more time was required to reach the maximum heat production rate (t_{Pmax}) in r-RSM (Table 6) compared to RSM (Table 1 in Publication III).

4.2.4.1 Utilization of carbohydrates and production of lactic acid in renneted RSM

Lactose consumption and lactate production per biomass formed (mmol/gdw) were calculated (see section 3.6) and are presented in Figures 19 A and B. Data presentation starts from the fourth hour to eliminate measurement errors for the determination of small amounts by HPLC. The calculated polysaccharide (PS) production per biomass produced (C), lactate yield per lactose consumed (D) and biomass yield per hexose consumed (E) are also presented in Figure 19. Figure 19 F provides a schematic illustration of lactose degradation; the dashed rectangular depicting the intracellular splitting of lactose and glycolysis, lactose, glucose_out, galactose_out and lactate are measured parameters, while polysaccharides (PS) is a calculated parameter.

During the growth of *St. thermophilus* ST12 in skim milk, lactose was consumed very fast (up to 1800 mmol/gdw in renneted RSM), and in RSM the lactose consumption rate exceeded that in irrRSM by several times. Lactose consumption led to the accumulation of galactose, as well as glucose, due to low glycolytic activity, reflected by the lactate yield per lactose below 0.4 mol/mol in irrRSM and 0.2 mol/mol in RSM (theoretical maximum 4 mol/mol), respectively. It can be assumed that galactose was not metabolized by this strain; however, the accumulation of galactose was lower than predicted compared to the consumption of lactose. This indicates that either galactose was consumed through glycolysis, or it was used for polysaccharide production. Usually polysaccharides are produced in growth-restricted conditions to synthesise carbon storage substances for the future. More limited availability of free amino acid in RSM than in irrRSM can be a stress factor for the cells, producing more unknown products (polysaccharides) in RSM than in irrRSM.

Taking into account that only glucose was metabolized through glycolysis, it can be seen that in RSM lactate yield per consumed glucose (up to 7 mol/mol) was higher than the theoretical value (2 mol/mol). This may indicate that lactate was also produced from amino acids and/or from galactose. In irrRSM, the lactate yield per consumed glucose was lower than the theoretical value and this can be explained assuming that such by-products as exopolysaccharides were formed from utilized glucose.

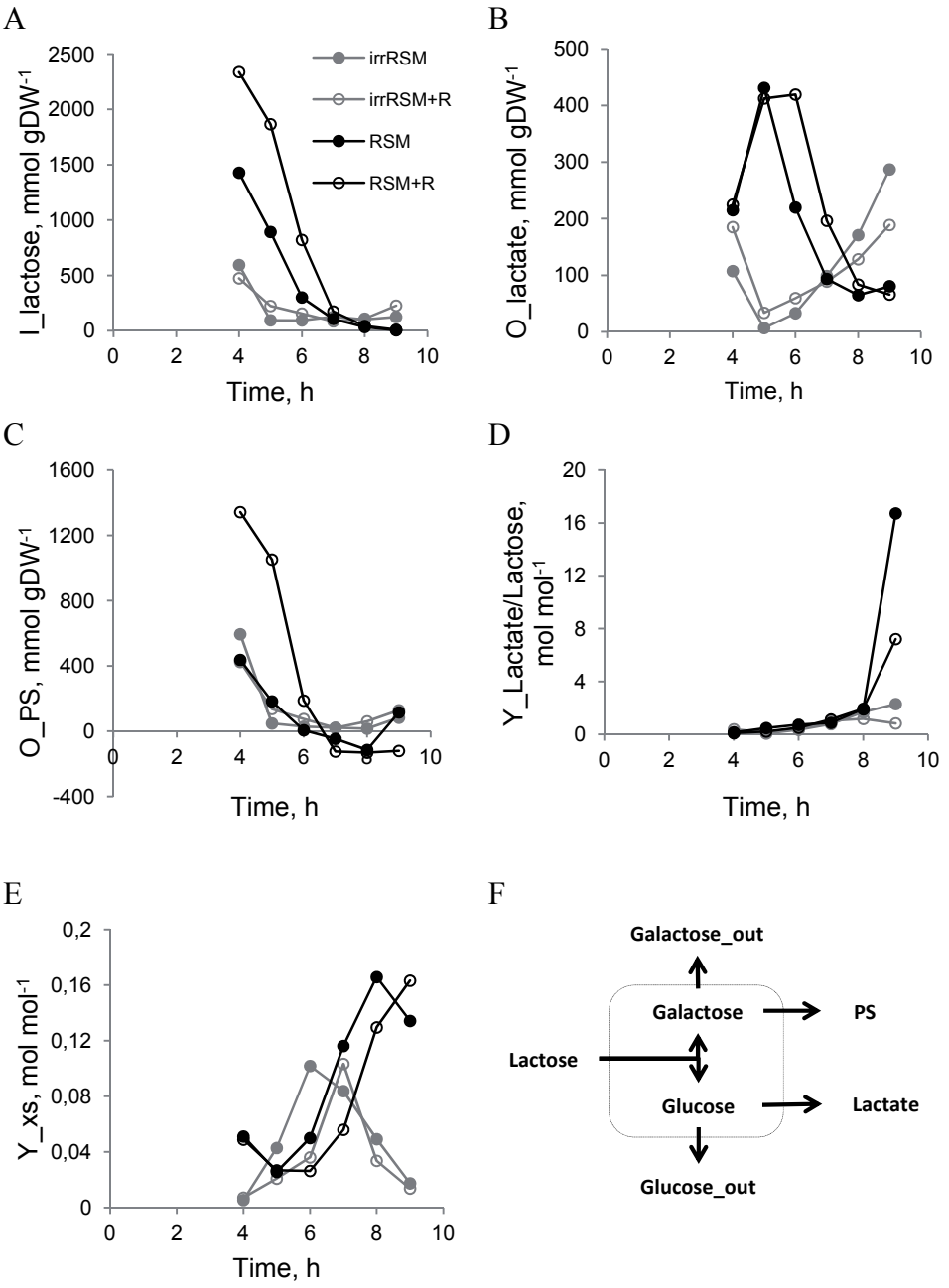

Figure 19. Metabolic characteristics of Streptococcus thermophilus ST12 during the growth in non-irradiated (black) and irradiated (grey) milk with (circles) and without (filled circles) rennet addition.

There was no clear difference between renneted and non-renneted milk. This may be due to the fact that fast acidification led to curd formation within the first four hours of growth. However, in the renneted skim milk, the lactose

consumption was delayed for about 1 h, which can be seen from the lower growth rate value and lactose consumption during the first 5 hours and vice versa between 6-9 h.

4.2.4.2 Change in free amino acids during fermentation in renneted RSM

Table 8 shows amino acid consumption during the 1st and 2nd growth phases. Although *St. thermophilus* has not been shown to be auxotrophic for any single amino acid, growth is abolished when both Glu/Gln and Cys/Met are removed (Letort et al., 2002). In addition, the omission of Leu and Val reduces the growth rate more than three times and only six amino acids (Asn, Ala, Ile, Gly, Ser and Thr) can be individually omitted without any effect on the growth rate.

Most of the trends of FAA during the growth of ST12 in RSM, irrRSM and those with rennet addition were comparable; however, during the first three hours more free amino acids per biomass formed were consumed in irrRSM (most of the amino acids were supplied in free form to satisfy requirements for biomass protein synthesis) than in RSM. The only amino acids consumed in amounts corresponding to the calculated requirements for biomass synthesis in the 1st exponential phase were Gly, Glx (Gln+Glu) and Asx. In irrRSM, the consumption of free Gly and Glx were also sufficient for biomass synthesis in the 2nd growth phase (~6 h). These amino acids were also the only amino acids whose concentrations in milk exceeded the amount required for biomass synthesis (Table 9). The amount of free His was also sufficient to satisfy biomass requirements, although the amount of this amino acid did not decrease during the growth. Most of the concentrations of other free amino acids could not support the growth of cells more than 30% if we look at the biomass concentration in irrRSM, which was 1.4 times higher at the 8th h than that in RSM (0.14 vs 1.0 g/L, respectively). Free Ala, Arg and Pro could support amino acid requirements for over 40% of the biomass synthesis; however, concentrations of the amino acids increased during the growth. The results showed that more free amino acids in irrRSM (initially) supported the faster growth of cells but the consumption per biomass (mmol/g) remained similar. In renneted milk samples more amino acids were released from proteins, especially in the second growth phase, which led to the accumulation of amino acids in the media and, due to this, it was not possible to analyse consumptions of amino acids in the second exponential growth phase.

Overconsumption of Glu(tamate) 5-7 times exceeding the need for biomass synthesis indicates the potential of the synthesis of other amino acids from it. In total, 4.6 mmol/g of amino acids were incorporated into the biomass. Of the total, 30% was covered by the consumption of free amino acids (excluding Glu). If we take into account Glu overconsumption and amino group transfer to other amino acids, Glu can provide ammonia for 25% of amino acids for biomass. The remaining 45% of amino acids must be derived from peptides.

Table 8. Relative consumption of free amino acids (RfAA)[a] compared to that required for the synthesis of biomass proteins (%). Green colour shows that free amino acids were consumed more than required for biomass synthesis. Amino acid composition of biomass is taken from the literature (Adamberg et al., 2012)

	RSM				irrRSM			
Time (h)	3	5	8	22	3	5	8	22
His	-579	-395	-18	165	-228	-33	-59	-292
Asn	2014	305	-9	-322	311	14	-55	98
Gln	61	-104	8	-14	126	-9	-45	51
Arg	-1550	-243	-17	-28	-1441	-94	-67	174
Gly	109	251	-5	-35	1142	99	110	-2
Asp	-249	-89	-134	-241	218	21	39	44
Glu	-302	375	-108	-481	2060	296	761	599
Thr	9	51	-2	-73	202	19	20	-102
Ala	-11	11	-2	-68	180	14	1	-265
Pro	-498	-241	-121	-680	201	-42	-348	-875
Cys	-5186	-381	91	618	-1320	-124	-125	261
Lys	69	-56	-49	-260	115	-1	-42	-69
Tyr	-52	-142	-79	-527	96	-22	-163	-160
Met	179	-28	-58	-342	60	5	-4	-6
Val	109	64	-32	-245	222	15	1	-38
Ile	105	26	-14	-203	160	11	5	-203
Leu	122	19	-30	-277	133	0	-46	-112
Phe	-9	-69	-43	-251	177	0	-71	-336
Trp	34	-76	-27	-163	-264	-34	-64	-203
	RSM+rennet				irrRSM+rennet			
His	-35	-587	-38	-60	668	88	-75	-77
Asn	402	231	-24	-110	-116	-30	-14	-10
Gln	3	-111	8	-7	285	29	-12	45
Arg	-287	-251	-100	-252	-888	-113	-143	-1451
Gly	39	257	4	4	1090	162	91	0
Asp	-38	31	-128	-161	433	53	8	88
Glu	69	13	-205	-27	2369	312	68	42
Thr	9	40	8	-5	150	22	9	-110
Ala	5	38	1	-13	178	26	11	-226
Pro	-76	-200	-85	-295	-339	-98	-159	-1220
Cys	-916	-692	247	126	-1730	-263	-78	-291
Lys	22	0	-34	-83	146	18	-1	21
Tyr	0	-33	-52	-161	-13	-26	-67	-844
Met	15	18	-38	-82	57	6	-10	-224
Val	26	79	-19	-37	229	33	13	-35
Ile	31	22	-15	-231	168	22	6	-151
Leu	1	-125	-81	-397	-240	-125	-251	-2720
Phe	-24	-335	-144	-506	-441	-167	-295	-3283
Trp	10	-207	-59	-211	-573	-82	-18	-588

[a]calculated as $R_{fAA} = I_{AA} \times 100 / B_{AA}$. B_{AA} is the amount of corresponding AA in gram of biomass

Table 9. Milk composition, maximal amount of biomass that can be obtained from free amino acids in milk and relative amount of free amino acids supporting the synthesis of biomass proteins

	Composition of milk (C_{AA}), µM				biom. (B_{AA}), mmol/g[a]	Theoretical from free AA (X_{theor}), g/L[b]				Exp. biomass (X_{exp}) at 8 h, g/L[c]				Max amount of free AA ($R_{fAA\ max}$), %[d]			
	irrRSM	RSM	r-irrRSM	r-RSM		irrRSM	RSM	r-irrRSM	r-RSM	irrRSM	RSM	r-irrRSM	r-RSM	irrRSM	RSM	r-irrRSM	r-RSM
His	18.1	10.4	18.5	10.5	0.08	0.22	0.12	0.22	0.12	0.17	0.12	0.20	0.18	124	100	113	70
Asn	40.6	70.1	40.6	69.9	0.22	0.19	0.33	0.19	0.32	0.17	0.12	0.20	0.18	109	262	96	181
Gln	21.1	14.6	20.9	14.4	0.19	0.11	0.08	0.11	0.08	0.17	0.12	0.20	0.18	66	64	58	43
Arg	15.0	14.4	15.2	15.0	0.22	0.07	0.06	0.07	0.07	0.17	0.12	0.20	0.18	39	52	35	37
Gly	86.0	55.7	85.9	56.0	0.45	0.19	0.12	0.19	0.12	0.17	0.12	0.20	0.18	110	99	97	69
Asp	43.7	26.9	43.7	26.5	0.22	0.20	0.12	0.20	0.12	0.17	0.12	0.20	0.18	117	101	104	69
Glu	306	270	307	271	0.19	1.65	1.46	1.65	1.46	0.17	0.12	0.20	0.18	954	1176	847	817
Thr	10.2	10.2	10.2	10.1	0.30	0.03	0.03	0.03	0.03	0.17	0.12	0.20	0.18	19	27	17	19
Ala	39.1	33.6	39.1	33.6	0.64	0.06	0.05	0.06	0.05	0.17	0.12	0.20	0.18	35	42	31	29
Pro	22.3	20.7	21.9	20.8	0.18	0.12	0.11	0.12	0.11	0.17	0.12	0.20	0.18	71	92	62	64
Cys	0.4	2.2	0.1	2.5	0.03	0.01	0.08	0.00	0.10	0.17	0.12	0.20	0.18	8	67	2	53
Lys	15.2	15.9	15.1	15.9	0.42	0.04	0.04	0.04	0.04	0.17	0.12	0.20	0.18	21	30	18	21
Tyr	2.5	2.7	2.4	2.7	0.12	0.02	0.02	0.02	0.02	0.17	0.12	0.20	0.18	12	18	10	12
Met	0.0	3.0	0.0	3.0	0.07	0.00	0.04	0.00	0.04	0.17	0.12	0.20	0.18	0	36	0	25
Val	14.8	15.1	14.8	14.9	0.36	0.04	0.04	0.04	0.04	0.17	0.12	0.20	0.18	24	33	21	23
Ile	5.6	6.0	5.6	6.0	0.27	0.02	0.02	0.02	0.02	0.17	0.12	0.20	0.18	12	18	11	12
Leu	10.7	8.3	10.4	8.1	0.39	0.03	0.02	0.03	0.02	0.17	0.12	0.20	0.18	16	17	14	12
Phe	3.8	3.0	3.4	2.9	0.18	0.02	0.02	0.02	0.02	0.17	0.12	0.20	0.18	12	13	10	9
Trp	1.0	3.2	0.8	3.3	0.07	0.01	0.04	0.01	0.05	0.17	0.12	0.20	0.18	8	36	6	26

[a]biomass composition is taken from literature (Adamberg et al., 2012).
[b]calculated as $X_{theor} = (C_{AA}/1000)/B_{AA}$ indicating how much biomass can be produced from free amino acid present in milk assuming that amino acid is used only for the synthesis of biomass proteins. AA refers to corresponding amino acid.
[c]experimentally determined biomass concentration assuming that heat produced per cell is 4.45×10^{-9} J per cell and cell mass is 0.2×10^{-12} g.
[d]calculated as $R_{fAA\ max} = X_{theor} \times 100/X_{exp}$ indicating relative amount of amino acid in biomass proteins that can be supported by consumed free amino acids. Percentage below indicates that free amino acid was less consumed than required for synthesis of biomass proteins.

4.2.5 Evaluation of microbiological quality of raw milk produced in Estonia (Publication IV)

Milk is a growth medium for starter bacteria. Therefore, it is important to know the physico-chemical and microbiological properties of raw milk produced on farms.

The microbiological quality of raw milk is usually evaluated by total bacterial count (TBC) and somatic cell count (SCC), which are regularly checked by independent authorities (at the farm level) and by dairies (pooled milk). The maximum levels of TBC and SCC are fixed by EU regulations (EEC, 1992, Council Directive 92/46/EEC).

The main aim of this study was the determination of specific bacterial groups (LABC, PBC and ASFBC) in the bulk-tank raw milk of dairy farms (Figure 20). These technologically important microbes are not analysed routinely by dairies; however, the results obtained give valuable information on the quality, processability and stability of raw milk in most of Estonian dairy farms during a period of four years.

In addition, the same bacterial groups were determined from dairy silos, which provides information about changes (growth) in microflora occurring during transportation and the pooling of farm milk in large volumes (Table 10).

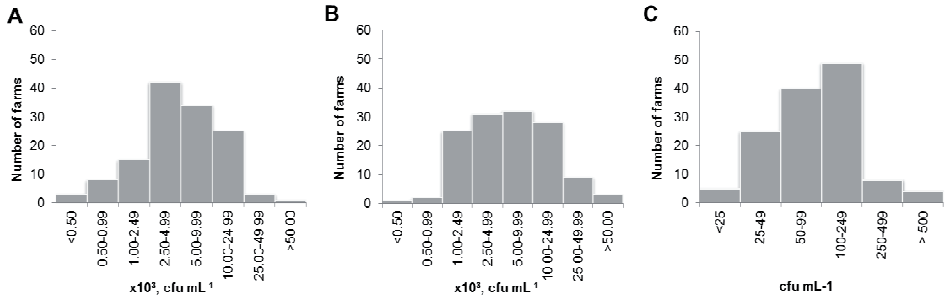

Figure 20. Histograms showing the relative distribution of three microbial groups in raw milk from Estonian farms between 2004-2007: A) lactic acid bacteria counts, B) psychrotrophic counts, C) aerobic spore-forming bacteria counts

4.2.5.1 Lactic acid bacteria

LAB are considered to be natural and generally desired microflora of raw milk. However, our results showed that lactic acid bacteria were the dominant bacterial group on only 24 farms (18% of the total) with an average of 7.6×10^3 CFU mL^{-1} (variation from 2.1×10^2 to 1.4×10^5 CFU mL^{-1}). In recent decades, the counts of lactic acid bacteria in farm bulk-tank raw milk have decreased by a factor of about 20 in Estonia (P.Elias, personal communication), possibly due to improved hygiene in milk production and fast cooling after milking.

Table 10. Average values for microbiological parameters of raw milk, from 2004 to 2007 (count CFU mL^{-1})

Parameter		Mean	Min	Max	Standard*
LABC	Dairy farm	7600	210	145000	(-)
	Auto	9274	1800	30436	
	Storage silos	12210	1350	134000	
PBC	Dairy farm	10000	424	64000	(-)
	Auto	17880	409	37295	
	Storage silos	56880	900	495000	
ASFBC	Dairy farm	131	5	836	(-)
	Auto	130	23	363	
	Storage silos	191	9	845	
TBC	Dairy farm	27000	5000	143000	100000
	Auto	29650	13000	60778	
	Storage silos	50421	20000	206000	
SCC	Dairy farm	277000	129000	514500	400000
	Auto	274880	165000	370830	
	Storage silos	279850	208500	339000	

LABC, lactic acid bacteria count; PBC, psychrotrophic bacteria count; ASFBC, aerobic spore-forming bacteria count; TBC, total bacterial count; SCC, somatic cell count.
* Current regulatory limits for total bacterial count (CFU mL^{-1}) and somatic cell count (cells mL^{-1}) in EU (EEC, 1992, Council Directive 92/46/EEC).
(-) No standard established.

4.2.5.2 Specific bacterial groups

Typical psychrotrophic microflora include *Pseudomonas* spp. and sporeforming bacteria.

Controlling the content of psychrotrophic bacteria is very important because these bacteria are able to produce heat-stable, extracellular proteases, as well as lipases. Proteases are associated with bitterness in milk, gelation of UHT sterilized milk, and reduced yields of soft cheese (Hantsis-Zacharov & Halpern 2007). Lipases, by hydrolyzing triglycerides, cause flavour defects associated with fat breakdown in cream, butter, cheese and UHT products (Hantsis-Zacharov & Halpern, 2007). In Europe, the psychrotrophic counts of raw milk can not exceed 50,000 CFU mL^{-1} (Cempírková & Mikulová, 2009). Lipolytic and proteolytic activities, supported by psychrotrophic bacteria, are considered insignificant at PC levels lower than 10^6 CFU mL^{-1} (Cempírková & Mikulová 2009). PBC levels in the majority of milk samples (98%) analysed by us were below 50,000 CFU mL^{-1}. Compared to farm bulk-tank raw milk, the PBC counts in dairy plant storage silos were higher (Table 10). The increased PBC count in plant storage silos may be caused by contamination during transportation and/or multiplication during cold storage of raw milk for two-three days. According to

molecular analyses, the genus *Pseudomonas* spp. was the dominant spoilage flora in Estonian farm milk, with *Pseudomonas fluorescens* as the prevailing species, which was also shown as true in France by Lafarge et al. (2004). The numbers of psychrotrophic bacteria were comparable to those of LAB.

Another group of undesirable microbes in raw milk include the aerobic spore-forming bacteria that may survive pasteurization and propagate in final products (Foltys & Kirchnerová, 2006). Barbano et al. (2006) stated that spores are typically present in low numbers in raw milk. The ASFBC found in our study were generally higher (a mean of 131 CFU mL^{-1}) than reported by Boor et al. (1998), with a mean of 49 CFU mL^{-1}. However, that study was conducted about 15 years ago. ASFB were found in raw milk samples from all farms during the whole period studied. The level of ASFBC from raw milk storage silos showed great variations over the sampling days (from 9 to 4.5×10^3 CFU mL^{-1}).

Although TBC is an important indicator of the overall microbial quality of raw milk produced, it does not provide additional information on the composition of this microflora. For example, if the majority of the microbes in milk with high TBC include psychrotrophic bacteria, there is a high risk of flavour defects and decreased yields in cheese or quark products. During 2004-2007, the average PBC exceeded that of LABC in Estonian farm milk.

The general microbiological quality of raw milk produced in Estonia is acceptable; however, there is a tendency for PBC to exceed the counts of LAB which may influence starter growth and product quality.

Based on the survey, dairy plants should better control their supply chains, and choose milk from the best farms (low PBC, ASFBC and SCC, and higher LABC) for the production of fermented products and cheese.

4.2.5.3 Detection of microrganisms in raw bulk milk using microcalorimetry

Calorimetric power-time curves of bulk raw milk from three different farms and acidification curves (measured by the iCINAC system) are presented in Figure 21. Measurements were made at 30°C.

It can be seen that the microcalorimetric method was able to detect bacterial growth in raw milk even if less than 5×10^5 CFU mL^{-1} were present. The result show that when CFU counts increase (and pH decreases), increased thermal power is also seen in the calorimetric measurement.

We suggest that calorimetry could be used as a complement to plate counts, for example to check for microbial growth in milk as a form of industrial quality control and for studies of the efficiency of different treatments.

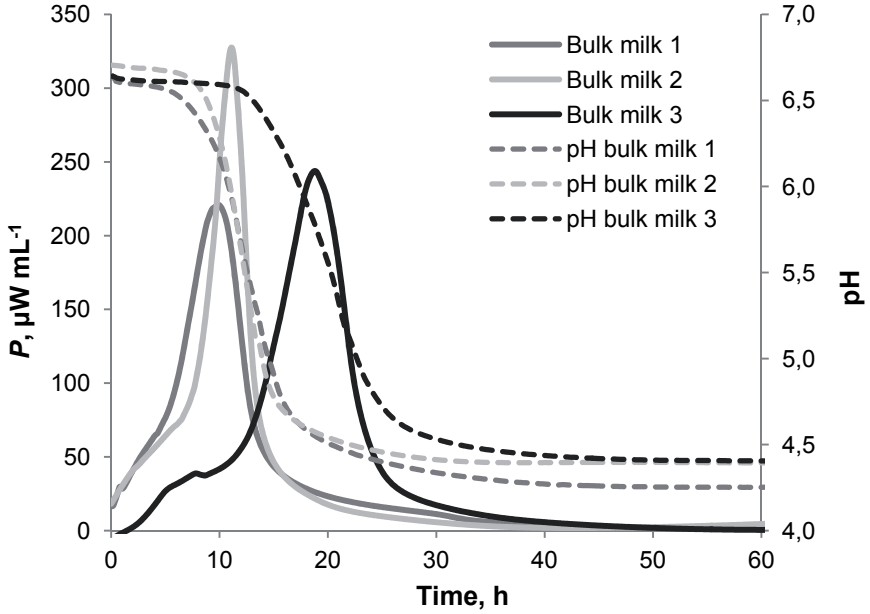

Figure 21. Calorimetric power-time curves of bulk raw milk from three different farms at 30°C

5. SUMMARY OF DISSERTATION

The main results from this dissertation, summarized in this section, are divided into five sections, related to the studied milk treatments:

I. Conclusions reached for the growth of LAB in heat-treated milk

a) It was shown that different heat treatments of milk changed the growth of LAB in milk: treatment at higher temperature led to more pronounced changes.

b) UHT treatment of milk led to a decrease in the growth rate of *St. thermophilus* ST12 and almost completely inhibited the growth of *Lb. paracasei* S1R1.

c) The fat content of milk had only a minor effect on the growth of LAB.

II. Conclusion reached for the growth of LAB in milk with removed oxygen

a) It was shown that the removal of oxygen by N_2 or the catalase treatment of milk resulted in faster growth with a shorter lag-phase of thermophilic starters in skim milk.

III. Conclusions reached for the growth of *St. thermophilus* ST12 in milk prepared from irradiated milk powder

a) It was shown that gamma irradiation of low-heat skim milk powder with 10 KGy doses led to distinct differences in growth patterns, and the metabolism of sugars and amino acids of *Streptococcus thermophilus* ST12 in reconstituted from irradiated powder skim milk. The data obtained were used for the analysis of peculiarities of changes in the intracellular metabolism of the bacteria observed.

b) 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. A lower μ_{max} was observed in irrRSM.

c) The power-time curves obtained for tenfold decreasing inoculation rates in the range 10^6–10^1 CFU ml^{-1} are strikingly similar (P_{max} value) and were shifted at practically regular intervals from each other.

d) The patterns of the hydrolysis of lactose, utilization of glucose and formation of lactic acid in irrRSM were very different from those in RSM. Practically all the lactose (~130 mmol L^{-1}) was hydrolysed to glucose during the active growth phase of the bacteria in RSM in comparison with the 30 mmol L^{-1} utilized in irrRSM. The accumulation of galactose was lower than predicted from the consumption of lactose. At the end of the exponential phase, the amount of lactic acid was twofold lower in irrRSM.

e) The initial total concentration of FAA in irrRSM was about 10% higher than in irrRSM. At the end of fermentations, twofold lower amounts of FAA were observed in irrRSM and the quantitatively dominant amino acid ratios were different: glutamic acid, proline and lysine in RSM, and proline and alanine in irrRSM.
f) It was shown that processing low-heat skim milk powder with gamma irradiation resulted, in addition to visible modification in colour (yellowish) and odour (pronounced off-flavour), substantial differences in SFS spectra (the intensity of the fluorescence maximum of tryptophan was threefold and twofold lower in irradiated powder and milk, respectively), as well as in the dynamic viscoelastic properties of gels (the time of the initiation of gel formation was about 36 min shorter, but the gels were considerably weaker).

IV. Conclusions reached for the growth of *St. thermophilus* in renneted milk

a) Two distinct types of power-time curves were measured in milk with and without rennet addition. The characteristic feature of the power-time curves observed in r-RSM was the "deviation moment", where the maximum specific growth rate had decreased.
b) No differences in μ_{max1} value were observed in the media studied, whereas μ_{max2} were significantly lower in r-RSM.
c) The acidification development was faster and led to a lower pH in liquid milk samples compared to renneted milk samples.
d) There were no clear differences in the hydrolysis of carbohydrates between renneted and non-renneted milks; however, in the r-RSM, the lactose consumption was delayed for about 1 h.
e) The increase in the total free amino acids (TFAA) during 22 h was higher in r-RSM than in RSM, possibly due to the enzymatic activity of rennet on casein.
f) *Streptococcus thermophilus* ST12 can be used as a fast-growing model organism for metabolic flux analyses (μ_{max} 2.2 h^{-1}), where the catalytic rates of enzymes can be significantly higher and the metabolism is organised in a different way than that of *L. lactis*.

V. Conclusions reached for the growth of LAB in raw milk

a) During the past decade LABC in farm bulk-tank raw milk in Estonia decreased by about a factor of 20 (500,000 to 50,000 CFU mL^{-1}).
b) Relatively stable counts of lactic acid bacteria over a 4-year period were found only in 24 (18%) of the farms studied.
c) It was shown that the outgrowth of even the low number of foreign bacteria detected could interfere with the growth patterns of LAB in milk.

6. CONCLUSIONS

- It was shown that the application of isothermal microcalorimetry combined with pH measurement and analyses of sugars and organic acids allow for the quantitative analysis of the growth of lactic acid bacteria in milk as the main substrate for dairy starters.
- Calculation methods were developed and validated for the analysis of complex power-time curves, together with auxiliary biochemical data on the growth of *Streptococcus thermophilus* ST12 in different milk samples. New information on the growth of LAB in milk was obtained.
- The effects of several milk compositional and treatment factors on the growth of *Streptococcus thermophilus* ST12 were elucidated.

BIBLIOGRAPHY

Adachi, S. (1962). Effect of gamma radiation on lactose. II. Isolation and identification of monosaccharides and their derivatives formed in irradiated lactose solution. *Journal of Dairy Science*, *45*, 12, 1427-1434.

Adamberg, K., Seiman, A., & Vilu, R. (2012). Increased biomass yield of *Lactococcus lactis* by reduced overconsumption of amino acids and increased catalytic activities of enzymes. *PLoS ONE*, *7*, 10, 1-14.

Aichinger, P.A., Michel, M., Servais, C., Dillmann, M.-L., Rouvet, M., D'Amico, N., Zink, R., Klostermeyer, H., & Horne, D.S. (2003). Fermentation of a skim milk concentrate with *Streptococcus thermophilus* and chymosin: structure, viscoelasticity and syneresis of gels. *Colloids and Surfaces B: Biointerfaces*, *31*, 243-255.

Antwi, M., Geeraerd, A.H., Vereecken, K.M., Jenné, R., Bernaerts, K., & Van Impe, J.F. (2006). Influence of a gel microstructure as modified by gelatin concentration on *Listeria innocua* growth. *Innovative Food Science and Emerging Technologies*, *7*, 124-131.

Arena, V. (1971). Ionizing Radiation and Life. Saint Louis, MO, USA: Mosby.

Axelsson, L. (2004). Lactic acid bacteria: classification and physiology. Lactic acid bacteria, Microbiological and functional aspects. (3rd ed.) edited by Seppo Salminen, Atte von Wright and Arthur Ouwehand. Marcel Dekker, Inc. USA.

Bandekar, J., Kamat, A., & Thomas, P. (1998). Microbiological quality of the dairy product pedha and its improvement using gamma radiation. *Journal of Food Safety*, *18*, 221-230.

Barbano, D.M., Ma, Y., & Santos, M.V. (2006). Influence of raw milk quality on fluid milk shelf life. *Journal of Dairy Science*, *89*, 15-19.

Becker, E. M., Christensen, J., Frederiksen, C. S., & Haugaard, V. K. (2003). Front-face fluorescence spectroscopy and chemometrics in analysis of yoghurt: rapid analysis of riboflavin. *Journal of Dairy Science*, *86*, 2508-2515.

Beresford, T.P., Fitzsimons, N.A., Brennan, N.L., Cogan, T.M. (2001). Recent advances in cheese microbiology. *International Dairy Journal*, *11*, 259-274.

Berridge, N.J., Cousins, C.M., &Cliffe, A.J. (1974). Microcalorimetry applied to sertain species of bacteria growing in sterilized separated milk. *Journal of Dairy Research*, *43*, 203.

Blecker, C., Habib-Jiwan, J.-M.., & Karoui, R. (2012). Effect of heat treatment of rennet skim milk induced coagulation on the rheological properties and molecular structure determined by synchronous fluorescence spectroscopy and turbiscan. *Food Chemistry*, *135*, 1809-1817.

Boor, K.J., Brown, D.P., Murphy, S.C., Kozlowski, S.M., Bandler, D.K. (1998). Microbiological and chemical quality of raw milk in New York State. *Journal of Dairy Science, 81*, 1743-1748.

Braissant, O., Wirz, D., Göpfert, B., & Daniels, A.U. (2010). Use of isothermalmicrocalorimetry tomonitormicrobial activities. *FEMS Microbiology Letters*, *303*, 1-8.

Braissant, O., Bonkat, G., Wirz, D., & Bachmann, A. (2013). Microbial growth and isothermal microcalorimetry: Growth models and their application to microcalorimetric data. *Thermochimica Acta*, *555*, 65-71.

Chamba, J. F., & Prost, F. (1989). Mesure de l'activité acidifiante des bactéries lactiques thermophiles utilisées pour les fabrications des fromages á pâte cuite. *Lait*, *69*, 417-431.

Champagne, C.P., Laing, R.R., Roy, D., & Mafu, A.A. (1994). Psychrotrophs in dairy products: their effects and their control. *Critical Rewiews in Food Science and Nutrition*, *34*, 1, 1-30.

Chauhan, S. K., Kumar, R., Nadanasabapathy, S., & Bawa, A. S. (2008). Detection methods for irradiated foods. *Comprehensive reviews in food science and food safety*, *8*, 4-16.

Cempírková, R., & Mikulová, M. (2009). Incidence of psychrotrophic lipolytic bacteria in cow's raw milk. *Czech Journal* of *Animal Science*, *54*, 65-73.

Cieśla, K., Salmieri, S., Lacroix, M., & Le Tien, C. (2004). Gamma irradiation influence on physical properties of milk proteins. *Radiation Physics and Chemistry*, *71*, 93-97.

Corrieu, G., Spinnler, H.E., Jomier, Y. and Picque, D. (1988) Automated system to follow up and control the acidification activity of lactic acid starters. French Patent 2 629 612, 6 October 1989.

Courtin, P., Monnet, V., & Rul, F. (2002). Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus*/*Lactobacillus bulgaricus* mixed cultures in milk. *Microbiology*, *148*, 3413-3421.

Galia, W., Perrin, C., Genay, M., & Dary, A. (2009). Variability and molecular typing of *Streptococcus thermophilus* strains displaying different proteolytic and acidifying properties. *International Dairy Journal*, *19*, 89-95.

Garault, P., Le Bars, D., Besset, C., & Monnet, V. (2002). Three oligopeptide-binding proteins are involved in the oligopeptide transport of *Streptococcus thermophilus*. *The Journal of Biological Chemistry*, *277*, 1, 32-39.

Gardea, A.A., Carvajal-Millán, E., Higuera-Ciapara, I., Figueroa, C., Molina-Corral, J., Rascón, A., Orozco, A., & Inda, A. (2002). Calorimetric assessment of microbial growth in milk as affected by different conditions. *Thermochimica Acta*, *394*, 179-184.

Giannino, M.L., Marzotto, M., Dellaglio, F., & Feligini, M. (2009). Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. *International Journal of Food Microbiology*, *130*, 188-195.

Gilliland, S. E., & Speck, M. L. (1969). Biological response of lactic streptococci and lactobacilli to catalase. *Applied Microbiology*, *17*, 797-800.

Giraffa, G., Chanishvili, N., Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Research in Microbiology*, *161*, 480-487.

Goh, Y., J., Goin, C., O´Flaherty, S., Altermann, E., & Hutkins, R. (2011). Specialized adaptation of a lactic acid bacterium to the milk environment: the comparative genomics of *Streptococcus thermophilus* LMD-9. *Microbial Cell Factories*, *10*, 1-17.

Greene, V.W., & Jezerski, J.J. (1957). Studies on starter metabolism. II. The influence of heating milk on the subsequent response of starter cultures. *Journal of Dairy Science*, *40*, 9, 1053-1061.

Guessas, B., & Kihal, M. (2004). Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats' milk. *African Journal of Biotechnology*, *3*, 6, 339-342.

Dandoy, D., Fremaux, C., de Frahan, M.H., Horvath, P., Boyaval, P., Hols, P., & Fontaine, L. (2011). The fast milk acidifying phenotype of *Streptococcus thermophilus* can be acquired by natural transformation of the genomic island encoding the cell-envelope proteinase PrtS. *Microbial Cell Factories*, *10*, 1-9.

Davies, K. J. A (1987). Protein damage and degradation by oxygen radicals. *The Journal of Biological Chemistry*, *262*, 9895-9901.

Davies, M. (2012). Free radicals, oxidants and protein damage. *Australian biochemist*, *43*, 8-12.

Day, E. A., Fross, D. A., & Patton, S. (1957). Flavor and odor defects of gamma-irradiated skimmilk. I. Preliminary observations and the role of volatile carbonyl compounds. *Journal of Dairy Science*, *40*, 922-931.

Day, E.A., & Papaioannou, S.E. (1963). Irradiation-induced changes in milk fat. *Journal of Dairy Science*, 46, 11, 1201-1206.

De la Hoz, L., & Netto, F. M. (2008). Structural modifications of β-lactoglobulin subjected to gamma radiation. *International Dairy Journal*, *18*, 1126-1132.

De Oliveira Silva, A.C., Mársico, E.T., de Jesus, E.F.O., Guimarães, C.F., & Cortes, M.A.S. (2011). Effect of gamma radiation on lipids by the TBARS and NMR. *Brasilian Archives of Biology and Technology*, *54*, 6, 1343-1348.

Desmasures, N., Bazin, F. & Guéguen, M. (1997a). Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. *Journal of Applied Microbiology*, *83*, 53-58.

Desmasures, N., Opportune, M. & Guéguen, M. (1997b). *Lactococcus* spp., yeasts and *Pseudomonas* spp. on teats and udders of milking cows as potential sources of milk contamination. *International Dairy Journal*, *7*, 10, 643-646.

Dewettinck, K., Rombaut, R., Thienpont, N., Le, T.T., Messens, K., & Camp, J.V. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal*, *18*, 436-457.

Diehl, J.F. (1983) Radiolytic effects in foods, in Preservation of Food by Ionizing Radiation, Vol. 1, (eds E.S. Josephson and M.S.Peterson), CRC Press Inc., Boca Raton, Florida, pp.279-357.

Doeven, M.K., Kok, J., & Poolman, B. (2005). Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms. *Molecular Microbiology*, *57*, 3, 640-649.

Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/κ-casein complexes in heated skim milk – A review. *Dairy Science and Technology*, *89*, 3-29.

Espeche, M.C., Pellegrino, M., Frola, I., Larriestra, A., Bogni, C., & Nader-Macías, M.E.F. (2012). Lactic acid bacteria from raw milk as potentially benefical strains to prevent bovine mastitis. *Anaerobe*, 18, 103-109.

Farkas, J. (2006). Irradiation for better foods – A Review. *Trends in Food Science & Technology*, *17*, 148-152.

Favrot, C., & Maubois, J. L. (1994). Growth of *Lactococcus lactis* subsp *lactis* in reconstituted irradiated milk powder. *Lait*, *74*, 253-266.

Favrot, C., & Maubois, J. L. (1996). Growth of *Lactococcus lactis* in milk and rennet curd: influence of the level of inoculation. *Lait*, *76*, 193-208.

Fernandez-Espla, M.D., Garault, P., Monnet, V., & Rul, F. (2000) *Streptococcus thermophilus* cell wall-anchored proteinase: release, purification, and biochemical and genetic characterization. *Applied and Environmental Microbiology*, *66*, 11, 4772-4778.

Foltys, V., & Kirchnerová, K. (2006). Mesophilic and psychrotrophic aerobic sporulating microorganisms in raw cow's milk. *Archiva Zootechica*, *9*, 41-55.

Foucaud, C., Kunji, E.R.S., Hagting, A., Richard, J., Konings, W.K., Desmazeaud, M., & Poolman, B. (1995). Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- anf tripeptides. *Journal of Bacteriology*, *177*, 16, 4652-4657.

Fox, P., F., Guinee, T.P., Cogan, T.M., & McSweeney, P.L.H. (2000). Fundamentals of cheese science. Gaithersburg, MD, USA: Aspen Publishers, Inc.

Frank, J.F., & Marth, E.H. (1999). Fermentations. In N.P.Wong (Ed.), Fundamentals of dairy chemistry (p. 656). Gaithersburg, MD, USA: Aspen Publishers, Inc.

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Annual Review of Pharmacology*, *23*, 239-257.

Fujita, T., Monk, P.R., & Wadsö, I. (1978). Calorimetric identification of several strains of lactic acid bacteria. *Journal of Dairy Research*, *45*, 457-463.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Chae, H. S., Yoo, Y. M., Kim, D. H., Lee, W. K., & Jo, C. (2009a). Irradiation effect on α- and β-caseins of milk and Queso Blanco cheese determined by capillary electrophoresis. *Radiation Physics and Chemistry*, *78*, 158-163.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Jang, A., Yoo, Y. M., Chae, H. S., Kim, D. H., Kim, H. J., Lee, W. K., & Jo, C. (2009b). Quality of irradiated plain yoghurt during storage at different temperatures. *Asian - Australasian Journal of Animal Sciences*, *22*, 289-295.

Ham, J. S., Noh, Y. B., Kim, S. I., Kim, H. S., Jeong, S. G., Chae, H. S., Ahn, J. N., Jo, C., & Lee, W. K. (2005). Changes of chemical, bacteriological, and allergenicity of raw milk by gamma irradiation. *Journal of Korean Dairy Technology Science*, *23*, 93-98.

Hassan, A.N., & Frank, J.F. (2001). Starter cultures and their use. In E.H. Marth & J.L.Steele (Ed.), Applied dairy microbiology (p. 157), New York, NY, USA: Marcel Dekker, Inc.

Hantsis-Zacharov, E., & Halpern, M. (2007). Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Applied and Environmental Microbiology*, *73*, 7162-7168.

Hayes, M.C., & Boor, K. (2001). Raw milk and fluid milk products. In E.H. Marth & J.L.Steele (Ed.), Applied dairy microbiology (pp. 59-76), New York, NY, USA: Marcel Dekker, Inc.

Herbert, S., Riaublanc, A., Bouchet, B., Gallant, D.J., & Dufour, E. (1999). Fluorescence spectroscopy investigation of acidor rennet-induced coagulation of milk. *Journal of Dairy Science*, *82*, 2056-2062.

Hoff, J.E., Wertheim, J.H., & Proctor, B.E. (1959). Radiation preservation of milk and milk products. V. Precursors to the radiation-induced oxidation flavor of milk fat. *Journal of Dairy Science*, *42*, 3, 468-475.

Hutkins, R.W., & Morris, H.A. (1987). Carbohydrate metabolism in *Streptococcus thermophilus*: a review. *Journal of Food Protection*, 50, 876–884.

Iyer, R., Tomar, S.K., Maheswari, T.U., & Singh, R. (2010). *Streptococcus thermophilus*: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*, 133-141.

Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H., & Toba, T. (2003). The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Current Microbiology*, *47*, 231-236.

Jang, H.D., & Swaisgood, H.E. (1990). Disulfide bond formation between thermally denaturated β-lactoglobulin and κ-casein in casein micelles. *Journal of Dairy Science*, *73*, 900-904.

Juffs, H. S., & Babel, F. J. (1975). Inhibition of psychrotrophic bacteria by lactic cultures in milk stored at low temperature. *Journal of Dairy Science*, *58*, 1612-1619.

Juille, O., Le Bars, D., & Juillard, V. (2005). The specifity of oligopeptide transport by Streptococcus thermophilus resembles that of *Lactococcus lactis* and not that of pathogenic streptococci. *Microbiology*, *151*, 1987-1994.

Kabanova, N., Stulova, I., & Vilu, R. (2012). Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels. *Food Microbiology*, *29*, 67-79.

Kask, S., Adamberg, K., Orłowski, A., Vogensen, F. K., Møller, P. L., Ardö, Y. & Paalme, T. (2003). Physiological properties of *L. paracasei*, *L. danicus* and *L. curvatus* strains isolated from Estonian semi-hard cheese. *Food Research International*, *36*, 1037-1046.

Khalid, K. (2011). An overview of lactic acid bacteria. *International Journal of Biosciences*, *1*, 3, 1-13.

Kemp, R.B. (2001). The application of heat conduction microcalorimetry to study the metabolism and pharmaceutical modulation of cultured mammalian cells. *Thermochimica Acta*, *380*, 229-244.

Kethireddipalli, P., Hill, A.R.H., & Dalgleish, D.G. (2010). Protein interactions in heat-treated milk and effect on rennet coagulation. *International Dairy Journal*, *20*, 838-843.

Konteles, S., Sinanoglou, V. J., Batrinou, A., & Sflomos, K. (2009). Effects of γ-irradiation on *Listeria monocytogenes* population, colour, texture and sensory properties of Feta cheese during cold storage. *Food Microbiology*, *26*, 157-165.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B., & Konings, W.N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek*, *70*, 197-221.

Lafarge, V., Ogier, J.-C., Girard, V., Madalen, V., Leveau, J.-Y., Gruss, A. & Delacroix-Buchet, A. (2004). Raw cow milk bacterial population shifts attributable to refrigeration. *Applied and Environmental Microbiology*, *70*, 9, 5644-5650.

Lamarque, M., Charbonnel, P., Aubel, D., Piard, J.-C., Atlan, D., & Juillard, V. (2004). A multifunction ABC transporter (Opt) contributes to diversity of peptide uptake specificity within the genus *Lactococcus*. *Journal of Bacteriology*, *186*, 19, 6492-6500.

Lante, A., Lomolino, G., Cagnin, M., & Spettoli, P. (2006). Content and characterisation of minerals in milk and in Crescenza and Squacquerone Italian fresh cheeses by ICP-OES. *Food Control*, *17*, 229-233.

Lanzanova, M., Mucchetti, G., & Neviani, E. (1993). Analysis of conductance changes as a growth index of lactic acid bacteria in milk. *Journal of Dairy Science*, *76*, 20-28.

Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology*, *15*, 67-78.

Letort, C., Nardi, M., Garault, P., Monnet, V., & Juillard, V. (2002). Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Applied and Environmental Microbiology*, *68*, 3162-3165.

Litz, B., & Schäffer, B. (2005). Indication of proliferation of lactic acid bacteria in mixed cultures. Estimation of their ratios by isoperibolic batch calorimetry. *Journal of Thermal Analysis and Calorimetry*, *82*, 261-264.

Liu, M., Nauta, A., Francke, C., & Siezen, R.J. (2008). Comparative genomics of enzymes in flavour-forming pathways from amino acids in lactic acid bacteria. *Applied and Environmental Microbiology*, 74, 15, 4590-4600.

Liu, X., & Metzger, L. E. (2007). Application of fluorescence spectroscopy for monitoring changes in nonfat dry milk during storage. *Journal of Dairy Science*, *90*, 24-37.

Lopez, C., & Dufour, E. (2001). The composition of the milk fat globule surface alters the structural characteristics of the coagulum. *Journal of Colloid and Interface Science*, *233*, 241-249.

Lucey, J. A. (2009). Milk protein gels. In A. Thompson, M. Boland, & H. Singh (Eds.), *Milk proteins: from expression to food* (pp. 449-481). San Diego, CA, USA: Elsevier Academic Press.

Martinussen, J., Solem, C., Koefoed, A., & Jensen, P.R. (2013). Engineering strategies aimed at control of acidification rate of lactic acid bacteria. *Current Opinion in Biotechnology*, *24*, 124-129.

Maskow, T., & Babel, W. (2003). Thermokinetic description of anaerobic growth of Halomonas halodenitrificans using a static microcalorimetric ampoule technique. *Journal of Biotechnology*, *101*, 267-274.

Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P. and Bardowski, J. (2010) Updates in the Metabolism of Lactic Acid Bacteria, in Biotechnology of Lactic Acid Bacteria: Novel Applications (eds F. Mozzi, R. R. Raya and G. M. Vignolo), Wiley-Blackwell, Oxford, UK.

McDonald, L.C., McFeeters, R.F., Daeschel, M.A., & Fleming, H.P. (1987). A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria. *Applied and Environmental Microbiology*, *53*, 6, 1382-1384.

McSweeney, P.L. (2004). Biochemistry of cheese ripening. International Journal of Dairy Technology, *57*, 2/3, 127-144.

Mende, S., Peter, M., Bartels, K., Rohm, H., & Jaros, D. (2013). Addition of purified exopolysaccharide isolates from *S. thermophilus* to milk and their impact on the rheology of acid gels. *Food Hydrocolloids*, *32*, 178-185.

Mertens, L., Van Derlinden, E., Van Impe, J.F. (2012). A novel method for high-throughput data collection in predictive microbiology: Optical density monitoring of colony growth as a function of time. *Food Microbiology, 32*, 196-201

Mitchell, D.A., von Meien, O.F., Krieger, N., & Dalsenter, F.D.H. (2004). A review of recent developments in modeling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. *Biochemical Engineering Journal*, *17*, 15-26.

Monk, P.R. (1979). Thermograms of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in single and mixed culture in milk medium. *Journal of Dairy Research*, *46*, 485-496

Morin, P., Pouliot, Y., & Britten, M. (2008). Effects of buttermilk made from creams with different heat treatment histories on properties of rennet gels and model cheeses. *Journal of Dairy Science*, *91*, 871-882.

Mucchetti, G., Gatti, M., & Neviani, E. (1994). Electrical conductivity changes in milk caused by acidification: determining factors. *Journal of Dairy Science*, *77*, 940-944.

Mäyrä-Mäkinen, A., & Bigret, M. (2004). Industrial use and production of lactic acid bacteria. Lactic Acid Bacteria. Microbiological and functional aspects. (3rd ed) edited by Seppo Salminen, Atte von Wright and Arthur Ouwehand. Marcel Dekker, Inc. USA.

Neves, A.R., Pool, W.A., Kok, J., Kuipers, O.P., & Santos, H. (2005). Overview on sugar metabolism and its control in *Lactococcus lactis* – The input from in vivo NMR. *FEMS Microbiology Reviews*, *29*, 531-554.

Nordmark, E.-L., Yang, Z., Huttunen, E., & Widmalm, G. (2005). Structural studies of an exopolysaccharide produced by *Streptococcus thermophilus* THS. *Biomacromolecules*, *6*, 105-108.

O'Brien, J. (1995) Heat-induced changes in lactose: isomerization, degradation, Maillard browning, in: Heat Induced Changes in Milk**.** (2nd ed.) Fox, P. F., ed. International Dairy Federation, Brussels, Belgium.

O'Farrell, H.M., Jimenez-Flores, J.R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L. K., Hicks, C. L., Hollar, C.M., Ng-Kwai-Hang, K. F., & Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk – sixth revision. *Journal of Dairy Science*, *87*, 1641-1674.

Parpinello, G. P., Chinnici, F., Versari, A., & Riponi, C. (2002). Preliminary study on glucose oxidaseecatalase enzyme system to control the browning of apple and pear purees. *LWT Food Science and Technology*, *35*, 239-243.

Phadungath, C. (2005). The mechanism and properties of acid-coagulated milk gels. *Journal of Science and Technology*, *27*, 2, 433-448.

Pernoud, S., Fremaux, C., Sepulchre, A., Corrieu, G., & Monnet, C. (2004). Effect of the metabolism of urea on the acidifying activity of *Streptococcus thermophilus*. *Journal of Dairy Science*, *87*, 550-555.

Poolman, B., Royer, T.J., Mainzer, S.E., & Schmidt, B.F. (1989). Lactose transport system of *Streptococcus thermophilus*: a hybrid protein with momology to the melibiose carrier and enzyme III of phosphoenolpyruvate-dependent phosphotransferase systems. *Journal of Bacteriology*, *171*, 1, 244-253.

Pouliot, Y., Boulet, M., & Paquin, P. (1989). An experimental technique for the study of milk milk salt balance. *Journal of Dairy Science*, *72*, 1, 36-40.

Pritchard, G.G., & Coolbear, T. (1993). The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiology Reviews*, *12*, 1-3, 179-206.

Quintans, N.G., Blancato, V., Repizo, C., & López, P. (2008). Citrate metabolism and aroma compound production in lactic acid bacteria. Molecular aspects of lactic acid bacteria for traditional and new applications, 65-88.

Raikos, V. (2010). Effect of heat reatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids*, 24, 259-265.

Rasmussen, T.B., Danielsen, M., Valina, O., Garrigues, C., Johansen, E., & Pedersen, M.B. (2008). *Streptococcus thermophilus* core genome: comparative genome hybridization study of 47 strains. *Applied and Environmental Microbiology*, *74*, 15, 4703-4710.

Reiter, B., & Härnulv, G. (1984). Lactoperoxidase antibacterial system: natural occurrence, biological functions and practical applications. *Journal of Food Protection*, *47*, 724-732.

Riva, M., Franzetti, L., Galli, A., & Schiraldi, A. (1997). Growth and fermentation activity of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus in milk: a calorimetric investigation. *Annali di Microbiologia ed Enzimologia*, *47*, 199-211.

Robinson, R.K. (2002). Dairy microbiology handbook. (3rd ed.), John Wiley and Sons, Inc., New York.

Routray, W., & Mishra, H. (2011). Scientific and technical aspects of yoghurt aroma and taste: a review. *Comprehensive Reviews in Food Science and Food Safety*, *10*, 208-220.

Rul, F., & Monnet, V. (1997). Presence of additional peptidases in *Streptococcus thermophilus* CNRZ 302 compared to *Lactococcus lactis*. *Journal of Applied Microbiology*, 82, 695-704.

Savijoki, K., Ingmer, H., & Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology*, *71*, 394-406.

Sadoun, D., Couvercelle, C., Strasser, A., Egler, A. & Hasselmann, C. (1991). Low dose irradiation of liquid milk, *Milchwissenschaft*, *46*, 5, 295-299.

Santos, M.J., Teixeira, J.A., & Rodrigues, L.R. (2012). Fractionation of the major whey proteins and isolation of β-lactoglobulin variants by anion exchange chromatography. *Separation and Purification Technology*, *90*, 133-139.

Shahbal, S., Hemme, D., & Desmazeaud, M. (1991). High cell wall-associated proteinase activity of some *Streptococcus thermophilus* strains (H-strains) correlated with a high acidification rate in milk. *Lait*, *71*, 351-357.

Sieuwerts, S., de Bok, F.A.M., Hugenholtz, J., van Hylckama Vlieg, E.T. (2008). Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Applied and Environmental Microbiology*, *74*, 16, 4997-5007.

Singh, G.P., & Sharma, R.R. (2009). Dominating species of lactobacilli and leuconostocs present among the lactic acid bacteria of milk of different cattles. *Asian Journal* of *Experimental Sciences*, *23*, 1, 173-179.

Smid, E.J., Poolman, B., & Konings, W.N. (1991). Casein utilization by lactococci. *Applied and Environmental Microbiology*, *57*, 9, 2447-2452.

Stadtman, E. R., & Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, *25*, 207-218.

Swinnen, I.A.M., Bernaerts, K., Dens, E.J.J., Geeraerd, A.H., Van Impe, J.F., (2004). Predictive modelling of the microbial lag phase: a review. *International Journal of Food Microbiology*, *94*, 137-159.

Tamime, A.Y. (2009). Milk processing and quality management, Blackwell Publishing Ltd., UK.

Thibessard, A., Leblond-Bourget, N., Fernandez, A., Gints, B., & Decaris, B. (2001). Response of *Streptococcus thermophilus* CNRZ368 and its colonial

variants to oxidative stress: evidence for an inducible defence system. *Lait*, *81*, 311-316.

Thomas, T.D., & Pritchard, G.G. (1987). Proteolytic enzymes of dairy starter cultures. *FEMS Microbiology Reviews*, *46*, 245-268.

Vaningelgem, F., Zamfir, M., Mozzi, F., Adriany, T., Vancanneyt, M., Swings, J., & De Vuyst, L. (2004). Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics. *Applied and Environmental Microbiology*, *70*, 2, 900-912.

Vasbinder, A.J., Rollema, H.S., & de Kruif, C.G. (2003). Impaired rennetability of heated milk; Study of enzymatic hydrolysis and gelation kinetics. *Journal of Dairy Science*, *86*, 1548-1555.

Zanatta, P., & Basso, A. (1992). A new approach to the characterization of Streptococcus salivarius subsp thermophilus based on acidification rates. *Lait*, *72*, 285-295.

Żegota, H., & Małolepszy, B. (2008). The decontamination of industrial casein and milk powder by irradiation. *Radiation Physics and Chemistry*, *77*, 1108-1111.

Zourari, A., Accolas, J.P., & Desmazeaud, M.J. (1992). Metabolism and biochemical characteristics of yoghurt bacteria. A review. *Lait*, *72*, 1-34.

Wertheim, J.H., Proctor, B.E., & Goldblith, S.A. (1956). Radiation preservation of milk and milk products. IV. Radiation-induced browning and some related chemical changes in milk. *Journal of Dairy Science*, *39*, 9, 1236-1246.

Wadsö, L., & Galindo, F.G. (2009). Isothermal calorimetry for biological applications in food science and technology. *Food Control*, *20*, 956-961.

Walstra, P. (1999) Dairy Technology: Principles of milk properties and processing, Marcel Dekker, New York.

Wilson, P.D.G., Brocklehurst, T.F., Arino, S., Thuault, D., Jakobsen, M., Lange, M., Farkas, J., Wimpenny, J.W.T., & Van Impe, J.F. (2002). Modelling microbial growth in structured foods: towards a unified approach. *International Journal of Food Microbiology*, *73*, 275-289.

ACKNOWLEDGMENTS

This work was conducted in the Institute of Chemistry (Chair of Biotechnology) at Tallinn University of Technology (TUT) and at the Competence Center of Food and Fermentation Technologies (CCFFT). Financial support for this work was provided by grant SF0140090s08 of the Estonian Ministry of Education and Research, and by the European Regional Development Fund projects EU28912 and EU29994.

I am deeply grateful to Prof. Raivo Vilu for being a great supervisor. His enthusiasm, expertise and encouragement were essential in the completion of this work.

My sincere thanks go to my colleagues at the Chair of Biotechnology at TUT and CCFFT for providing a pleasant working atmosphere and for all the help during the study and research work.

Special thanks are extended to Dr. Tiiu-Maie Laht and Dr. Signe Adamberg for introducing me to the field of dairy science (milk and cheese microbiology) and for fruiful cooperation.

My deepest thanks go to Dr. Tiina Kriščiunaite and Dr. Natalja Kabanova for their help, motivation and support, both personal and professional. I am sincerely grateful to Dr. Anna Mihhalevski for her moral support.

I would like to express my appreciation to Dr. Kaarel Adamberg and Dr. Andrus Seiman for their invaluable help in data processing.

I would like to thank Anastassia Taivosalo for her technical assistance on the UPLC analyses and Viktoria Põzjanova for SFS analyses.

I thank Prof. Toomas Paalme for reviewing the thesis.

I am also grateful to the Archimedes Foundation and the DoRa programme for enabling me to introduce my results and broaden my knowledge at conferences and seminars. The financial support of the Estonian Science Foundation (grant 7636) for the research at Tallinn University of Technology is also gratefully acknowledged.

My warmest thanks go to my family, relatives and friends, who believed in me and supported me throughout these years. In particular, I would like to mention my mother for her valuable support, and express my gratitude to my sweet daughter Maria for giving me a chance to develop.

Самые сердечные слова благодарности моей семье за их любовь и веру в меня. Я искренне признательна моей маме за ее поддержку, понимание оптимизм и терпение.

ABSTRACT

The ability to grow in milk is an important feature of the lactic acid bacteria (LAB) used as starters for fermented milk products. The activity of starters is a consequence of many factors, some of which are difficult to quantify, such as the physiological state of cultures, growth conditions, harvesting and storage conditions. Usually activity measurements are confined to the ability of starter cultures to acidify milk, and the classic way to determine this activity is by measuring the pH of the culture at different time intervals. In the majority of the studies reported, a more detailed analysis of the acidification process has not been carried out. Calorimetry, in particular isothermal calorimetry, is ideally suited for the detailed study of acidification processes, as it offers many unique advantages.

In the present work, the peculiarities of the growth of thermophilic starter bacteria in differently pretreated milk samples were studied using the isothermal batch microcalorimeter TAMIII. Maximal specific growth rates μ_{max} (W h^{-1}), heat produced during different growth stages Q_{tot} (J mL^{-1}) and Q_{exp} (J mL^{-1}), and lag–phase λ (h) duration were obtained by processing calorimetric curves. Along with calorimetric measurements, changes in the concentrations of amino acids, sugars and lactic acid were determined and pH measurements of culture media were carried out in order to obtain additional information for the interpretation of calorimetric power-time curves. The rheological properties of milk gels using low amplitude oscillatory measurements and fluorescence spectra using the spectral fluorescence signature technique were additionally measured for the investigation of the effect of γ-irradiation on skim milk powder. The applied combination of novel methods made it possible to study, in unprecedented detail, the quantitative growth of LAB in milk samples.

The microbiology of fluid milk impacts the production and consumption of dairy products in several different ways. Numerous types of bacteria (*Pseudomonas* spp., for instance) can degrade milk components, creating negative sensory attributes, decreasing processed product shelf life and adversely affecting cultured dairy product yield. Therefore, it is important to know the microbiological properties of raw milk produced on farms. The microbiological quality of raw milk is usually evaluated by total bacterial count (TBC) and somatic cell count (SCC). The main contribution of this study is the determination of specific bacterial groups (LABC, ASFBC and PBC) not routinely analysed by dairies in the bulk-tank raw milk of dairy farms. The results obtained provide valuable information on the milk quality, processability and stability of raw milk on large number of Estonian dairy farms over a four-year period. We suggest, in addition to analysing TBC, analysing the proportions of different bacterial groups that have technological impacts on better milk grading.

KOKKUVÕTE

Võime kasvada piimas on piimhappebakterite tähtis omadus, mis võimaldab neid kasutada juuretistena piimatoodete valmistamisel. Juuretisebakterite aktiivsus on määratud mitmete faktorite poolt, mida polegi iga kord kerge määrata – bakterikultuuride füsioloogiline seisund, kasvutingimused, eraldamise ja säilitamise tingimused jne. Tavaliselt hinnatakse piimhappebakterite aktiivsust nende piima hapustamise võime järgi mõõtes keskkonna pH muutust. Enamikus siianitehtud uuringutes pole hapnemisprotsesse detailsemalt uuritud. Kalorimeetria on aga meetod, mis sobib väga hästi piimhappebakterite kasvu ja piima hapendumisprotsesside uurimiseks.

Käesolevas töös uuriti termofiilsete juuretisebakterite kasvu erinevalt eeltöödeldud piimas, kasutades isotermilist mikrokalorimeetrit TAM III. Mõõdetud kalorimeetriliste kasvukõverate analüüs võimaldas määrata bakterite maksimaalset kasvukiirust μ_{max} (W h^{-1}), erinevates kasvufaasides toodetud soojushulki Q_{tot} (J mL^{-1}), Q_{exp} (J mL^{-1}), lag-faasi pikkust λ (h) jne. Lisaks kalorimeetriliste kasvukõverate mõõtmisele ja analüüsile mõõdeti ka aminohapete, suhkrute, piimhappe jms kontsentratsioone, samuti kasvukeskkonna pH muutumist eesmärgiga koguda täiendavat teavet bakterite ainevahetuse ja kasvuprotsesside iseärasuste kohta. Piimageelide reoloogilisi omadusi ja fluorestsentsspektrite muutusi mõõdeti, et täiendavalt iseloomustada γ-kiirguse poolt esilekutsutud muutusi piimas kui bakterite kasvukeskkonnas. Kirjeldatud meetodite kooskasutamine tegi võimalikuks pretsedenditult detailse termofiilsete juuretisebakterite kasvuprotsesside uurimise.

Piimas leiduvad võõrbakterid mõjutavad piimatoodete tootmisprotsesse ja nende kasutamist mitmel viisil. Näiteks bakterid *Pseudomonas spp* võivad hüdrolüüsida piimavalke ning sellega mõjuda negatiivselt toodete sensoorsetele omadustele, lühendada nende säilivusaega ning vähendada juuretisebakterite toimimise efektiivsust. Eelöeldu tõttu on tähtis teada võimalikult täpselt piima võõrmikrofloora koostist. Piima mikrobioloogilise kvaliteedi hindamiseks määratakse regulaarselt ja tavaliselt tsentraliseeritult bakterite üldarvu ning somaatiliste rakkude arvu. Käesolevas töös hinnati lisaks neile parameetritele ka spetsiifiliste bakterigruppide LABC, ASFBC, PBC sisaldust Eesti farmide piimas nelja aasta jooksul. Saadud tulemused võimaldavad hinnata detailselt nimetatud bakterite mõju piima kui juuretisebakterite kasvukeskkonna omadustele.

CURRICULUM VITAE

1. Personal data

Name	Irina Stulova
Date of birth	26.02.1978
Place of birth	Tallinn, Estonia
Citizenship	Estonian

2. Contact information

Address	AS TFTAK, Akadeemia tee 15a, Tallinn 12618, Estonia
Contact phone	+37254551307
E-mail	irina.stulova@tftak.eu

3. Education

2003– present	Tallinn University of Technology**,** Faculty of Science, doctoral studies
2001–2003	Tallinn University of Technology, Faculty of Chemicals and Materials Technology, MSc in food and biotechnology
1996–2000	Tallinn University of Technology, Faculty of Chemicals and Materials Technology, BSc in food and biotechnology
1985–1996	Tallinn 14th Secondary School (*silver medal*)

4. Professional Employment

11.2011 – 12.2012	Tallinn University of Technology, Faculty of Science, Department of Chemistry, Chair of Biotechnology; Researcher
2009 – present	Tallinn University of Technology, Faculty of Science, Department of Chemistry; Engineer
2004 – present	Competence Center of Food and Fermentation Technologies; Researcher
2001 – 2004	Baltic Bread Ltd; Food hygiene specialist
02.2001 – 09.2001	Tallinn University of Technology, Department of Chemistry, Chair of Analytical Chemistry; Engineer

5. Special courses

27.03–02.06.2006	Research work in KVL (Research title: Microscopical interactions between *Lactobacillus paracasei* and *Lactococcus lactis*). The Royal Veterinary and Agriculture University (KVL), Copenhagen, Denmark
21.–25.08.2006	PhD course: From Milk to Cheese, Norwegian University of Life Sciences, Äs, Norway
26.–30.08.2010	PhD course: Advanced Food Microbiology, University of Copenhagen, Copenhagen, Denmark

6. Defended thesis

Master thesis	Supervisor Dr. Anne Orav. Title: "Effect of technological processing and retaining to the composition of essential oils of some spices"
Bachelor thesis	Supervisor Dr. Tiiu Liebert. Title: "Nutrition of diabetic patients"

7. Theses supervised

2004	Supervising BSc thesis of Kaja Kattai "Factors affecting formation of farm milk microbial communities, effect to technology"
2005	Supervising BSc thesis of Mariane Koplimaa „Lactic acid bacteria as possible inhibitors of spoilage microflora in milk microbiota"

8. Awards

2012	Research paper award. In recognition of outstanding research contributions by a young investigator in the profession of food microbiology and hygiene at the FoodMicro 2012 Symposium in Istanbul, Turkey

9. Current grant funding

Project EU29994 – support for Competence Center of Food and Fermentation Technologies

ELULOOKIRJELDUS

1. Isikuandmed

Nimi	Irina Stulova
Sünniaeg	26.02.1978
Sünnikoht	Tallinn, Eesti
Kodakondsus	Eesti

2. Kontaktandmed

E-mail	irina.stulova@tftak.eu
Telefon	+37254551307
Adress	Akadeemia tee 15A, Tallinn 12618, Eesti

3. Hariduskäik

2003– …	Tallinna Tehnikaülikool Matemaatika-loodusteaduskond, doktoriõpe
2001–2003	Tallinna Tehnikaülikool Keemia- ja materjalitehnoloogia teaduskond MSc bio-ja toiduainetetehnoloogias
1996–2000	Tallinna Tehnikaülikool Keemia- ja materjalitehnoloogia teaduskond BSc bio-ja toiduainetetehnoloogias
1985–1996	Tallinna 14. Keskkool (*hõbemedal*)

4. Teenistuskäik

11.2011–12.2012	Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut, Biotehnoloogia õppetool; teadur
01.03.2009– …	Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut, Biotehnoloogia õppetool; insener
2004– …	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus AS; teadur
2001–2004	AS Balti Sepik; toiduhügieenispetsialist
05.2001–09.2001	Tallinna Tehnikaülikool, Keemiainstituut, Analüütilise keemia õppetool; insener

5. Täiendusõpe

27.03–02.06.2006	Välispraktikum KVL-is (Uurimustöö teema: *Lactobacillus paracasei* ja *Lactococcus lactis'*e interaktsioonide uurimine kasutades fluorestsents-mikroskoopiat) The Royal Veterinary and Agriculture University (KVL), Kopenhaagen, Taani
21.–25.08.2006	PhD kursus: Piimast juustuni, Norwegian University of Life Sciences, Äs, Norra
26.–30.08.2010	PhD kursus: Kaasaegne toidumikrobioloogia, University of Copenhagen, Kopenhaagen, Taani

6. Kaitstud lõputööd

Magistritöö	Juhendaja Dr. Anne Orav, "Tehnoloogilise töötlemise ja säilitamise mõju mõnede vürtside eeterliku õli koostisele"
Bakalaureusetöö	Juhendaja Dr. Tiiu Liebert, "Diabeetikute toitumine"

7. Juhendatud väitekirjad

2004	Kaja Kattai BSc töö "Farmipiimade mikroobikooslusi kujundavad tegurid, seos tehnoloogiaga" juhendamine
2005	Mariane Koplimaa BSc töö „Piimhappebakterid saaste-mikrofloora potentsiaalsete inhibiitoritena piima mikroobikoosluses" juhendamine

8. Tunnustused

2012	Teadustöö auhind (FoodMicro 2012 Symposium in Istanbul, Turkey)

9. Jooksvad grandid

Projekt EU29994 – finantstoetus Toidu- ja Fermentatsioonitehnoloogia Arenduskeskusele

PUBLICATION I

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

The effect of milk heat treatment on the growth characteristics of lactic acid bacteria.

Agronomy Research, 9, 473 – 478 (2011)[1]

[1] Copyright 2011

Agronomy Research **9** (Special Issue II), 473–478, 2011

The Effect of Milk Heat Treatment on the Growth Characteristics of Lactic Acid Bacteria

I. Stulova [1, 2], N. Kabanova [1, 2], T. Kriščiunaite [1, 2], T.-M. Laht [1, 2] and R. Vilu [1,2]

[1] Tallinn University of Technology, Ehitajate tee 5, 19086, Tallinn, Estonia
[2] Competence Center of Food and Fermentation Technologies (CCFFT), Akadeemia tee 15B, 12618, Tallinn, Estonia; e-mails: irina.stulova@tftak.eu; natalja@tftak.eu; tiina@tftak.eu; tiiu@tftak.eu; raivo@tftak.eu

Abstract. The ability to growth in milk is an important feature for lactic acid bacteria (LAB) used as starters for fermented milk products. Several decades ago the results of the studies varied widely: some of them showed that LAB grew better in raw milk and others demonstrated improved growth of the bacteria in heat-treated milk (Foster et al., 1952). The effectiveness of heat treatment of milk as a tool for modifying the functional properties of protein components has been extensively documented in the literature (Raikos, 2010), but the information on the influence of heat treatment of milk on the growth of LAB is not exhaustive. Peculiarities of growth of *Streptococcus thermophilus* ST12 and *Lactobacillus paracasei* S1R1 were studied using isothermal batch microcalorimeter TAMIII. Bacterial growth was monitored in pasteurized and ultra-high temperature (UHT) treated milk with different fat content, and also in reconstituted skim milk (RSM) prepared from low-heat skim milk powder (LHSMP). Heat produced during different growth stages (Q_{tot}, Q_{exp}), maximal specific growth rate (μ_{max}) and lag–phase (λ) duration were determined by processing calorimetric curves, and detailed analysis of growth of the bacteria in differently pretreated milks were carried out on the basis of these data. The results of the experiments showed that primarily heat treatment and, to a minor extent, fat content of milk influenced the growth parameters of both bacterial strains, especially Lb. *paracasei*, growth of which was almost completely inhibited in UHT milk

Key words: bacterial growth, *Lactobacillus paracasei*, microcalorimetry, milk treatment, *Streptococcus thermophilus*

INTRODUCTION

Milk as a raw material has a relatively short shelf life but it can be prolonged by heat treatment, which is an essential step adopted by the dairy industry (Raikos, 2010). For a high proportion of cheese varieties, pasteurization is the sole treatment applied to the cheese-milk (Kelly et al., 2008). The temperature/time combinations for the batch heat treatments used in yoghurt manufacture are 85°C for 30 min or 90–95°C for 5 min. However, very high temperature short time (100–130 °C for 4 to 16 s) or ultra-high temperature (UHT) 140°C for 4 to 16 s treatments are also sometimes used (Lee & Lucey, 2010). Heat treatment of milk during commercial processing operations not only inactivates the microorganisms (Odriozola-Serrano et al., 2007), but also results in a number of physico-chemical changes in the milk constituents. The rheological

properties of milk gels, both chymosin and acid induced, are affected by the heat treatment applied to milk (Parnell et al., 1988), and can result in irreversible changes in milk protein structure. Some of the changes involved are whey protein denaturation and aggregation, interactions of whey proteins with casein micelles, reactions between lactose and proteins, changes in casein micelle structure, transfer of soluble calcium and phosphate to colloidal phase, changes in fat globule membranes, and decrease in pH (Singh & Waungana, 2001).

The thermophilic bacteria *St. thermophilus* are widely used in the dairy industry in the production of yoghurt and hard 'cooked' cheeses (Emmental, Gruyere, Grana). In the industrial implementations of *St. thermophilus*, fast growth of the bacteria is crucial in intense acidification of milk (Derzelle et al., 2005).

The species of *Lb. casei/paracasei* and *Lb. plantarum* are the main components of mesophilic nonstarter microflora (Laht et al., 2002) and become important as adjunct cultures for the production of fermented milk products (Dupont et al., 2000). As the starter bacteria decrease in number, a secondary microbial flora grows in the maturing cheese and it becomes dominant after 1–3 months of ripening (Laht et al., 2002).

Calorimetry is especially helpful in the studies of the growth of the bacteria in opaque media and is a useful method to obtain kinetic and thermodynamic information on microbial growth (Kabanova et al., 2009; Kriščiunaite et al., 2011). The objectives of this investigation were to study the effect of milk heat treatment on the growth parameters of thermophilic starter and non-starter lactic acid bacteria using isothermal batch microcalorimetry.

MATERIALS AND METHODS

Bacterial culture and preparation of growth inocula

The strain of *St. thermophilus* ST12 used in this work was provided by Chr. Hansen (Denmark). Frozen cultures of *St. thermophilus* ST12 culture were thawed and pre-grown on Petri dishes with M17 Agar (LAB M, UK) for 24 h at 40°C. One colony from a pre-grown Petri dish was used as an inoculum for a 10 mL culture in sterilized RSM (Kalev Paide Tootmine AS, Paide, Estonia) at 40°C and left till coagulation. 1% of pre-grown culture was used as inoculum for the next 10 mL of RSM, left until coagulation and further used for inoculation of differently heat-treated milk samples.

A frozen *Lb. paracasei* S1R1 culture isolated from Estonian cheese (Kask et al., 2003) was thawed and pre-cultured on Petri dishes with MRS Agar (LAB M) medium for 24 h at 35°C. One colony from a pre-grown Petri dish was used as an inoculum for a 10 mL culture in liquid sterilized MRS Broth (LAB M) at 35°C. 1 mL of bacterial suspension grown overnight was used as inoculum for the next liquid 10 mL of MRS Broth and further used for inoculation of differently heat-treated milk samples.

Milk samples

Low-heat skim milk powder (Kalev Paide Tootmine AS, Paide, Estonia) was reconstituted in distilled water with thorough mixing for 1 h to yield a final concentration of 10% (w/v) milk solids. Commercial pasteurized milk with 3.5%, 2.5% and 0.05% fat content (Tere AS, Tallinn, Estonia) and commercial UHT milk with 3.5% and 0.05% fat content (Kalev Paide Tootmine AS) used in the study were obtained from retail sellers.

Calorimetric equipment and measurements

After the addition of bacteria, samples were stirred and 2 mL were transferred into the autoclaved ampoules. Isothermal batch microcalorimeter TAM III Thermal Activity Monitor (Thermometric, Järfälla, Sweden) equipped with 24 channels was used for the study of the growth of *St. thermophilus* ST12 and *Lb. paracasei* S1R1 in various milk substrates. Data analysis was accomplished using TAM Assistant program (v 0.9.1012.40, SciTech Software AB, Thermometric AB).

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.

RESULTS AND DISCUSSION

The power-time curves describing the growth of *St. thermophilus* ST12 at 40°C and *Lb. paracasei* S1R1 at 35°C in differently pretreated milk samples at the same initial inoculation rate of 10^5 cfu mL^{-1} are presented in Fig. 1 and Fig. 2, respectively. Each curve is the average of three power-time curves, obtained with replicated samples.

The power-time curves were processed as described by Kabanova et al., 2009 and the numerical results were presented in Table 1. ANOVA of the data showed that both milk thermal processing and fat content significantly affected the growth characteristics of both bacteria ($P < 0.05$).

Table 1. Parameters describing *St. thermophilus* ST12 growth in differently pretreated milk samples: means of maximum specific growth rate (μ_{max}), heat evolved during the exponential growth phase (Q_{exp}), total heat (Q_{tot}) and time at maximum heat production rate ($t_{(dQ/dt)max}$) obtained from microcalorimetric power-time curves.

Sample	μ_{max}, h^{-1}		*Qexp*, J mL^{-1}		*Qtot*, J mL^{-1}	$t_{(dQ/dt)max}$, h
	1st exp. phase	2nd exp. phase	1st exp. phase	2nd exp. phase		end of 2nd exp. phase
RSM	1.72^{a}	1.38^{a}	0.44^{a}	2.43^{a}	6.73^{a}	6.69^{a}
Past 0.05%	2.03^{b}	1.80^{b}	0.07^{b}	1.89^{b}	6.51^{a}	5.88^{b}
Past 2.5%	2.03^{b}	1.55^{c}	0.08^{c}	2.05^{c}	6.51^{a}	5.88^{b}
Past 3.5%	1.94^{c}	1.47^{c}	0.07^{b}	2.01^{bc}	6.58^{a}	6.05^{b}
UHT 0.05%	2.03^{b}	1.22^{d}	0.10^{d}	2.39^{a}	5.98^{b}	15.87^{c}
UHT 3.5%	1.99^{bc}	0.98^{e}	0.11^{e}	2.41^{a}	5.88^{b}	16.02^{c}

The dual-peak power-time curve of diauxic growth of *St. thermophilus* ST12 was registered in reconstituted milk prepared from LHSMP, also in pasteurized and UHT milk. Two peaks observed correspond to two growth phases: the first exponential growth phase (a shoulder of the curve), and the second (major) exponential growth phase. However, power-time curves in the milk subjected to various heat treatments were completely different. In RSM the heat evolved during the first exponential phase was 6.5% of the total, whereas the contribution of this phase was 1.1–1.9% in other

milks. There were no significant differences in Q_{tot} (6.5–6.7 J mL^{-1}) between pasteurized milk with 0.05%, 2.5%, 3.5% fat content and RSM ($P > 0.05$), but the total evolved heat Q_{tot} was the smallest in UHT milk (5.9 J mL^{-1}).

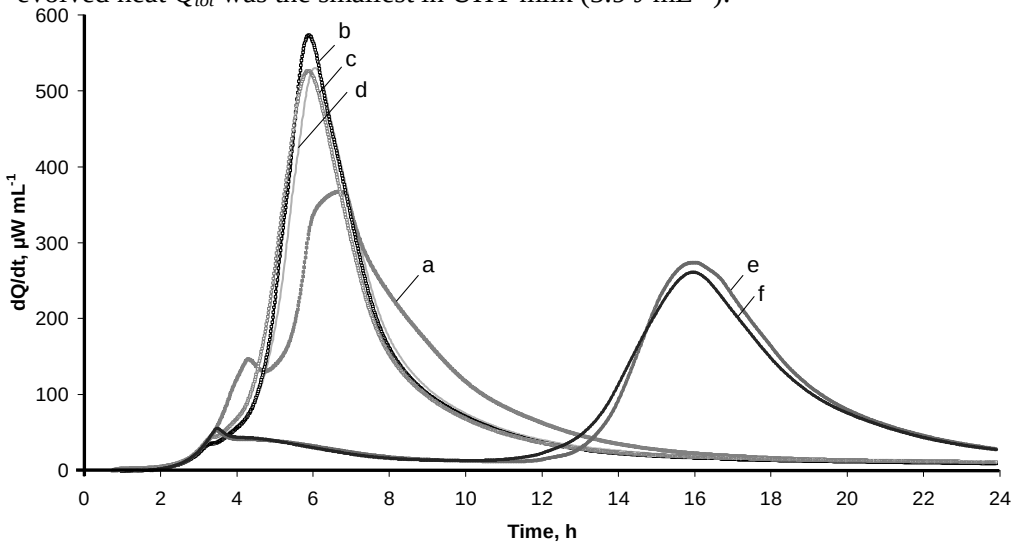

Figure1. Thermal profiles of differently pretreated milk inoculated with thermophilic lactic starter bacteria *St.thermophilus* ST12. Mean power-time curves (n = 3) of milk prepared from low-heat skim milk powder (a), pasteurized milk with 0.05% (b), 2.5% (c), 3.5% (d) fat content, and UHT milk with 0.05% (e) and 3.5% (f) fat content.

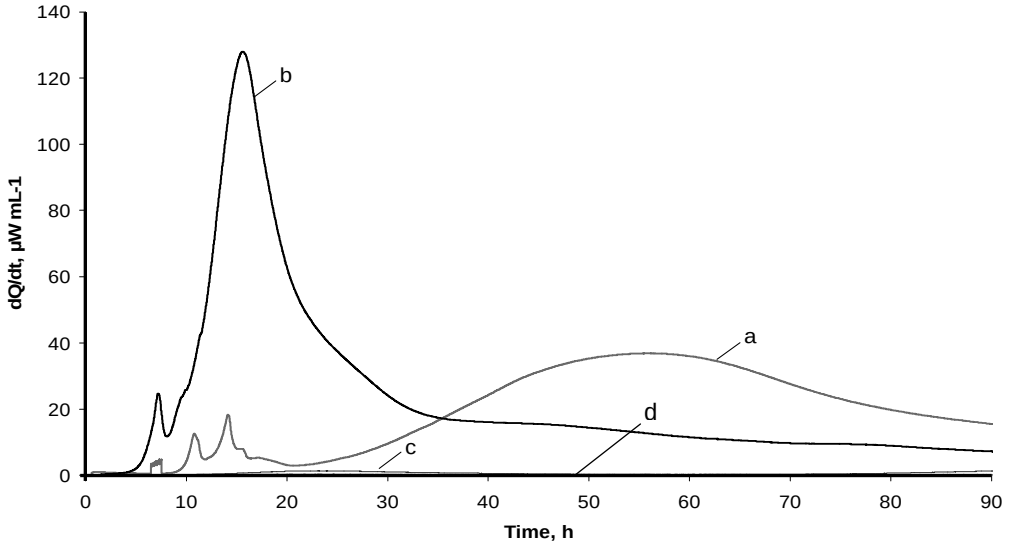

Figure 2. Thermal profiles of differently pretreated milk inoculated with non-starter lactic acid bacteria *Lb.paracasei* S1R1. Mean power-time curves (n = 2) of milk prepared from low-heat skim milk powder (a), pasteurized milk with 2.5% fat content (b), UHT milk with 3.5% fat content (c) and UHT milk with 0.05% fat content (d) – no heat production detected.

The differences in time scales and heat evolution scales on Fig. 1 and Fig. 2 reflect the differences in growth of the bacteria.

The mean fermentation times needed to reach the maximum heat production rate ($t_{(dQ/dt)max}$) on the power-time curves were equal to approximately 6 h for pasteurized milk with 0.05%, 2.5% and 3.5% fat content, and they were different from the UHT milk with 0.05% and 3.5% fat content, where $t_{(dQ/dt)max}$ was reached in 16 h.

It was shown for *St. thermophilus* ST12 that the calculated μ_{max} in the first exponential growth phase was higher than in the second exponential phase irrespective of milk substrate. These results are in agreement with the results obtained earlier (Letort et al., 2002; Kriščiunaite et al., 2011). After inoculation, the cells start growing exponentially presumably using the amino acids, dipeptides, tripeptides, and oligopeptides that are freely available in milk. Subsequently, amino acids become limiting, and the culture enters a post-exponential growth phase in which the synthesis of extracellular protease is needed and initiated for the production of free amino acids. Finally, in a second exponential phase, the proteolytic system is able to supply sufficient peptides for exponential growth, but here the growth rate is lower than in the first exponential phase, probably due to the limited capacity of the peptide uptake systems (Letort et al., 2002; Sieuwerts et al., 2008).

It has been reported that the growth of several strains of LAB was identical in whole milk and in the same milk with fat removed, but the organisms grew much better in milk heated at 115°C for 15 min compared to milk heated at 80°C for 10 min (Foster, 1952). As seen from our data, *St. thermophilus* ST12 had higher values of μ_{max} in the first exponential growth phase growing in milk with 0.05% fat content than in milk with 3.5% fat content. A remarkable decrease of the value of μ_{max} in the second exponential growth phase was also observed in both pasteurized and UHT milks with 3.5% fat content, compared with low-fat samples – from 1.94 ± 0.02 to 1.47 ± 0.01 in the case of 3.5% pasteurized milk, and from 1.99 ± 0.05 to 0.98 ± 0.05 in the case of 3.5% UHT milk.

The duration of the lag-phase was the same in all milk substrates, but the start of the second exponential growth phase in UHT milk was markedly delayed. Poor growth of *St. thermophilus* ST12 in UHT milk could be explained by specific amino acid requirements which cannot be met by the proteolytic action of the bacteria on casein, taking into account that casein micelles in heated milk are coated with denatured whey proteins (Vasbinder et al., 2003).

It was shown for *Lb. paracasei* S1R1 that the power-time curves corresponded to the two-stage growth pattern in pasteurized milk and multiphase growth in RSM. *Lb. paracasei* S1R1 was characterized by low μ_{max} in the second exponential growth phase in pasteurized milk with 2.5% fat content and three times lower in reconstituted milk (data not shown). No heat production was recorded during 90 h in UHT milk with 0.05% fat content and very negligible growth occurred in UHT milk with 3.5% fat content.

CONCLUSIONS

It has been shown that milk thermal processing significantly affected the growth characteristics of starter culture and NSLAB during fermentation, whereas fat content had minor effect on bacterial growth. It was demonstrated in the present study that UHT-treatment of milk led to a decrease of growth rate of *St. thermophilus* ST12 and almost completely inhibited the growth of *Lb. paracasei* S1R1. The values of

maximum growth rates depended notably on the milk fat content, especially in the second exponential phase. The results obtained showed also that microcalorimetry is a very powerful instrument in studying quantitative detailed peculiarities of fermentation processes in milk.

ACKNOWLEDGEMENTS: This investigation was financially supported by the grant No.7636 of Estonian Science Foundation, by the European Regional Development Fund project EU29994, and Ministry of Education, Estonia, through the grant SF0140090s08.

REFERENCES

Derzelle, S., Bolotin, A., Mistou, M-Y. & Rul, F. 2005. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. *App. and Environ. Microbiol*. **71**(12), 8597–8605.

Dupont, I., Roy, D. & Lapointe, G. 2000. Comparison of exopolysaccharide production by strains of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* grown in chemically defined medium and milk. *J Ind Microbiol Biotechnol*. **24**, 251–255.

Foster, E. M. 1952 The effect of heat on milk as a culture medium for lactic acid bacteria. *J. Dairy Sci*. **35**(12), 988–997.

Kabanova, N., Kazarjan, A., Stulova I. & Vilu R. 2009. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentration in broth. *Thermochim Acta*. **496**, 87–92.

Kask, S., Adamberg, K., Orłowski, A., Vogensen, F. K., Møller, P. L., Ardö, Y. & Paalme, T. 2003. Physiological properties of *L. paracasei*, *L. danicus* and *L. curvatus* strains isolated from Estonian semi-hard cheese. *Food Res Int*. **36**, 1037–1046.

Kelly, A. L., Huppertz, T. & Sheehan, J. J. 2008. Pre-treatment of cheese milk: principles and developments. *Dairy Sci.Technol*. **88**, 549–572.

Kriščiunaite, T., Stulova, I., Kabanova, N., Laht, T-M. & Vilu, R. 2011. The effect of hydrogen peroxide on the growth of thermophilic lactic starter and acid gelation of UHT milk. *Int. Dairy J*. **21**, 239–246.

Laht, T-M., Kask, S., Elias, P., Adamberg, K. & Paalme, T. 2002. Role of arginine in the development of secondary microflora in Swiss-type cheese. *Int. Dairy J*. **12**, 831–840.

Lee, W. J. & Lucey, J. A. 2010. Formation and physical properties of yogurt. *Asian-Aust. J. Anim. Sci.* **23**(9), 1127–1136.

Letort, C., Nardi, M., Garault, P., Monnet & V., Juillardi, V. 2002 Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *App. and Environ. Microbiol*. **68**(6), 3162–3165.

Odriozola-Serrano, I., Bendicho-Porta, S. & Martín-Belloso, O. 2006. Comparative study on shelf life of whole milk processed by high-intensity pulsed electric field or heat treatment. *J. Dairy Sci.* **89**, 905–911.

Parnell-Clunies, E., Kakuda, Y., deMan, J.M. & Cazzola, F. 1988. Gelation profiles of yogurt as affected by heat treatment of milk. *J. Dairy Sci.* **71**, 582–588.

Raikos, V. 2010. Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocoll*. **24**, 259–265.

Sieuwerts, S., de Bok, F. A. M., Hugenholtz, J. & van Hylckama Vlieg, J. E. T. 2008 Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *App. and Environ. Microbiol*. **74**(16), 4997–5007.

Singh, H. & Waungana, A. 2011. Influence of heat treatment of milk on cheese-making properties. *Int. Dairy J*. **11**, 543–551.

Vasbinder, A. J., Rollema, H. S. & de Kruif, C. G. 2003. Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *J. Dairy Sci*. **86**, 1548–1555.

PUBLICATION II

Kriščiunaite, T., Stulova, I., Kabanova, N., Laht, T.-M., 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)[1]

[2] Copyright 2010, reprinted with permission from Elsevier

International Dairy Journal 21 (2011) 239–246

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

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

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

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

ARTICLE INFO

Article history:
Received 17 August 2010
Received in revised form
26 November 2010
Accepted 29 November 2010

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

* Corresponding author. Competence Centre of Food and Fermentation Technologies (CCFFT), Akadeemia tee 15b, 12618 Tallinn, Estonia. Tel.: +372 6 202 831; fax: +372 6 202 828.
E-mail address: tiina@tftak.eu (T. Kriščiunaite).

0958-6946/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2010.11.012

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).

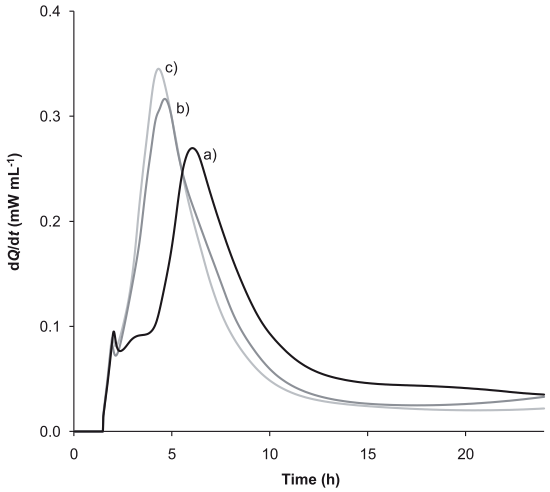

Fig. 1. Characteristic thermal profiles of milk inoculated with thermophilic lactic starter. Mean power-time curves ($n = 3$) of a) UHT 3.5% control samples, b) UHT 3.5% samples treated with catalase and c) UHT 3.5% samples sparged with N_2 before inoculation.

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.

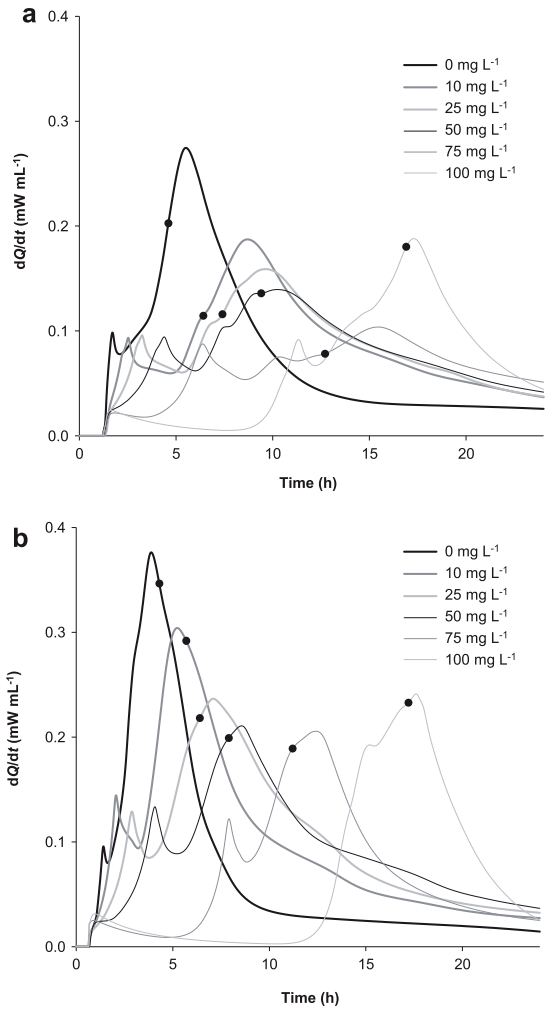

Fig. 2. Mean power-time curves of inoculated milk samples pre-treated with H_2O_2 (0–100 mg L^{-1}) 1 h before starter addition: a) UHT 3.5% ($n = 3$) and b) UHT 0.05% ($n = 2$) milk samples. Mean ($n = 3$) gelation times (●) of the samples are marked on the power-time curves.

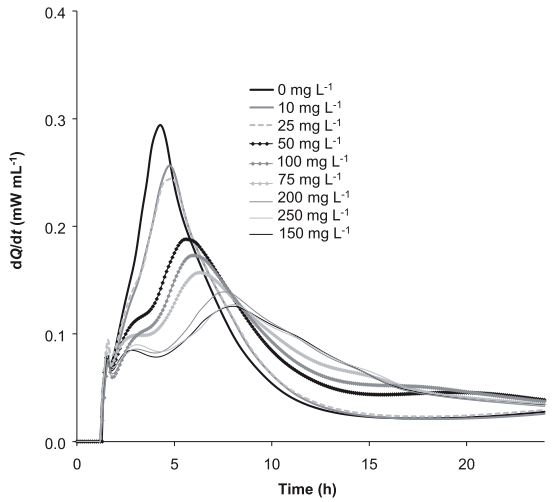

Fig. 3. Mean power-time curves ($n = 3$) of UHT 3.5% inoculated milk samples, incubated with H_2O_2 (0–250 mg L^{-1}) for 1 h and subsequently treated with catalase before starter addition.

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.

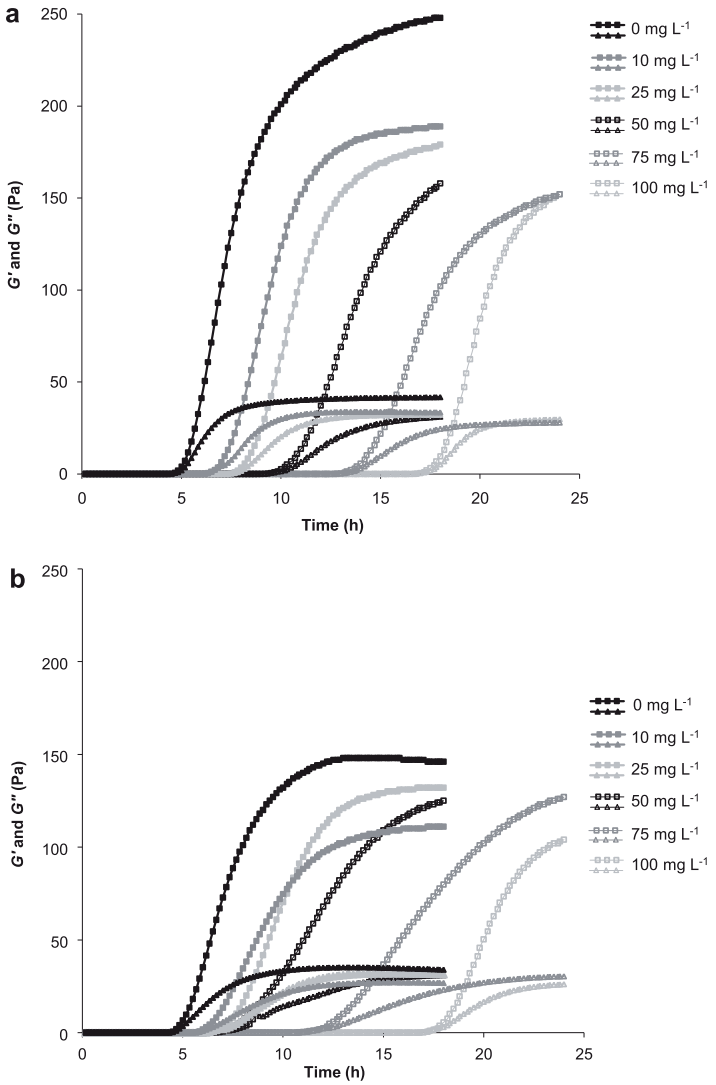

Fig. 4. Mean curves of storage (G') (■) and loss (G'') moduli (▲) as a function of time in inoculated milk samples pre-treated with H_2O_2 (0–100 mg L^{-1}) 1 h before starter addition: a) UHT 3.5% and b) UHT 0.05% milk samples. Curves are means of three replicates.

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.

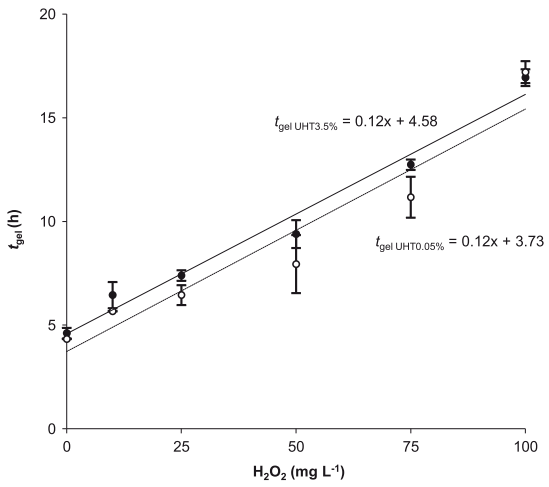

Fig. 5. Gelation initiation times of UHT 3.5% (●) and UHT 0.05% (○) milk samples as a function of H_2O_2 concentration. Values are means of three replicates with vertical bars for the standard deviations.

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

This investigation was financially supported through the grant ETF7636 of Estonian Science Foundation, through the grant SF0140090s08 of Estonian Ministry of Education and Research, and by the European Regional Development Fund projects EU22704, EU27607, EU28668, and EU29994.

References

Amin, V. M., & Olson, N. F. (1967). Effect of temperature on stability of hydrogen peroxide in milk. *Journal of Dairy Science, 50*, 1336–1338.
Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry, 272*, 20313–20316.
Björck, L. (1987). Preservation of milk by chemical means. *Bulletin of the International Dairy Federation, 221*, 57–61.
Codex Alimentarius Commission. (1991). Guidelines for the preservation of raw milk by use of the lactoperoxidase system (CAC/GL 13-1991). http://www.codexalimentarius.net/download/standards/29/CXG_013e.pdf Accessed 30.06.2010.
Cosio, M. S., Mannino, S., & Buratti, S. (2000). Electrochemical sensor detecting free sulfhydryl droups: evaluation of milk heat treatment. *Journal of Dairy Science, 83*, 1933–1938.
Courtin, P., Monnet, V., & Rul, F. (2002). Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus/Lactobacillus bulgaricus* mixed cultures in milk. *Microbiology, 148*, 3413–3421.
Fish, N. L., Pinkston, P. J., Claydon, T. J., & Mickelsen, R. (1969). Effect of hydrogen peroxide treatment of milk on its proteolysis by *Pseudomonas fluorescens*. *Journal of Dairy Science, 52*, 619–624.
Fonteh, F. A., Grandison, A. S., & Lewis, M. J. (2005). Factors affecting lactoperoxidase activity. *International Journal of Dairy Technology, 58*, 233–236.
Fox, P. F., & Kosikowski, F. V. (1967). Some effects of hydrogen peroxide on casein and its implications in cheese making. *Journal of Dairy Science, 50*, 1183–1188.
Gardea, A. A., Carvajal-Millán, E., Higuera-Ciapara, I., Figueroa, C., Molina-Corral, J., Rascón, A., et al. (2002). Calorimetric assessment of microbial growth in milk as affected by different conditions. *Thermochimica Acta, 394*, 179–184.
Gilliland, S. E. (1969). Enzymatic determination of residual hydrogen peroxide in milk. *Journal of Dairy Science, 52*, 321–324.
Gilliland, S. E., & Speck, M. L. (1969). Biological response of lactic streptococci and lactobacilli to catalase. *Applied Microbiology, 17*, 797–800.
Giulivi, C., Traaseth, N. J., & Davies, K. J. A. (2003). Tyrosine oxidation products: analysis and biological relevance. *Amino Acids, 25*, 227–232.
Henno, M., & Olkonen, A. (1998). Eestis turustatavad lüpsiseadmete pesu- ja desoained. *Tõuloomakasvatus, 4*, 18–20.
Hirano, R., Hirano, M., Oooka, M., Dosako, S., Nakajima, I., & Igoshi, K. (1998a). Lactoperoxidase effects on rheological properties of yogurt. *Journal of Food Science, 63*, 35–38.
Hirano, R., Hirano, M., Oooka, M., & Hatanaka, K. (1998b). Effect of lactoperoxidase on gelation properties of yogurt. *Food Research International, 31*, 1–6.
Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H., & Toba, T. (2003). The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Current Microbiology, 47*, 231–236.
Juffs, H. S., & Babel, F. J. (1975). Inhibition of psychrotrophic bacteria by lactic cultures in milk stored at low temperature. *Journal of Dairy Science, 58*, 1612–1619.
Kabanova, N., Kazarjan, A., Stulova, I., & Vilu, R. (2009). Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth. *Thermochimica Acta, 496*, 87–92.
Kosikowski, F. V., & Fox, P. F. (1968). Low heat, hydrogen peroxide, and bactofugation treatments of milk to control coliforms in Cheddar cheese. *Journal of Dairy Science, 51*, 1018–1022.
Kussendrager, K. D., & van Hooijdonk, A. C. M. (2000). Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *British Journal of Nutrition, 84*, S19–S25.
Letort, C., Nardi, M., Garault, P., Monnet, V., & Juillard, V. (2002). Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Applied and Environmental Microbiology, 68*, 3162–3165.
Maskow, T., & Babel, W. (2003). Thermokinetic description of anaerobic growth of *Halomonas halodenitrificans* using a static microcalorimetric ampoule technique. *Journal of Biotechnology, 101*, 267–274.
Østdal, H., Bjerrum, M. J., Pedersen, J. A., & Andersen, H. J. (2000). Lactoperoxidase-induced protein oxidation in milk. *Journal of Agricultural and Food Chemistry, 48*, 3939–3944.
Özer, B. H. (1999). Natural anti-microbial systems - lactoperoxidase and lactoferrin. In R. K. Robinson (Ed.), *Encyclopedia of food microbiology* (pp. 1587–1591). Oxford: Elsevier.
Özer, B. H., & Atamer, M. (1999). Some properties of yoghurts produced from milk preserved by hydrogen peroxide. *Milchwissenschaft, 54*, 83–86.
Özer, B., Grandison, A., Robinson, R., & Atamer, M. (2003). Effects of lactoperoxidase and hydrogen peroxide on rheological properties of yoghurt. *Journal of Dairy Research, 70*, 227–232.
Özer, B., Kirim, B., & Atamer, M. (2000). Effect of hydrogen peroxide on the quality of raw cream. *International Journal of Dairy Technology, 53*, 83–86.
Özer, B., Kirmaci, H. A., Oztekin, S., Hayaloglu, A., & Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal, 17*, 199–207.
Parpinello, G. P., Chinnici, F., Versari, A., & Riponi, C. (2002). Preliminary study on glucose oxidase–catalase enzyme system to control the browning of apple and pear purees. *LWT Food Science and Technology, 35*, 239–243.
Reiter, B., & Härnulv, G. (1984). Lactoperoxidase antibacterial system: natural occurrence, biological functions and practical applications. *Journal of Food Protection, 47*, 724–732.
Roundy, Z. D. (1958). Treatment of milk for cheese with hydrogen peroxide. *Journal of Dairy Science, 41*, 1460–1465.
Schmidt, R. H., Morris, H. A., & Morr, C. V. (1969). Action of rennet on casein as influenced by hydrogen peroxide-catalase treatment. *Journal of Dairy Science, 52*, 1727–1732.
Seifu, E., Buys, E. M., & Donkin, E. F. (2005). Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review. *Trends in Food Science and Technology, 16*, 137–154.
Siegenthaler, E. J., & Kosikowski, F. V. (1969). Electrochemical method for detecting hydrogen peroxide-catalase-treated milk. *Journal of Dairy Science, 52*, 1922–1927.
Subramanian, C. S., & Olson, N. F. (1968). Effect of hydrogen peroxide on activity of lactic cultures in milk. *Journal of Dairy Science, 51*, 517–519.
Takasaki, S., Kato, Y., Murata, M., Homma, S., & Kawakishi, S. (2005). Effects of peroxidise and hydrogen peroxide on the dityrosine formation and the mixing characteristics of wheat-flour dough. *Bioscience, Biotechnology, and Biochemistry, 69*, 1686–1692.
Tamime, A. Y., & Robinson, R. K. (1999). *Yoghurt science and technology* (2nd ed.). Cambridge, UK: Woodhead Publishing Limited.
Uchida, K. (2003). Histidine and lysine as targets of oxidative modifications. *Amino Acids, 25*, 249–257.

PUBLICATION III

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

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

International Dairy Journal, 31, 139–149 (2013)[3]

[3] Copyright 2010, reprinted with permission from Elsevier

International Dairy Journal 31 (2013) 139–149

Contents lists available at SciVerse ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

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

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

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

ARTICLE INFO

Article history:
Received 25 July 2012
Received in revised form
28 January 2013
Accepted 1 February 2013

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).

* Corresponding author. Tel.: +372 6 202 831.
E-mail addresses: irina.stulova@tftak.eu, irinastulova@yahoo.com (I. Stulova).

0958-6946/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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

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

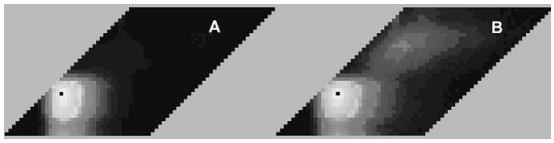

Fig. 1. Spectral fluorescence signatures of (A) low-heat skim milk powder (LHSMP) and (B) irradiated LHSMP; the black dot corresponds to the fluorescence intensity maximum.

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.

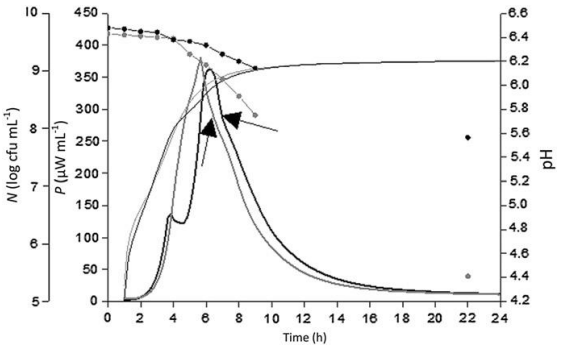

Fig. 2. Calorimetric power–time (P) curves (bold lines) of the growth of *Streptococcus thermophilus* ST12, number of bacteria (N) calculated from the power–time curves (thin lines), and pH changes (circles) in reconstituted skim milk powder (RSM; black) and in irradiated RSM (grey) at an inoculation 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.

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 mL^{-1})	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

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

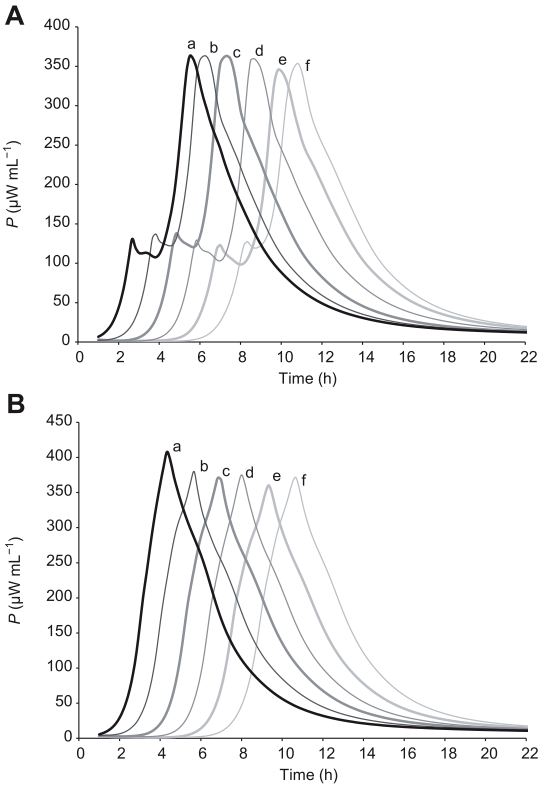

Fig. 3. Calorimetric power–time (P) curves of the growth of *Streptococcus thermophilus* ST12 in (A) reconstituted skim milk powder (RSM) and (B) irradiated RSM at different inoculation rates (cfu mL^{-1}): a, 10^6; b, 10^5; c, 10^4; d, 10^3; e, 10^2; f, 10^1.

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

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 10^5–10^9 bacteria (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,

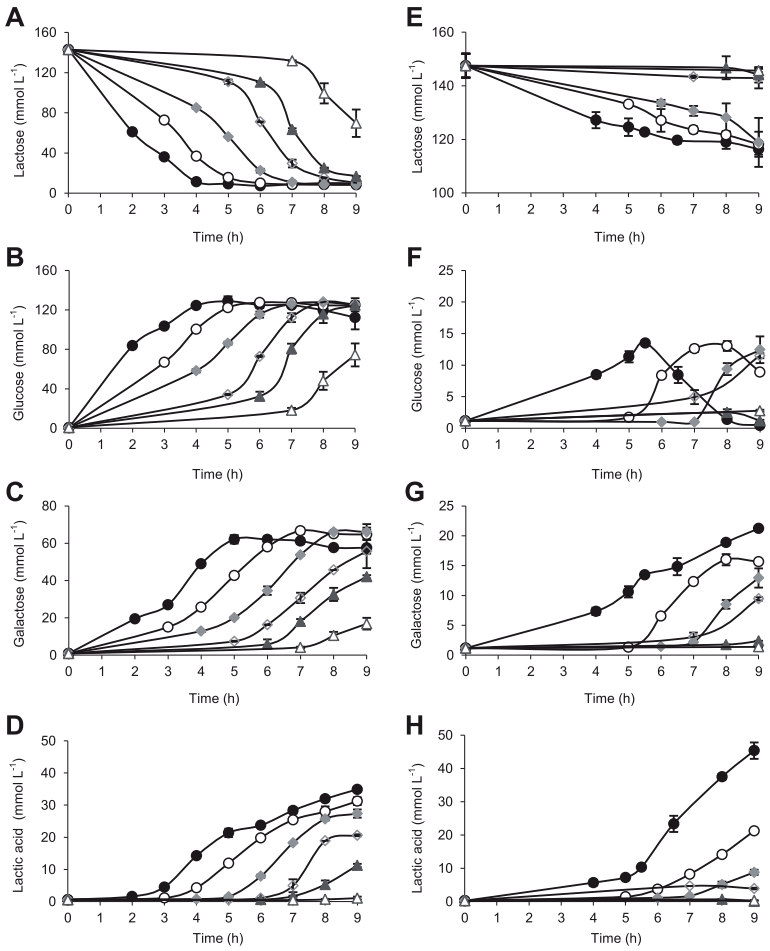

Fig. 4. Comparison of lactose (A, E), glucose (B, F), galactose (C, G) and lactic acid (D, H) profiles during growth of *Streptococcus thermophilus* ST12 in (A, B, C, D) reconstituted skim milk (RSM) and (E, F, G, H) irradiated RSM at different inoculation rates (cfu mL^{-1}): ●, 10^6; ○, 10^5; ◆, 10^4;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

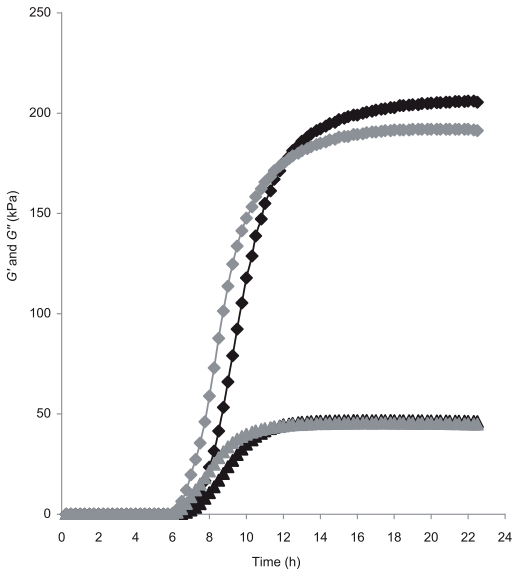

Fig. 5. Development of storage (G'; diamonds) and loss (G''; triangles) moduli during fermentation of reconstituted skim milk powder (RSM; black symbols) and irradiated RSM (grey symbols) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}.

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.

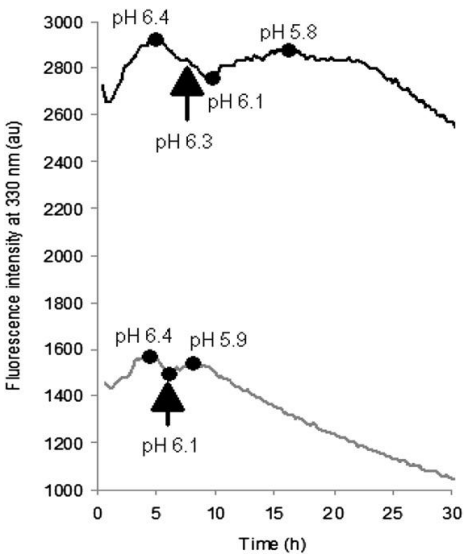

Fig. 6. Development of fluorescence intensity at 285/330 nm during fermentation of reconstituted skim milk powder (RSM; black line) and irradiated RSM (grey line) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}. Arrows mark the mean gelation times determined rheologically.

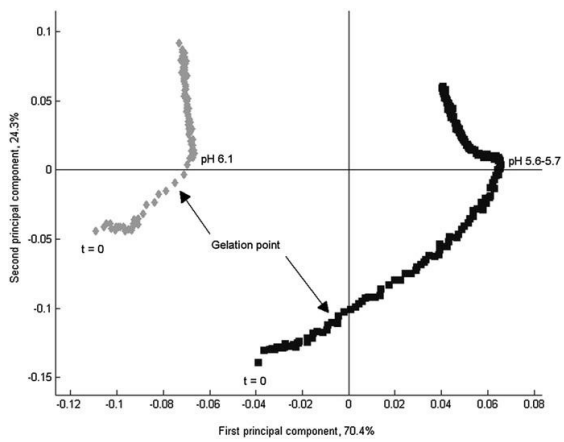

Fig. 7. Principal component analysis map defined by two first principal components for spectral fluorescence signatures data recorded during fermentation of reconstituted skim milk (RSM; black squares) and irradiated RSM (grey diamonds) with *Streptococcus thermophilus* ST12 at an inoculation rate of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

Considering PC1, 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, 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.

Acknowledgements

The financial support for this research was financially supported through the grant ETF7636 of Estonian Science Foundation, through the grant SF0140090s08 of Estonian Ministry of Education and Research, and by the EU Regional Development Fund projects EU28912, EU29994 and 3.2.0701.11-0018.

References

Adachi, S. (1962). Effect of gamma irradiation on lactose. II. Isolation and identification of monosaccharides and their derivatives formed in irradiated lactose solution. *Journal of Dairy Science, 45*, 1427–1434.

Arena, V. (1971). *Ionizing radiation and life*. Saint Louis, MO, USA: Mosby.

Bandekar, J., Kamat, A., & Thomas, P. (1998). Microbiological quality of the dairy product pedha and its improvement using gamma radiation. *Journal of Food Safety, 18*, 221–230.

Becker, E. M., Christensen, J., Frederiksen, C. S., & Haugaard, V. K. (2003). Front-face fluorescence spectroscopy and chemometrics in analysis of yogurt: rapid analysis of riboflavin. *Journal of Dairy Science, 86*, 2508–2515.

Bhattacharya, D., Saha, A., & Mandal, P. C. (2000). Radiation induced modification of tryptophan and tyrosine residues in flavocytochrome b_2 in dilute aqueous solution. *Radiation Physics and Chemistry, 59*, 71–80.

Braissant, O., Wirz, D., Göpfert, B., & Daniels, A. U. (2010). Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiology Letters, 303*, 1–8.

Chamba, J. F., & Prost, F. (1989). Mesure de l'activité acidifiante des bactéries lactiques thermophiles utilisées pour les fabrications des fromages á pâte cuite. *Lait, 69*, 417–431.

Champagne, C. P., Gagnon, D., St-Gelais, D., & Vuillemard, J. C. (2009). Interactions between *Lactococcus lactis* and *Streptococcus thermophilus* strains in Cheddar cheese processing conditions. *International Dairy Journal, 19*, 669–674.

Chauhan, S. K., Kumar, R., Nadanasabapathy, S., & Bawa, A. S. (2008). Detection methods for irradiated foods. *Comprehensive Reviews in Food Science and Food Safety, 8*, 4–16.

Cho, Y. S., Song, K. B., & Yamda, K. (2010). Effect of ultraviolet irradiation on molecular properties and immunoglobulin production-regulating activity of β-lactoglobulin. *Food Science and Biotechnology, 19*, 595–602.

Christensen, J., Povlsen, V. T., & Sørensen, J. (2003). Application of fluorescence spectroscopy and chemometrics in the evaluation of processed cheese during storage. *Journal of Dairy Science, 86*, 1101–1107.

Christopherson, A. T., & Zottola, E. A. (1989). Growth and activity of mesophilic lactic acid streptococci in ultrafiltered skim milk and in reconstituted nonfat dry milk of differing total solids contents. *Journal of Dairy Science, 72*, 2856–2861.

Cieśla, K., Salmieri, S., Lacroix, M., & Le Tien, C. (2004). Gamma irradiation influence on physical properties of milk proteins. *Radiation Physics and Chemistry, 71*, 93–97.

Davies, K. J. A. (1987). Protein damage and degradation by oxygen radicals. *Journal of Biological Chemistry, 262*, 9895–9901.

Davies, M. (2012). Free radicals, oxidants and protein damage. *Australian Biochemist, 43*, 8–12.

Day, E. A., Fross, D. A., & Patton, S. (1957). Flavour and odor defects of gamma-irradiated skimmilk. I. Preliminary observations and the role of volatile carbonyl compounds. *Journal of Dairy Science, 40*, 922–931.

De la Hoz, L., & Netto, F. M. (2008). Structural modifications of β-lactoglobulin subjected to gamma radiation. *International Dairy Journal, 18*, 1126–1132.

Delorme, C. (2008). Safety assessment of dairy microorganisms: *Streptococcus thermophilus*. *International Journal of Food Microbiology, 126*, 274–277.

Farkas, J. (2006). Irradiation for better foods – a review. *Trends in Food Science and Technology, 17*, 148–152.

Favrot, C., & Maubois, J. L. (1994). Growth of *Lactococcus lactis* subsp *lactis* in reconstituted irradiated milk powder. *Lait, 74*, 253–266.

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Annual Review of Pharmacology, 23*, 239–257.

Gaisford, S., O'Neill, M. A. A., & Beezer, A. E. (2009). Shelf life prediction of complex food systems by quantitative interpretation of isothermal calorimetric data. In G. Kaletunç (Ed.), *Calorimetry in food processing: Analysis and design of food systems* (pp. 237–263). New York, NY, USA: John Wiley & Sons Publishers.

Grolichová, M., Dvořák, P., & Musilová, H. (2004). Employing ionizing radiation to enhance food safety – a review. *Acta Veterinaria Brno, 73*, 143–149.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Chae, H. S., Yoo, Y. M., et al. (2009a). Irradiation effect on α- and β-caseins of milk and Queso Blanco cheese determined by capillary electrophoresis. *Radiation Physics and Chemistry, 78*, 158–163.

Ham, J. S., Jeong, S. G., Lee, S. G., Han, G. S., Jang, A., Yoo, Y. M., et al. (2009b). Quality of irradiated plain yogurt during storage at different temperatures. *Asian - Australasian Journal of Animal Sciences, 22*, 289–295.

Ham, J. S., Noh, Y. B., Kim, S. I., Kim, H. S., Jeong, S. G., Chae, H. S., et al. (2005). Changes of chemical, bacteriological, and allergenicity of raw milk by gamma irradiation. *Journal of Korean Dairy Technology Science, 23*, 93–98.

Herbert, S., Riaublan, A., Bouchet, B., Gallant, D. J., & Dufour, E. (1999). Fluorescence spectroscopy investigation of acid- or rennet-induced coagulation of milk. *Journal of Dairy Science, 82*, 2056–2062.

Hols, P., Hancy, F., Fontaine, L., Grossiord, B., Prozzi, D., Leblond-Bourget, N., et al. (2005). New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiology Reviews, 29*, 435–463.

Horrall, B. E., Elliker, P. R., & Kensler, G. (1950). The use of reconstituted non-fat dry milk solids for propagating mother and batch starter cultures. *Journal of Dairy Science, 33*, 250–256.

Juillard, V., Le Bars, D., Kunji, E. R. S., Konings, W. N., Gripon, J. C., & Richard, J. (1995). Oligopeptides are the main source of nitrogen for *Lactococcus lactis* growth in milk. *Applied and Environmental Microbiology, 61*, 3024–3030.

Juille, O., Le Bars, D., & Juillard, V. (2005). The specifity of oligopeptide transport by *Streptococcus thermophilus* resembles that of *Lactococcus lactis* and not that of pathogenic streptococci. *Microbiology, 151*, 1987–1994.

Kabanova, N., Kazarjan, A., Stulova, I., & Vilu, R. (2009). Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentration in broth. *Thermochimica Acta, 496*, 87–92.

Kabanova, N., Stulova, I., & Vilu, R. (2012). Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels. *Food Microbiology, 29*, 67–79.

Konteles, S., Sinanoglou, V. J., Batrinou, A., & Sflomos, K. (2009). Effects of γ-irradiation on *Listeria monocytogenes* population, colour, texture and sensory properties of Feta cheese during cold storage. *Food Microbiology, 26*, 157–165.

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

Laligant, A., Famelart, M.-H., Paquet, D., & Brulé, G. (2003). Fermentation by lactic bacteria at two temperatures of pre-heated reconstituted milk. II-Dynamic approach of the gel construction. *Lait, 83*, 307–320.

Leroy, F., & De Vuyst, L. (2003). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology, 15*, 67–78.

Letort, C., Nardi, M., Garault, P., Monnet, V., & Juillard, V. (2002). Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Applied and Environmental Microbiology, 68*, 3162–3165.

Liu, X., & Metzger, L. E. (2007). Application of fluorescence spectroscopy for monitoring changes in nonfat dry milk during storage. *Journal of Dairy Science, 90*, 24–37.

Lopez, C., & Dufour, E. (2001). The composition of the milk fat globule surface alters the structural characteristics of the coagulum. *Journal of Colloid and Interface Science, 233*, 241–249.

Martin, G. J. O., Williams, R. P. W., & Dunstan, D. E. (2007). Comparison of casein micelles in raw and reconstituted skim milk. *Journal of Dairy Science, 90*, 4543–4551.

Michel, V., & Martley, F. G. (2001). *Streptococcus thermophilus* in cheddar cheese-production and fate of galactose. *Journal of Dairy Research, 68*, 317–325.

Mihhalevski, A., Sarand, I., Viiard, E., Salumets, A., & Paalme, T. (2011). Growth characterization of individual rye sourdough bacteria by isothermal microcalorimetry. *Journal of Applied Microbiology, 110*, 529–540.

Mills, S., O'Sullivan, O., Hill, C., Fitzgerald, G., & Ross, R. P. (2010). The changing face of dairy starter culture research: from genomics to economics. *International Journal of Dairy Technology, 63*, 149–170.

Mohamed, M. O., Morris, H. A., & Breene, W. M. (1982). A comparison of stirred curd cheeses made from normal milks and reconstituted nonfat dry milk with vegetable oils. *Journal of Food Processing and Preservation, 6*, 15–30.

Niven, G. W., Knight, D. J., & Mulholland, F. (1998). Changes in the concentrations of free amino acids in milk during growth of *Lactococcus lactis* indicate biphasic nitrogen metabolism. *Journal of Dairy Research, 65*, 101–107.

Peters, J. J. (1960). Ripening of Cheddar cheese made from raw, pasteurized homogenized and reconstituted milk. *Milk Products Journal, 51*, 20–21.

Ranken, M. D., Kill, R. C., & Baker, C. G. J. (1997). *Food industries manual* (24th ed.). London, UK: Blackie Academic & Professional. Chap. 3.

Riva, M., Franzetti, L., Galli, A., & Schiraldi, A. (1997). Growth and fermentation activity of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in milk: a calorimetric investigation. *Annali di Microbiologia ed Enzimologia, 47*, 199–211.

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products – a review. *International Dairy Journal, 15*, 95–109.

Stadtman, E. R., & Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids, 25*, 207–218.

Thibessard, A., Leblond-Bourget, N., Fernandez, A., Gints, B., & Decaris, B. (2001). Response of *Streptococcus thermophilus* CNRZ368 and its colonial variants to oxidative stress: evidence for an inducible defence system. *Lait, 81*, 311–316.

Thomas, T. D., & Crow, V. L. (1984). Selection of galactose-fermenting *Streptococcus thermophilus* in lactose-limited chemostat cultures. *Applied and Envoronmental Microbiology, 48*, 186–191.

Wang, W., Zhang, L., Li, Y., Zhang, L., Luo, X., & Wang, S. (2012). Improvement of cheese produced by reconstituted milk powder: effects of *Streptococcus thermophilus* on the texture and microstructure. *Advanced Materials Research, 396–398*, 1541–1544.

Whitehead, W. E., Ayres, J. W., & Sandine, W. E. A. (1993). Review of starter media for cheese making. *Journal of Dairy Science, 76*, 2344–2353.

White, C. H., & Ryan, J. M. (1983). Defining optimal conditions for making cottage cheese from reconstituted milk powder. *Journal of Food Protection, 46*, 686–692.

Zanatta, P., & Basso, A. (1992). A new approach to the characterization of *Streptococcus salivarius* subsp *thermophilus* based on acidification rates. *Lait, 72*, 285–295.

Żegota, H., & Małolepszy, B. (2008). The decontamination of industrial casein and milk powder by irradiation. *Radiation Physics and Chemistry, 77*, 1108–1111.

PUBLICATION IV

Stulova, I., Adamberg, S., Kriščiunaite, T., Kampura, M., Blank, L., Laht, T.-M.

Microbiological quality of raw milk produced in Estonia.

Letters in Applied Microbiology, 51 (6), 683 – 690 (2010)[4]

[4] Copyright 2010, reprinted with permission from John Wiley and Sons

Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Microbiological quality of raw milk produced in Estonia

I. Stulova[1,2], S. Adamberg[1], T. Kriščiunaite[1,2], M. Kampura[1], L. Blank[1,2] and T-M. Laht[1,2]

1 Tallinn University of Technology, Tallinn, Estonia
2 Competence Centre of Food and Fermentation Technologies (CCFFT), Tallinn, Estonia

Keywords
aerobic spore-forming bacteria, farm bulk-tank raw milk, lactic acid bacteria, microbiological quality, *Pseudomonas*, psychrotrophic bacteria.

Correspondence
Irina Stulova, Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia.
E-mail: irinastulova@yahoo.com

2010/0760: received 6 May 2010, revised 8 September 2010 and accepted 28 September 2010

doi:10.1111/j.1472-765X.2010.02951.x

Abstract

Aims: The microbial quality of farm bulk-tank raw milk produced in Estonia during years 2004–2007 was investigated.
Methods and Results: Bulk-tank milk samples were analysed for lactic acid bacteria count (LABC), psychrotrophic bacteria count (PBC), aerobic spore-forming bacteria count (ASFBC), total bacterial counts using BactoScan and somatic cell count (SCC) using Fossomatic. Randomly selected psychrotrophic isolates were subjected to 16S–23S PCR-ribotyping. LABC remained below 10^4 CFU ml^{-1} in most samples, while psychrotrophic micro-organisms dominated in 60% of farms. PBC ranged from $4{\cdot}2 \times 10^2$ to $6{\cdot}4 \times 10^4$ CFU ml^{-1}, and ASFBC varied from 5 to 836 CFU ml^{-1}.
Conclusions: In general, the microbiological quality of the farm bulk-tank milk was good – more than 91% of samples contained <50 000 CFU ml^{-1}, and SCC in the majority of samples did not exceed the internationally recommended limits. Genus *Pseudomonas* spp. was the dominating spoilage flora with *Pseudomonas fluorescens* as the prevailing species.
Significance and Impact of the Study: Specific bacterial groups (LABC, PBC and ASFBC), not analysed routinely by dairies, were determined in bulk-tank raw milk of numerous dairy farms during 4-year period. Based on the survey, dairy plants can better control their supply chains and select farms (milk) for the production of specific products, i.e. milk with low PBC and high LABC for cheesemaking.

Introduction

Factors affecting yield and quality of fermented dairy products are of major concern for the dairy industry. The primary prerequisite for making high quality fermented milk products is the production of high quality raw milk by dairy farms. Milk from different farms varies in the physico-chemical characteristics and microbiological quality. These parameters are especially technologically important when the milk is used for the manufacture of fermented milk products, cheese or liquid milk products with long shelf life. Peculiarities of the changes of the content and composition of bacterial flora in raw milk and its implications on the dairy technologies need to be described and taken into account (Niku-Paavola *et al.* 1999). Closed milking systems, better sanitary design of equipment, more efficient cleaning of cows (udder, teats) and effective 'clean in place' systems enable to produce raw milk with extremely low microbial contamination (Barbano *et al.* 2006). However, rapid cooling and long storage of raw milk at low temperature can be a favour for the growth of psychrotrophic bacteria. Raw cow milk offered for sale within the European Union (EU) has to be produced according to the requirements of Commission Directive 89/362/EEC and to meet quality standards described in Council Directive 92/46/EEC. Microbiological quality of raw milk is assessed in large extend by total bacterial count (TBC) and somatic cell count (SCC), and these parameters are routinely measured and compared. Milk for human consumption should have a 3-month geometric mean SCC of <400 000 cells ml^{-1} and plate count at 30°C less than 100 000 CFU ml^{-1} [Regulation (EC) No 853/2004]. In Estonia, the milk received by

dairy plants is classified in three quality categories according to TBC and SCC: premium class with TBC not exceeding 50 000 CFU ml^{-1} and SCC <300 000 cells ml^{-1}; extra class with TBC ≤100 000 CFU ml^{-1} and SCC <400 000 cells ml^{-1} and first class with TBC ≤200 000 CFU ml^{-1} and SCC ≤600 000 cells ml^{-1} (Estonian Ministry of Agriculture 2008).

Lactic acid bacteria (LAB) are widely distributed in the nature and considered generally as indigenous microflora of raw milk. Representatives of genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* are usually found in milk. Decrease in LAB together with other contaminating bacteria in raw milk should be considered probably the most significant change of the milk quality caused by the technological progress (Guessas and Kihal 2004).

It has been shown that more than 50% of psychrotrophs in refrigerated raw milk at the first day of storage belong to the genus *Pseudomonas*, with *Pseudomonas fluorescens* being the predominant species (Lafarge *et al.* 2004). Psychrotrophic (Gram-negative) bacteria can produce heat-stable proteases and lipases that are potentially harmful for technological quality of milk. Significant levels of extracellular enzymes and the consequent defects in end products generally appear when bacterial counts exceed 10^6 CFU ml^{-1} (Kumaresan *et al.* 2007). Conversely, it has been shown that heat-stable proteases produced by even low numbers of *Ps. fluorescens* could activate the plasmin system in milk and accelerate proteolysis in cheese (Frohbieter *et al.* 2005). Until recently, the bacterial community of raw milk was described only by classical microbiological methods (Ercolini *et al.* 2009). Modern DNA-based techniques have provided new possibilities for bacterial identification and taxonomy. For example, ribotyping has been widely used for identifying Gram-negative bacteria isolated from milk or dairy products (Martins *et al.* 2006).

Aerobic spore-forming bacteria (ASFB) belonging to the genus *Bacillus* are frequently present in raw milk. Their spores survive pasteurization, germinate, outgrow, and they have been linked to spoilage of raw and pasteurized milk and dairy products such as fermented and ultra heat treated (UHT) milk (McGuiggan *et al.* 2002; Coorevits *et al.* 2008). The increased aerobic spore concentration in farm tank milk during the spring and summer months has been associated with the contamination of teats with soil during grazing (Vissers *et al.* 2007). During the housing period, the teats can be contaminated by faeces, feed and bedding material: hay and dust have been considered important sources of contamination in winter (Magnusson *et al.* 2007; Christiansson *et al.* 1999).

The aim of the current study was to investigate microbial quality of farm bulk-tank raw milk produced in Estonia during years 2004–2007 and to identify the predominant bacterial groups: LAB and technologically potentially harmful psychrotrophic and spore-forming bacteria. These data can help dairy farms to improve milk quality as well as dairies to select milk for different applications – either drinking milk or products made with starter cultures like yoghurt, quark and cheese.

Materials and methods

Sampling

A total of 970 bulk-tank raw milk samples were collected from 131 farms at geographically different locations in Estonia by licensed milk haulers between February 2004 and June 2007. Samples were also taken from dairy silos in the same days. The samples were held on ice until arrival to the laboratory, and all microbiological analyses were performed within 36 h from sampling.

Enumeration of micro-organisms

The numbers of LAB, psychrotrophic bacteria and mesophilic ASFB were determined by plate count method. Milk samples were diluted according to the IDF standard (Anon. 1996). LAB were enumerated on De Man, Rogosa and Sharpe agar (MRS) – inoculated plates were incubated at 30°C for 72 h. Samples for the determination of psychrotrophic bacteria count (PBC) were spread on plate count agar (PCA), and plates were incubated at 7°C for 7 days (IDF Standard 101A:1991). For the enumeration of mesophilic ASFB, aliquots of milk were heated at 80°C for 10 min, cooled in ice bath, plated on PCA supplemented with 0·1% skim milk powder (Kalev Paide Tootmine AS, Paide, Estonia) and incubated at 30°C for 72 h.

State milk quality control analysis

For regular milk quality control, all collected samples were analysed for TBC using BactoScan (FOSS Analytical A/S, Hillerød, Denmark) and SCC using Fossomatic (FOSS Analytical A/S) in the Laboratory of Animal Breeding (Tartu, Estonia) according to the supplier's manual.

Characterization of psychrotrophic bacteria

Selected isolates from psychrotrophic agar plates were identified at species level by PCR-ribotyping, and their lipolytic and proteolytic activity and pigment production were determined.

Lipolytic activity. Lipolytic activity was tested by agar diffusion assay in tributyrin agar (1·0% tributyrin, 0·5% peptone, 0·3% yeast extract, 1·4% agar) at 30°C for

3 days. Colonies surrounded by clear zones were identified as lipolytic isolates.

Proteolytic activity. Proteolytic activity was estimated by agar diffusion assay using skim milk agar (10% skim milk powder, 0·5% peptone, 1·5% agar) incubated at 7°C for 14 days and at 25°C for 7 days. The presence of clear zones around the colonies was indicative of proteolysis.

Production of fluorescent pigments. Psychrotrophic bacteria with proteolytic activity were further examined for fluorescent pigment production using King B medium (20·0 g l^{-1} proteose peptone, 10·0 g l^{-1} glycerol, 1·5 g l^{-1} K_2HPO_4, 1·5 g l^{-1} $MgSO_4 \times 7\ H_2O$, 15·0 g l^{-1} agar), and colony fluorescence was confirmed under UV light (260 nm).

DNA extraction. DNA was extracted using PureLink Genomic DNA Purification kit (Invitrogen, Carlsbad, CA, USA) or using method described by Scarpellini *et al.* (2004). Bacterial suspension (200 μl) was boiled in MilliQ (OD_{600} = 0·6; Millipore SAS, Molsheim, France) for 10 min in Eppendorf tubes. The tubes were cooled on ice and centrifuged (20 000 ***g*** for 10 min at 5°C); the supernatants were kept on ice or −20°C. One microlitre of template DNA suspension was used for each reaction.

PCR-ribotyping. PCR-ribotyping uses PCR to amplify the 16S–23S intergenic spacer region of the bacterial rRNA operon. DNA amplification was performed using the primer set 23SR7 (5′-GGTACTTAGATGTTTCAGTTC-3′) and 16SR2 (5′-TTGTACACACCGCCCGTCA-3′) as described by Wang and Jayarao (2001). All PCRs were performed in total volume of 25 μl containing 50–100 ng of bacterial genomic DNA solution, 2·5 μl of 10× PCR buffer, 100 μmol l^{-1} of each dNTP, 2·5 μl of $MgCl_2$, 1·25 μl of each primer, 0·1 μl of Taq DNA polymerase (5 U μl^{-1}) and 15 μl of distilled water. Each PCR cycle consisted of 95°C for 1 min, 52°C for 1 min and 75°C for 1 min. A total of 25 cycles were run in thermocycler (Biorad DNA Engine Thermal Cycler; BioRad, Hercules, CA). The amplified DNA was electrophoresed in a 2% agarose gel containing 1·5 μg ml^{-1} ethidium bromide using 1× TAE buffer at 90 V for 1·5 h. The amplified DNA was visualized and photographed using Healthcare Image Quant 400 system. *Pseudomonas fluorescens* Live A506 and *Pseudomonas putida* Live KT 2701 were used as reference strains (Institute of Molecular and Cell Biology, University of Tartu).

Statistical evaluation of results

Statistical analyses were performed on 604 of 970 raw milk samples. Three hundred and sixty-six samples were excluded from correlation analyses because one or more of the microbiological parameters were not available. Results expressed as CFU ml^{-1} were converted to decimal logs. The log10 transformed SCC and bacterial counts [LABC, PBC and aerobic spore-forming bacteria count (ASFBC)] in samples from each farm were averaged and subjected to correlation coefficient analysis. Linear regression was calculated using Microsoft Excel software. All correlation coefficients remained below 0·5.

Results

The results of the microbiological studies of bulk-tank raw milk samples are presented in Table 1. Distribution of bacterial counts in the samples is shown in Fig. 1. In 119 (91%) of 131 farms, the TBC of the milk samples did not exceed 50 000 CFU ml^{-1}, attesting that the milk was of good quality. During the period 2004–2007, the TBC of the bulk-tank raw milk showed a clear declining tendency (Table 2).

Lactic acid bacteria count (LABC)

Mean LABC in bulk-tank raw milk samples collected from separate farms varied roughly from 10^2 to10^5 CFU ml^{-1} with the mean value of all analysed bulk-milk samples of $7{\cdot}6 \times 10^3$ CFU ml^{-1} (Table 1). Mean LABC in the milk of separate farms $<10^4$ CFU ml^{-1} was obtained in 102 farms (78%). Our study showed that LAB were the dominant bacterial group only in 24 farms (18% of total).

LABC in bulk-tank raw milk decreased in 21 farms (16%) during observed time period, while in three farms, it decreased even more than 20 times; in 13 farms (10%), LABC increased. LABC of 60 farms (46%) was fairly

Table 1 Overall mean and boundary values of microbiological parameters and SCCs found in bulk-tank raw milk from separate farms from 2004 to 2007 (CFU ml^{-1})

Parameter	Mean	Min*	Max*	Standard†
LABC	7600	210	145 000	(–)
PBC	10 000	424	64 000	(–)
ASFBC	131	5	836	(–)
TBC	27 000	5000	143 000	100 000
SCC	277 000	129 000	514 500	400 000

TBC, total bacterial count; SCC, somatic cell count; PBC, psychrotrophic bacteria count; LABC, lactic acid bacteria count; ASFBC, aerobic spore-forming bacteria count; (–), No standard established.

*Boundary values are given as mean values of LABC, PBC, ASFBC, TBC and SCC established for separate farms through the studied period.

†Current regulatory limit for TBC (CFU ml^{-1}) and SCC (cells ml^{-1}) in EU (EEC, 1992, Council Directive 92/46/EEC).

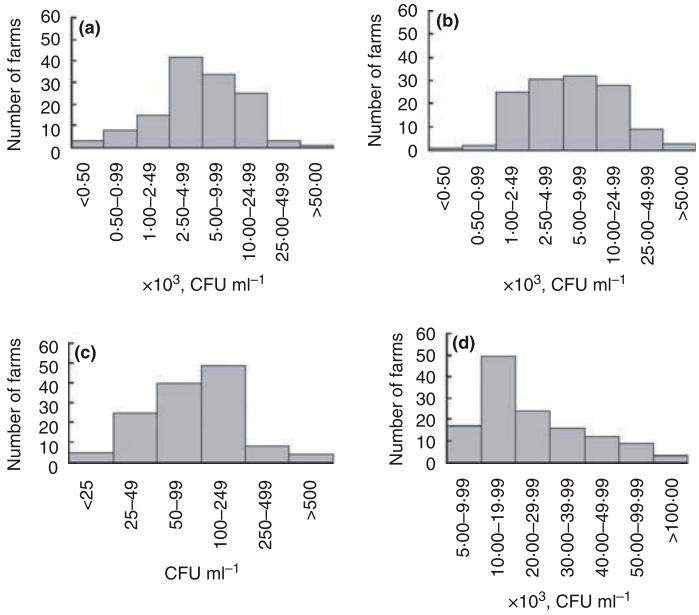

Figure 1 Histograms showing the relative distribution of bacterial numbers among numerical intervals for the four microbiological analyses: (a) lactic acid bacteria count, (b) psychrotrophic bacteria count, (c) aerobic spore-forming bacteria count, (d) total bacterial count.

Table 2 Annual trend for the mean values of microbiological parameters (LABC, PBC, ASFBC, TBC) of bulk-tank raw milk samples from farms and storage silos at dairy plant (CFU ml^{-1})

	LABC		PBC		ASFBC		TBC	
Year	Dairy farm	Raw milk storage silos	Dairy farm	Raw milk storage silos	Dairy farm	Raw milk storage silos	Dairy farm	Raw milk storage silos
2004	23 700	66 800	22 000	135 500	5197	627	36 500	35 000
2005	9070	6200	6400	25 600	73	138	22 500	101 500
2006	8750	4600	13 600	68 700	434	214	23 700	54 000
2007	8650	10 400	10 600	12 600	135	132	21 000	44 000

TBC, total bacterial count; PBC, psychrotrophic bacteria count; LABC, lactic acid bacteria count; ASFBC, aerobic spore-forming bacteria count.

constant, and counts in 37 farms (28%) changed essentially randomly 1–2 log units.

The highest mean annual LABC level calculated on the basis of individual bulk-tank raw milk samples was observed in 2004 (Table 2); high LABC was determined mainly during the spring months (March–May) (Fig. 2). Mean LABC level was lower in 2005 compared to 2004, with the maximum values observed in November ($7{\cdot}8 \times 10^5$ CFU ml^{-1}), what is possibly attributed to two farms with maximum values of $3{\cdot}1 \times 10^5$–$6{\cdot}2 \times 10^5$ CFU ml^{-1} and three farms with values of $9{\cdot}0 \times 10^4$–$1{\cdot}0 \times 10^5$ CFU ml^{-1}. Annual mean value of LABC decreased during observed time period approx. 2–3 times from $2{\cdot}4 \times 10^4$ CFU ml^{-1} in 2004 to below 10^4 CFU ml^{-1} in 2007 (Table 2).

Psychrotrophic bacteria count (PBC)

Mean PBC in bulk-tank raw milk samples from separate farms ranged from $4{\cdot}2 \times 10^2$ to $6{\cdot}4 \times 10^4$ CFU ml^{-1} with the overall mean value of 10^4 CFU ml^{-1} (Table 1). Mean PBC in milk of separate farms $<10^4$ CFU ml^{-1} was observed in 91 farms (69%).

The numbers of psychrotrophic bacteria decreased in 23 farms (18%) during the study period, among them three farms showed a decrease in PBC more than 20 times, while a slight increase in PBC was reported in 13 farms (10%). PBC of milk from 52 farms (40%) remained fairly constant: considerable fluctuation (1–2 log units) in PBC was observed in 43 farms (33%) during the 4-year period.

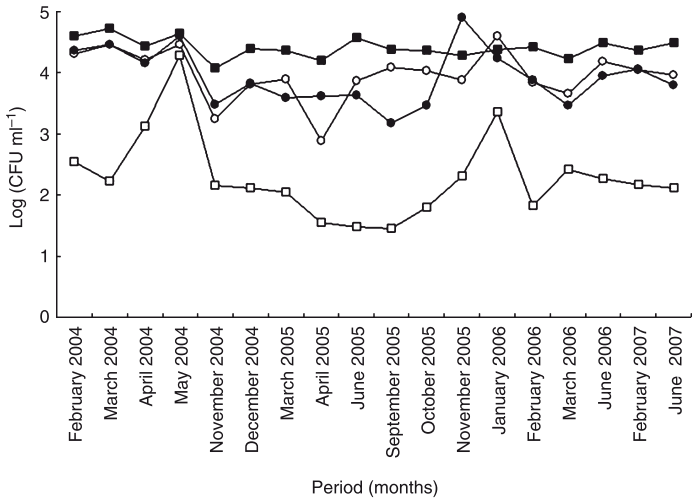

Figure 2 Monthly changes in the microbial counts in bulk-tank raw milk expressed as log CFU ml^{-1}: (●) lactic acid bacteria count, (○) psychrotrophic bacteria count, (□) aerobic spore-forming bacteria count, (■) total bacterial count. Monthly values are means of all samples analysed in each month.

The highest annual mean of PBC ($2{\cdot}2 \times 10^4$ CFU ml^{-1}) was found in 2004 (Table 2), and the lowest annual mean ($6{\cdot}4 \times 10^3$ CFU ml^{-1}) was determined in 2005, with the lowest monthly mean value of 765 CFU ml^{-1} in April (Fig. 2). An increase in PBC over 10^4 CFU ml^{-1} was observed again in 2006; relatively high PBC in January was caused by four bulk-tank raw milk samples from separate farms with high PBC over 10^5 CFU ml^{-1} (Fig. 2). The annual trend in PBC during 2004–2007 was not consistent, but a general tendency for PBC was decreasing with time (Table 2).

Based on the morphology of the colonies, more than 1000 randomly selected Gram-negative psychrotrophic isolates from bulk-tank raw milk samples were examined. From those, 120 representative isolates were further characterized for proteolytic and lipolytic activities. Lipolytic activity was not detected in any of the selected isolates, while 80 were proteolytically active both at room temperature and in the refrigerator at 7°C. Among them, 60 isolates produced fluorescent pigment. According to PCR-ribotyping patterns, *Ps. fluorescens* was the dominating psychrotrophic species in bulk-tank raw milk from Estonian farms and in dairy silos.

Aerobic spore-forming bacteria count (ASFBC)

The mean numbers of ASFB in bulk-tank raw milk samples collected from separate farms varied from 5 to 836 CFU ml^{-1} with the overall mean value of 131 CFU ml^{-1}. ASFBC remained always below 100 CFU ml^{-1} in 70 farms (53%); among them, 30 farms (23% of total) had ASFB mean values under or equal to 49 CFU ml^{-1}. ASFBC was in range 100–499 CFU ml^{-1} in 57 farms (44%). Significantly high ASFB levels (over 500 CFU ml^{-1}) were determined in four farms (Fig. 1).

A decrease in ASFBC in bulk-tank raw milk samples was observed in 23 farms (18%), during the study period, while in seven farms, the decrease in ASFBC was more than ten times. ASFBC was fairly constant in 32 farms (24% of total) and unstable in 76 farms (58%).

The highest annual mean of ASFBC ($5{\cdot}2 \times 10^3$ CFU ml^{-1}) was observed in 2004 (Table 2). From April to May 2004, the monthly mean of ASFBC in farm bulk-tank milk was remarkably high (Fig. 2), which could possibly be attributed to one farm with maximum value of 10^5 CFU ml^{-1} in April and five farms with maximum values of 10^4–10^6 CFU ml^{-1} in May. In 2005, the mean annual ASFBC in milk samples was low (73 CFU ml^{-1}). An increase in ASFBC was observed in 2006 (434 CFU ml^{-1}); relatively high numbers in January can be attributed to two farms with high ASFBC near 10^4 CFU ml^{-1}). Twelve farms showed high ASFBC (≥1000 CFU ml^{-1}) steadily during winter months.

Total bacterial count (TBC) and somatic cell count (SCC)

During the period 2004–2007, mean TBC in farm bulk-tank raw milk from separate farms ranged from $5{\cdot}0 \times 10^3$ to $1{\cdot}4 \times 10^5$ CFU ml^{-1} with the mean value of $2{\cdot}7 \times 10^4$ CFU ml^{-1} (Table 1). Sixty-seven farms of 131 (51%) had TBC $\leq 2{\cdot}0 \times 10^4$ CFU ml^{-1}, while TBC greater than of 100 000 CFU ml^{-1} was detected only in three farms. Across all 4 years, the highest values of TBC were

recorded in June and the lowest values in November. The maximum annual mean value of TBC ($3{\cdot}6 \times 10^4$ CFU ml^{-1}) was observed in 2004. Generally, high TBC was found mainly during the spring months (March to May) (Fig. 2). During 4 years, TBC of the raw milk decreased clearly in 35 farms (27%); among them, six farms showed decrease in TBC more than 20 times. A slight increase in TBC was reported in 11 farms (8%). TBC was relatively stable in 34 farms (26%) throughout the study period, and it changed randomly in 51 farms (39%).

Mean SCC in farm bulk-tank raw milk samples from separate farms was in the range from 129 000 to 514 500 cells ml^{-1}. Mean SCC of eight farms tested was >400 000 cells ml^{-1} but the majority of samples (93%) remained below this level, yielding an average SCC of 277 000 cells ml^{-1} for all bulk-tank milk samples (Table 1). In general, the farm milk contained the highest SCCs in June, the lowest in November and December (Fig. 2). Seventeen of 131 farms examined (13%) had SCC <200 000 cells ml^{-1}. SCCs between 200 000 and 400 000 cells ml^{-1} were detected in 106 farms (81%). In seven farms (5%), the average SCC was constantly high: 400 000–499 999 cells ml^{-1} and in one farm, it exceeded 500 000 cells ml^{-1}. A decrease of 2–3 times in SCC in 31 farms (24%) and an increase in 14 farms (11%) were observed during 4-year period. The SCC level was stable in 31 farms (24%) and changed randomly in 55 farms (39%).

Discussion

The main priority of the Estonian agriculture is milk production. During the recent years, farmers and dairy enterprises have made considerable investments to increase milk production and improve milk quality. Most of the milk in Estonia is produced in relatively big dairy farms: about 55% of milk is supplied by farms keeping 100–600 cows, 26% of milk is produced by farms with over 600 cows (Estonian Dairy Association 2007). Farm bulk-tank raw milk is refrigerated quickly below 4°C after milking and collected by large refrigerated trucks mostly on the following day. Distances for transportation from farm to dairy plant are 100 km on average. Milk storage time comprises two distinct periods: in the bulk-milk tank at farm and in the dairy silo. Microbiological quality of raw milk is usually evaluated by TBC and SCC.

The microbial counts determined once a week in farm bulk-tank raw milk samples with Bactoscan showed that TBC in Estonian farm milk have decreased about ten times during the last decades – from nearly 500 000 CFU ml^{-1} in average in 1990 (T-M. Laht, personal communication) to nearly 5000–10 000 CFU ml^{-1} in 2004–2007 according to our data. Milk also contains a certain level of somatic cells, and frequently their content and effects on milk quality are considered together with the bacterial contamination. If there is a bacterial infection, tissue damage or other inflammation processes affecting the mammary tissue, the SCC in milk increases abruptly (Gargouri *et al.* 2008). SCC in the majority of analysed bulk-tank raw milk samples did not exceed the internationally recommended limits. Several studies have implicated high SCC as a causative factor of the reduced shelf life of fluid milk (Berry *et al.* 2006) as well as reduced cheese quality (Barbano *et al.* 1991), lower cheese yield, increased fat and casein loss in whey and compromised sensory quality (Ma *et al.* 2000). According to TBC and SCC content and the classification of milk quality by Estonian Ministry of Agriculture, 94% of the analysed farm bulk-tank raw milk samples belonged to extra class (among them 65% to premium class) and 6% to class I.

In Europe, PBC of raw milk should not exceed 50 000 CFU ml^{-1} (Cempírková and Mikulová 2009). Lipolytic and proteolytic activities, supported by psychrotrophic bacteria, are considered insignificant at PBC lower than 10^6 CFU ml^{-1} (Cempírková and Mikulová 2009). In the present study, PBC remained below 50 000 CFU ml^{-1} in the majority of milk samples (98%). According to molecular analyses, genus *Pseudomonas* spp. was the dominating spoilage flora in farm bulk-tank raw milk with *Ps. fluorescens* as the prevailing species, as also shown by Lafarge *et al.* (2004). Controlling the content of psychrotrophic bacteria is very important because these bacteria are able to produce heat-stable extracellular proteases, as well as lipases. Proteases are associated with bitterness in milk, gelation of UHT sterilized milk and reduced yield of soft cheese (Hantsis-Zacharov and Halpern 2007). Lipases, by hydrolysing triglycerides, cause flavour defects associated with fat breakdown in cream, butter, cheese and UHT products (Hantsis-Zacharov and Halpern 2007). Compared to farm bulk-tank raw milk, the PBC in dairy plant storage silos was higher (Table 2). The increased PBC in dairy plant storage silos may be caused by contamination during transportation and/or storage of raw milk at low temperatures for 2–3 days before processing.

Spore-forming micro-organisms have a special position among total microflora of raw milk with regard to their ability to survive pasteurization and subsequently to propagate in final products (Foltys and Kirchnerová 2006). Barbano *et al.* (2006) stated that spores are typically present in low numbers in raw milk. The numbers of ASFB found in bulk-tank raw milk in our study were generally higher (mean of 131 CFU ml^{-1}) than reported by Boor *et al.* (1998), with mean of 49 CFU ml^{-1}. ASFB were found in raw milk samples from all farms during

the whole studied period. The counts of ASFB from raw milk storage silos showed great variations over sampling days (from 9 to $4{\cdot}5 \times 10^3$ CFU ml^{-1}).

LABC in farm bulk-tank raw milk has decreased during the past decade about 20 times in Estonia (P. Elias, personal communication). Content of LAB in raw milk has decreased possibly because of improved hygiene of milk production and rapid cooling of milk after milking. LAB could be considered as normal microflora of raw milk. Taking into account relatively high levels of psychrotrophic bacteria in milk, unfavourable proportion between LAB and technologically harmful microflora could be expected.

Today, the first selection of milk in dairies is based on express and routine microbiological methods; however, the analyses of specific microbial groups performed in the present study are rather time and labour consuming for a dairy plant. The results of the present study have helped dairies in Estonia to choose milk for specific products. For example, we found one farm that produced milk with the highest average SCC ($5{\cdot}3 \times 10^5$ cells ml^{-1}), with the highest average TBC level ($7{\cdot}5 \times 10^5$ CFU ml^{-1}) and highest average PBC level ($2{\cdot}4 \times 10^5$ CFU ml^{-1}). Microbiological and somatic cells parameters in the samples from this farm were higher than the critical level defined for farm bulk-tank raw milk. Another farm in analysed set produced milk with high average PBC ($1{\cdot}5 \times 10^5$ CFU ml^{-1}) and with the highest average ASFBC (over 700 CFU ml^{-1}), exceeding the permissible hygienic limit for PBC and ASFBC. Both of these farms were excluded from the supply chain.

In conclusion, the results of the current study clearly indicate good microbiological quality of raw milk produced in Estonia: in average 94% of the raw milk satisfies the requirements of international and Estonian standards. However, LABC tends to decrease and was found relatively stable over time only in 24 (18%) farms studied. Generally, content of psychrotrophic organisms did not exceed established international limits. *Pseudomonas* spp. was the dominating spoilage flora in Estonian farm milk with *Ps. fluorescens* as the prevailing species. Although milk selection for different products in dairies is based on express and routine microbiological methods, proportions of different microbial groups may differ between raw milk samples with similar TBC numbers. Our study gives information about stability of the microbiological quality and content of technologically important bacterial groups in raw bulk-tank milk from individual farms that can be used by dairy plants for the better selection of milk for specific products. Milk from dairy farms producing constantly high bacteriological quality milk (low PBC, ASFBC, SCC and higher LABC) is preferred for the production of fermented dairy products and cheese.

Acknowledgements

The authors thank Kristi Pärn from Tallinn Dairy Ltd. for valuable assistance and Prof. Raivo Vilu for critically reading the manuscript. This investigation was financially supported by the grant ETF7636 of Estonian Science Foundation, by the European Regional Development Fund projects EU22704 and EU29994, and Estonian Ministry of Education and Research, Estonia, through the grant SF0140090s08.

References

Anon (1996) *IDF standard 122C. Milk and Milk Products. Preparation of samples and Dilutions for Microbiological Examination*. Brussels: International Dairy Federation.

Barbano, D.M., Rasmussen, R.R. and Lynch, J.M. (1991) Influence of milk somatic cell count and milk age on cheese yield. *J Dairy Sci* **74**, 369–388.

Barbano, D.M., Ma, Y. and Santos, M.V. (2006) Influence of raw milk quality on fluid milk shelf life. *J Dairy Sci* **89**, 15–19.

Berry, D.P., O'Brien, B., O'Callaghan, E.J., Sullivan, K.O. and Meaney, W.J. (2006) Temporal trends in bulk tank somatic cell count and total bacterial count in Irish dairy herds during the past decade. *J Dairy Sci* **89**, 4083–4093.

Boor, K.J., Brown, D.P., Murphy, S.C., Kozlowski, S.M. and Bandler, D.K. (1998) Microbiological and chemical quality of raw milk in New York State. *J Dairy Sci* **81**, 1743–1748.

Cempírková, R. and Mikulová, M. (2009) Incidence of psychrotrophic lipolytic bacteria in cow's raw milk. *Czech J Anim Sci* **54**, 65–73.

Christiansson, A., Bertilsson, J. and Svensson, B. (1999) *Bacillus cereus* spores in raw milk: factors affecting the contamination of milk during the grazing period. *J Dairy Sci* **82**, 305–314.

Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. and Heyndrick, M. (2008) Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126–140.

Ercolini, D., Russo, F., Ferrocino, I. and Villani, F. (2009) Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiol* **26**, 228–231.

Foltys, V. and Kirchnerová, K. (2006) Mesophilic and psychrotrophic aerobic sporulating microorganisms in raw cow's milk. *Arch Zootech* **9**, 41–55.

Frohbieter, K.A., Ismail, B., Nielsen, S.S. and Hayes, K.D. (2005) Effects of *Pseudomonas fluorescens* M3/6 bacterial protease on plasmin system and plasminogen activation. *J Dairy Sci* **88**, 3392–3401.

Gargouri, A., Hamed, H. and ElFeki, A. (2008) Total and differential bulk cow milk somatic cell counts and their relation with lipolysis. *Livest Sci* **113**, 274–279.

Guessas, B. and Kihal, M. (2004) Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats' milk. *Afr J Biotechnol* **3**, 339–342.

Hantsis-Zacharov, E. and Halpern, M. (2007) Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Appl Environ Microbiol* **73**, 7162–7168.

Kumaresan, G., Annalvilli, R. and Sivakumar, K. (2007) Psychrotrophic spoilage of raw milk at different temperatures of storage. *J Appl Sci Res* **3**, 1383–1387.

Lafarge, V., Ogier, J.-C., Girard, V., Madalen, V., Leveau, J.-Y., Gruss, A. and Delacroix-Buchet, A. (2004) Raw cow milk bacterial population shifts attributable to refrigeration. *Appl Environ Microbiol* **70**, 9, 5644–5650.

Ma, Y., Ryan, C., Barbano, D.M., Galton, D.M., Rudan, M.A. and Boor, K.J. (2000) Effects of somatic cell count on quality and shelf-life of pasteurized fluid milk. *J Dairy Sci* **83**, 264–274.

Magnusson, M., Christiansson, A. and Svensson, B. (2007) *Bacillus cereus* spores during housing of dairy cows: factors affecting contamination of raw milk. *J Dairy Sci* **90**, 2745–2754.

Martins, M.L., Pinto, C.L.O., Rocha, R.B., de Araújo, E.F. and Vanetti, M.C.D. (2006) Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. *Int J Food Microbiol* **111**, 144–148.

McGuiggan, J.T.M., McCleery, D.R., Hannan, A. and Gilmour, A. (2002) Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. *Int J Dairy Technol* **55**, 100–107.

Niku-Paavola, M.-L., Laitila, A., Mattila-Sandholm, T. and Haikara, A. (1999) New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *J Appl Microbiol* **86**, 29–35.

Scarpellini, M., Franzetti, L. and Galli, A. (2004) Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. *FEMS Microbiol Lett* **236**, 257–260.

Vissers, M.M.M., Te Giffel, M.C., Driehuis, F., De Jong, P. and Lankveld, J.M.G. (2007) Minimizing the level of *Bacillus cereus* spores in farm tank milk. *J Dairy Sci* **90**, 281–292.

Wang, L. and Jayarao, B.M. (2001) Phenotypic and genotypic characterization of *Pseudomonas fluorescens* isolated from bulk tank milk. *J Dairy Sci* **84**, 1421–1429.

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

1. **Olav Kongas**. Nonlinear Dynamics in Modeling Cardiac Arrhytmias. 1998.

2. **Kalju Vanatalu**. Optimization of Processes of Microbial Biosynthesis of Isotopically Labeled Biomolecules and Their Complexes. 1999.

3. **Ahto Buldas**. An Algebraic Approach to the Structure of Graphs. 1999.

4. **Monika Drews**. A Metabolic Study of Insect Cells in Batch and Continuous Culture: Application of Chemostat and Turbidostat to the Production of Recombinant Proteins. 1999.

5. **Eola Valdre**. Endothelial-Specific Regulation of Vessel Formation: Role of Receptor Tyrosine Kinases. 2000.

6. **Kalju Lott**. Doping and Defect Thermodynamic Equilibrium in ZnS. 2000.

7. **Reet Koljak**. Novel Fatty Acid Dioxygenases from the Corals *Plexaura homomalla* and *Gersemia fruticosa.* 2001.

8. **Anne Paju**. Asymmetric oxidation of Prochiral and Racemic Ketones by Using Sharpless Catalyst. 2001.

9. **Marko Vendelin**. Cardiac Mechanoenergetics *in silico*. 2001.

10. **Pearu Peterson**. Multi-Soliton Interactions and the Inverse Problem of Wave Crest. 2001.

11. **Anne Menert**. Microcalorimetry of Anaerobic Digestion. 2001.

12. **Toomas Tiivel**. The Role of the Mitochondrial Outer Membrane in *in vivo* Regulation of Respiration in Normal Heart and Skeletal Muscle Cell. 2002.

13. **Olle Hints**. Ordovician Scolecodonts of Estonia and Neighbouring Areas: Taxonomy, Distribution, Palaeoecology, and Application. 2002.

14. **Jaak Nõlvak**. Chitinozoan Biostratigrapy in the Ordovician of Baltoscandia. 2002.

15. **Liivi Kluge**. On Algebraic Structure of Pre-Operad. 2002.

16. **Jaanus Lass**. Biosignal Interpretation: Study of Cardiac Arrhytmias and Electromagnetic Field Effects on Human Nervous System. 2002.

17. **Janek Peterson**. Synthesis, Structural Characterization and Modification of PAMAM Dendrimers. 2002.

18. **Merike Vaher**. Room Temperature Ionic Liquids as Background Electrolyte Additives in Capillary Electrophoresis. 2002.

19. **Valdek Mikli**. Electron Microscopy and Image Analysis Study of Powdered Hardmetal Materials and Optoelectronic Thin Films. 2003.

20. **Mart Viljus**. The Microstructure and Properties of Fine-Grained Cermets. 2003.

21. **Signe Kask**. Identification and Characterization of Dairy-Related *Lactobacillus.* 2003

22. **Tiiu-Mai Laht**. Influence of Microstructure of the Curd on Enzymatic and Microbiological Processes in Swiss-Type Cheese. 2003.

23. **Anne Kuusksalu**. 2–5A Synthetase in the Marine Sponge *Geodia cydonium*. 2003.

24. **Sergei Bereznev**. Solar Cells Based on Polycristalline Copper-Indium Chalcogenides and Conductive Polymers. 2003.

25. **Kadri Kriis**. Asymmetric Synthesis of C_2-Symmetric Bimorpholines and Their Application as Chiral Ligands in the Transfer Hydrogenation of Aromatic Ketones. 2004.

26. **Jekaterina Reut**. Polypyrrole Coatings on Conducting and Insulating Substracts. 2004.

27. **Sven Nõmm**. Realization and Identification of Discrete-Time Nonlinear Systems. 2004.

28. **Olga Kijatkina**. Deposition of Copper Indium Disulphide Films by Chemical Spray Pyrolysis. 2004.

29. **Gert Tamberg**. On Sampling Operators Defined by Rogosinski, Hann and Blackman Windows. 2004.

30. **Monika Übner**. Interaction of Humic Substances with Metal Cations. 2004.

31. **Kaarel Adamberg**. Growth Characteristics of Non-Starter Lactic Acid Bacteria from Cheese. 2004.

32. **Imre Vallikivi**. Lipase-Catalysed Reactions of Prostaglandins. 2004.

33. **Merike Peld**. Substituted Apatites as Sorbents for Heavy Metals. 2005.

34. **Vitali Syritski**. Study of Synthesis and Redox Switching of Polypyrrole and Poly(3,4-ethylenedioxythiophene) by Using *in-situ* Techniques. 2004.

35. **Lee Põllumaa**. Evaluation of Ecotoxicological Effects Related to Oil Shale Industry. 2004.

36. **Riina Aav**. Synthesis of 9,11-Secosterols Intermediates. 2005.

37. **Andres Braunbrück**. Wave Interaction in Weakly Inhomogeneous Materials. 2005.

38. **Robert Kitt**. Generalised Scale-Invariance in Financial Time Series. 2005.

39. **Juss Pavelson**. Mesoscale Physical Processes and the Related Impact on the Summer Nutrient Fields and Phytoplankton Blooms in the Western Gulf of Finland. 2005.

40. **Olari Ilison**. Solitons and Solitary Waves in Media with Higher Order Dispersive and Nonlinear Effects. 2005.

41. **Maksim Säkki**. Intermittency and Long-Range Structurization of Heart Rate. 2005.

42. **Enli Kiipli**. Modelling Seawater Chemistry of the East Baltic Basin in the Late Ordovician–Early Silurian. 2005.

43. **Igor Golovtsov**. Modification of Conductive Properties and Processability of Polyparaphenylene, Polypyrrole and polyaniline. 2005.

44. **Katrin Laos**. Interaction Between Furcellaran and the Globular Proteins (Bovine Serum Albumin β-Lactoglobulin). 2005.

45. **Arvo Mere**. Structural and Electrical Properties of Spray Deposited Copper Indium Disulphide Films for Solar Cells. 2006.

46. **Sille Ehala**. Development and Application of Various On- and Off-Line Analytical Methods for the Analysis of Bioactive Compounds. 2006.

47. **Maria Kulp**. Capillary Electrophoretic Monitoring of Biochemical Reaction Kinetics. 2006.

48. **Anu Aaspõllu.** Proteinases from *Vipera lebetina* Snake Venom Affecting Hemostasis. 2006.

49. **Lyudmila Chekulayeva**. Photosensitized Inactivation of Tumor Cells by Porphyrins and Chlorins. 2006.

50. **Merle Uudsemaa**. Quantum-Chemical Modeling of Solvated First Row Transition Metal Ions. 2006.

51. **Tagli Pitsi**. Nutrition Situation of Pre-School Children in Estonia from 1995 to 2004. 2006.

52. **Angela Ivask**. Luminescent Recombinant Sensor Bacteria for the Analysis of Bioavailable Heavy Metals. 2006.

53. **Tiina Lõugas**. Study on Physico-Chemical Properties and Some Bioactive Compounds of Sea Buckthorn (*Hippophae rhamnoides* L.). 2006.

54. **Kaja Kasemets**. Effect of Changing Environmental Conditions on the Fermentative Growth of *Saccharomyces cerevisae* S288C: Auxo-accelerostat Study. 2006.

55. **Ildar Nisamedtinov**. Application of ^{13}C and Fluorescence Labeling in Metabolic Studies of *Saccharomyces* spp. 2006.

56. **Alar Leibak**. On Additive Generalisation of Voronoï's Theory of Perfect Forms over Algebraic Number Fields. 2006.

57. **Andri Jagomägi**. Photoluminescence of Chalcopyrite Tellurides. 2006.

58. **Tõnu Martma**. Application of Carbon Isotopes to the Study of the Ordovician and Silurian of the Baltic. 2006.

59. **Marit Kauk**. Chemical Composition of $CuInSe_2$ Monograin Powders for Solar Cell Application. 2006.

60. **Julia Kois**. Electrochemical Deposition of $CuInSe_2$ Thin Films for Photovoltaic Applications. 2006.

61. **Ilona Oja Açik**. Sol-Gel Deposition of Titanium Dioxide Films. 2007.

62. **Tiia Anmann**. Integrated and Organized Cellular Bioenergetic Systems in Heart and Brain. 2007.

63. **Katrin Trummal**. Purification, Characterization and Specificity Studies of Metalloproteinases from *Vipera lebetina* Snake Venom. 2007.

64. **Gennadi Lessin**. Biochemical Definition of Coastal Zone Using Numerical Modeling and Measurement Data. 2007.

65. **Enno Pais**. Inverse problems to determine non-homogeneous degenerate memory kernels in heat flow. 2007.

66. **Maria Borissova**. Capillary Electrophoresis on Alkylimidazolium Salts. 2007.

67. **Karin Valmsen**. Prostaglandin Synthesis in the Coral *Plexaura homomalla*: Control of Prostaglandin Stereochemistry at Carbon 15 by Cyclooxygenases. 2007.

68. **Kristjan Piirimäe**. Long-Term Changes of Nutrient Fluxes in the Drainage Basin of the Gulf of Finland – Application of the PolFlow Model. 2007.

69. **Tatjana Dedova**. Chemical Spray Pyrolysis Deposition of Zinc Sulfide Thin Films and Zinc Oxide Nanostructured Layers. 2007.

70. **Katrin Tomson**. Production of Labelled Recombinant Proteins in Fed-Batch Systems in *Escherichia coli*. 2007.

71. **Cecilia Sarmiento**. Suppressors of RNA Silencing in Plants. 2008.

72. **Vilja Mardla**. Inhibition of Platelet Aggregation with Combination of Antiplatelet Agents. 2008.

73. **Maie Bachmann**. Effect of Modulated Microwave Radiation on Human Resting Electroencephalographic Signal. 2008.

74. **Dan Hüvonen**. Terahertz Spectroscopy of Low-Dimensional Spin Systems. 2008.

75. **Ly Villo**. Stereoselective Chemoenzymatic Synthesis of Deoxy Sugar Esters Involving *Candida antarctica* Lipase B. 2008.

76. **Johan Anton**. Technology of Integrated Photoelasticity for Residual Stress Measurement in Glass Articles of Axisymmetric Shape. 2008.

77. **Olga Volobujeva**. SEM Study of Selenization of Different Thin Metallic Films. 2008.

78. **Artur Jõgi**. Synthesis of 4'-Substituted 2,3'-dideoxynucleoside Analogues. 2008.

79. **Mario Kadastik**. Doubly Charged Higgs Boson Decays and Implications on Neutrino Physics. 2008.

80. **Fernando Pérez-Caballero**. Carbon Aerogels from 5-Methylresorcinol-Formaldehyde Gels. 2008.

81. **Sirje Vaask**. The Comparability, Reproducibility and Validity of Estonian Food Consumption Surveys. 2008.

82. **Anna Menaker**. Electrosynthesized Conducting Polymers, Polypyrrole and Poly(3,4-ethylenedioxythiophene), for Molecular Imprinting. 2009.

83. **Lauri Ilison**. Solitons and Solitary Waves in Hierarchical Korteweg-de Vries Type Systems. 2009.

84. **Kaia Ernits**. Study of In_2S_3 and ZnS Thin Films Deposited by Ultrasonic Spray Pyrolysis and Chemical Deposition. 2009.

85. **Veljo Sinivee**. Portable Spectrometer for Ionizing Radiation "Gammamapper". 2009.

86. **Jüri Virkepu**. On Lagrange Formalism for Lie Theory and Operadic Harmonic Oscillator in Low Dimensions. 2009.

87. **Marko Piirsoo**. Deciphering Molecular Basis of Schwann Cell Development. 2009.

88. **Kati Helmja**. Determination of Phenolic Compounds and Their Antioxidative Capability in Plant Extracts. 2010.

89. **Merike Sõmera**. Sobemoviruses: Genomic Organization, Potential for Recombination and Necessity of P1 in Systemic Infection. 2010.

90. **Kristjan Laes**. Preparation and Impedance Spectroscopy of Hybrid Structures Based on $CuIn_3Se_5$ Photoabsorber. 2010.

91. **Kristin Lippur**. Asymmetric Synthesis of 2,2'-Bimorpholine and its 5,5'-Substituted Derivatives. 2010.

92. **Merike Luman**. Dialysis Dose and Nutrition Assessment by an Optical Method. 2010.

93. **Mihhail Berezovski**. Numerical Simulation of Wave Propagation in Heterogeneous and Microstructured Materials. 2010.

94. **Tamara Aid-Pavlidis**. Structure and Regulation of BDNF Gene. 2010.

95. **Olga Bragina**. The Role of Sonic Hedgehog Pathway in Neuro- and Tumorigenesis. 2010.

96. **Merle Randrüüt**. Wave Propagation in Microstructured Solids: Solitary and Periodic Waves. 2010.

97. **Marju Laars**. Asymmetric Organocatalytic Michael and Aldol Reactions Mediated by Cyclic Amines. 2010.

98. **Maarja Grossberg**. Optical Properties of Multinary Semiconductor Compounds for Photovoltaic Applications. 2010.

99. **Alla Maloverjan**. Vertebrate Homologues of Drosophila Fused Kinase and Their Role in Sonic Hedgehog Signalling Pathway. 2010.

100. **Priit Pruunsild**. Neuronal Activity-Dependent Transcription Factors and Regulation of Human *BDNF* Gene. 2010.

101. **Tatjana Knjazeva**. New Approaches in Capillary Electrophoresis for Separation and Study of Proteins. 2011.

102. **Atanas Katerski**. Chemical Composition of Sprayed Copper Indium Disulfide Films for Nanostructured Solar Cells. 2011.

103. **Kristi Timmo.** Formation of Properties of $CuInSe_2$ and $Cu_2ZnSn(S,Se)_4$ Monograin Powders Synthesized in Molten KI. 2011.
104. **Kert Tamm**. Wave Propagation and Interaction in Mindlin-Type Microstructured Solids: Numerical Simulation. 2011.
105. **Adrian Popp**. Ordovician Proetid Trilobites in Baltoscandia and Germany. 2011.
106. **Ove Pärn**. Sea Ice Deformation Events in the Gulf of Finland and This Impact on Shipping. 2011.
107. **Germo Väli**. Numerical Experiments on Matter Transport in the Baltic Sea. 2011.
108. **Andrus Seiman**. Point-of-Care Analyser Based on Capillary Electrophoresis. 2011.
109. **Olga Katargina**. Tick-Borne Pathogens Circulating in Estonia (Tick-Borne Encephalitis Virus, *Anaplasma phagocytophilum*, *Babesia* Species): Their Prevalence and Genetic Characterization. 2011.
110. **Ingrid Sumeri**. The Study of Probiotic Bacteria in Human Gastrointestinal Tract Simulator. 2011.
111. **Kairit Zovo**. Functional Characterization of Cellular Copper Proteome. 2011.
112. **Natalja Makarytsheva**. Analysis of Organic Species in Sediments and Soil by High Performance Separation Methods. 2011.
113. **Monika Mortimer**. Evaluation of the Biological Effects of Engineered Nanoparticles on Unicellular Pro- and Eukaryotic Organisms. 2011.
114. **Kersti Tepp**. Molecular System Bioenergetics of Cardiac Cells: Quantitative Analysis of Structure-Function Relationship. 2011.
115. **Anna-Liisa Peikolainen**. Organic Aerogels Based on 5-Methylresorcinol. 2011.
116. **Leeli Amon**. Palaeoecological Reconstruction of Late-Glacial Vegetation Dynamics in Eastern Baltic Area: A View Based on Plant Macrofossil Analysis. 2011.
117. **Tanel Peets**. Dispersion Analysis of Wave Motion in Microstructured Solids. 2011.
118. **Liina Kaupmees**. Selenization of Molybdenum as Contact Material in Solar Cells. 2011.
119. **Allan Olspert**. Properties of VPg and Coat Protein of Sobemoviruses. 2011.
120. **Kadri Koppel**. Food Category Appraisal Using Sensory Methods. 2011.
121. **Jelena Gorbatšova**. Development of Methods for CE Analysis of Plant Phenolics and Vitamins. 2011.
122. **Karin Viipsi**. Impact of EDTA and Humic Substances on the Removal of Cd and Zn from Aqueous Solutions by Apatite. 2012.
123. **David Schryer**. Metabolic Flux Analysis of Compartmentalized Systems Using Dynamic Isotopologue Modeling. 2012.
124. **Ardo Illaste**. Analysis of Molecular Movements in Cardiac Myocytes. 2012.
125. **Indrek Reile**. 3-Alkylcyclopentane-1,2-Diones in Asymmetric Oxidation and Alkylation Reactions. 2012.
126. **Tatjana Tamberg**. Some Classes of Finite 2-Groups and Their Endomorphism Semigroups. 2012.

127. **Taavi Liblik**. Variability of Thermohaline Structure in the Gulf of Finland in Summer. 2012.
128. **Priidik Lagemaa**. Operational Forecasting in Estonian Marine Waters. 2012.
129. **Andrei Errapart**. Photoelastic Tomography in Linear and Non-linear Approximation. 2012.
130. **Külliki Krabbi**. Biochemical Diagnosis of Classical Galactosemia and Mucopolysaccharidoses in Estonia. 2012.
131. **Kristel Kaseleht**. Identification of Aroma Compounds in Food using SPME-GC/MS and GC-Olfactometry. 2012.
132. **Kristel Kodar**. Immunoglobulin G Glycosylation Profiling in Patients with Gastric Cancer. 2012.
133. **Kai Rosin**. Solar Radiation and Wind as Agents of the Formation of the Radiation Regime in Water Bodies. 2012.
134. **Ann Tiiman**. Interactions of Alzheimer`s Amyloid-Beta Peptides with Zn(II) and Cu(II) Ions. 2012.
135. **Olga Gavrilova**. Application and Elaboration of Accounting Approaches for Sustainable Development. 2012.
136. **Olesja Bondarenko**. Development of Bacterial Biosensors and Human Stem Cell-Based *In Vitro* Assays for the Toxicological Profiling of Synthetic Nanoparticles. 2012.
137. **Katri Muska**. Study of Composition and Thermal Treatments of Quaternary Compounds for Monograin Layer Solar Cells. 2012.
138. **Ranno Nahku**. Validation of Critical Factors for the Quantitative Characterization of Bacterial Physiology in Accelerostat Cultures. 2012.
139. **Petri-Jaan Lahtvee**. Quantitative Omics-level Analysis of Growth Rate Dependent Energy Metabolism in *Lactococcus lactis*. 2012.
140. **Kerti Orumets**. Molecular Mechanisms Controlling Intracellular Glutathione Levels in Baker's Yeast *Saccharomyces cerevisiae* and its Random Mutagenized Gluthatione Over-Accumulating Isolate. 2012.
141. **Loreida Timberg**. Spice-Cured Sprats Ripening, Sensory Parameters Development, and Quality Indicators. 2012.
142. **Anna Mihhalevski**. Rye Sourdough Fermentation and Bread Stability. 2012.
143. **Liisa Arike**. Quantitative Proteomics of *Escherichia coli*: From Relative to Absolute Scale. 2012.
144. **Kairi Otto**. Deposition of In_2S_3 Thin Films by Chemical Spray Pyrolysis. 2012.
145. **Mari Sepp**. Functions of the Basic Helix-Loop-Helix Transcription Factor TCF4 in Health and Disease. 2012.
146. **Anna Suhhova**. Detection of the Effect of Weak Stressors on Human Resting Electroencephalographic Signal. 2012.
147. **Aram Kazarjan**. Development and Production of Extruded Food and Feed Products Containing Probiotic Microorganisms. 2012.
148. **Rivo Uiboupin**. Application of Remote Sensing Methods for the Investigation of Spatio-Temporal Variability of Sea Surface Temperature and Chlorophyll Fields in the Gulf of Finland. 2013.
149. **Tiina Kriščiunaite**. A Study of Milk Coagulability. 2013.

150. **Tuuli Levandi**. Comparative Study of Cereal Varieties by Analytical Separation Methods and Chemometrics. 2013.
151. **Natalja Kabanova**. Development of a Microcalorimetric Method for the Study of Fermentation Processes. 2013.
152. **Himani Khanduri**. Magnetic Properties of Functional Oxides. 2013.
153. **Julia Smirnova**. Investigation of Properties and Reaction Mechanisms of Redox-Active Proteins by ESI MS. 2013.
154. **Mervi Sepp**. Estimation of Diffusion Restrictions in Cardiomyocytes Using Kinetic Measurements. 2013.
155. **Kersti Jääger**. Differentiation and Heterogeneity of Mesenchymal Stem Cells. 2013.
156. **Victor Alari**. Multi-Scale Wind Wave modeling in the Baltic Sea. 2013.
157. **Taavi Päll**. Studies of SD44 Hyaluronan Binding Domain as Novel Angiogenesis Inhibitor. 2013.