THESIS ON NATURAL AND EXACT SCIENCES B151

Development of a Microcalorimetric Method for the Study of Fermentation Processes

NATALJA KABANOVA

TUT PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

Dissertation was accepted for the defence of the degree of Doctor of Philosophy (Chemistry) on March 7th, 2013.

Supervisor: Professor Raivo Vilu,
Department of Chemistry,
Tallinn University of Technology (TUT)

Reviewed by: Senior Scientist Kaarel Adamberg,
Department of Food Processing,
Tallinn University of Technology (TUT)

Opponents: Professor Lars Wadsö,
Department of Building and Environmental Technology
Lund University, Sweden

Dr. Vlad T. Popa,
"Ilie Murgulescu" Institute of Physical Chemistry,
Romanian Academy, Romania

Defence of the thesis: April 19th, 2013

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

ISSN 1406-4723
ISBN 978-9949-23-451-6 (publication)
ISBN 978-9949-23-452-3 (PDF)

LOODUS- JA TÄPPISTEADUSED B151

Kalorimeetrilise meetodi väljatöötamine fermentatsiooniprotsesside uurimiseks

NATALJA KABANOVA

TTÜ
KIRJASTUS

CONTENTS

ABSTRACT

In homogeneous liquid cultures, every bacterium grows essentially in the same environment, which makes the study and characterization of the growth processes considerably simple. However, in solid matrices bacteria grow in colonies and the restricted diffusion of substrates and putative toxic products etc. lead to different and more complex growth curves/laws than those in broth. Microcalorimetry is an especially useful method for the study of bacterial growth in solid and opaque media where the applicability of optical and other physical methods is limited due to the non-transparent or structured media. Since the heat effects are not specific, the results obtained using microcalorimetry have to be combined with the results of parallel measurements of the concentration of substrates and products, e.g. outplating, for the determination of the number of bacteria etc. in a series of experiments. The results of these series of parallel experiments make it possible to develop a more detailed interpretation of the growth processes and add a special meaning to the results of calorimetric measurements. In this study, we developed a microcalorimetric method for the study of bacterial growth in liquid cultures based on the use of serial dilutions, and further implemented the method for fermentation processes in different solid matrices.

For the *Lactococcus lactis* IL1403 in liquid transparent CRM (*Carbohydrate-restricted medium*), the heat yield coefficient Y_Q was determined as $Y_Q = (2.58\pm0.44)*10^{-9}$ J cfu^{-1}. It was shown that the exponential growth of bacteria was stopped when pH reached approximately 4.6 and the growth of the cultures ended either because of the exhaustion of glucose at lower initial glucose concentrations (2 g L^{-1}), or because of the formation of lactate inhibiting the growth and lowering the pH value to 4.0 in the case of higher initial glucose concentrations (10 and 20 g L^{-1}). When the growth of *L. lactis* was further studied in solid agar 1% gels, the changes in pH and lactic acid concentrations, together with the density and geometry of colonies, were identified as factors determining the peculiarities of bacterial growth. The data obtained, together with the calculated heat yield coefficient Y_Q (J cfu^{-1}) and microscopic measurements, made it possible to analyse and describe quantitatively the growth of individual colonies, to develop a model distribution of colonies in a solid matrix and to construct the growth curve of a typical colony of *L. lactis* in 1% agar gel.

Furthermore, the microcalorimetric method was implemented for the study of bacterial growth in opaque liquid and solid media. The peculiarities of the growth of *Streptococcus thermophilus* ST12 were studied in reconstituted milk prepared from 10 kGy gamma-irradiated and non-irradiated low-heat skim milk powder ((irrLHSMP and LHSMP, respectively), both with and without rennet added. The growth of the bacteria at inoculation rates from 10^1 to 10^6 cfu mL^{-1} in 10-fold increments was investigated at two different temperatures: at 35˚C and 40˚C. The heat yield coefficient $Y_Q = (4.45\pm0.15)*10^{-9}$ J cfu^{-1} was determined

for *Streptococcus thermophilus* ST12 growth in skim milk. The diauxic growth of *St. thermophilus* was registered in reconstituted milk prepared from LHSMP at both 35˚C and 40˚C. The power-time curves obtained in irrLHSMP were remarkably different and no diauxie was observed at 40˚C. The consumption profiles of the sugars measured demonstrated remarkable differences in growth in milk samples reconstituted from non-irradiated versus irradiated powders. It was shown that the growth rate of bacteria decreased in the renneted curd in comparison with liquid cultures, starting from a certain number of bacteria in the samples, in colonies. The differences in the growth of *St. thermophilus* in the liquid and solid samples were more clearly observed at lower inoculation rates, obtaining lower μ_{max} values, and the shape of the calorimetric curves became lower and prolonged in structured samples. The microcalorimetric method was also successfully used to monitor the growth of probiotic bacteria *Enterococcus faecium* NCIMB10145 in the complex matrices of extruded food and feed products.

It was concluded that the microcalorimetric method, in combination with other relevant methods, is a very powerful tool in the study of solid state fermentation and the growth of bacteria in various opaque media, such as milk and extruded products, and can be used for the optimization of industrial solid state fermentation processes. Calorimetric measurements are very precise and easy to carry out in comparison with other methods currently used.

KOKKUVÕTE

Homogeensetes vedelates keskkondades kasvavad kõik bakterid samades tingimustes ning see lihtsustab oluliselt kasvuprotsesside uurimist ja kirjeldamist. Bakterite kasv on märksa keerulisem tahketes maatriksites, kus bakterid on sunnitud kasvama kolooniatena. Kolooniate kasv on mõjutatud substraadi ja produktide difusioonikiirustest nii kolooniate sees kui ka keskkonnas, samas omavad ka kolooniatevahelised interaktsioonid suurt rolli. Mikrokalorimeetria on tundlik käepärane meetod bakterite kasvu uurimiseks tahketes ja mitteläbipaistvates keskkondades – igal pool, kus bakterite kasvu klassikaliste mikrobioloogiliste uurimismeetodite (optiliste, väljakülvide jt.) kasutamine võib olla piiratud. Kalorimeetriliste kõverate paremaks interpreteerimiseks tuleb meetodit kasutada koos teiste meetoditega, mis võimaldavad jälgida tarbitud substraadi ja toodetud produktide kontsentratsioonide muutusi, pH muutumist ajas, bakterite arvukuse suurenemist jne. Paralleelkatsete andmed võimaldavad paremini aru saada bakterite kasvuprotsessist ja kasvu eripärasustest, detailsemalt ja rikkamalt seletada kasvu pidurdamismehhanisme, lisades kalorimeetrilistele andmetele detaile. Antud töö tähtsaimaks eesmärgiks oli mikrokalorimeetrilise meetodi väljatöötamine bakterite kasvu iseloomustamiseks esialgu vedelates keskkondades ning seejärel saadud teadmiste ja meetodite kasutamine bakterite kasvu uurimisel tahkefaasiliste fermentatsiooniprotsesside jooksul erinevates maatriksites.

Piimhappebakteri *Lactococcus lactis* IL1403 jaoks määrati saagise-koefitsiendi Y_Q väärtuseks $Y_Q = (2.58\pm0.44)*10^{-9}$ J cfu^{-1}. Nimetatud bakterite kultiveerimiskatsete jooksul täheldati, et eksponentsiaalne kasv lõpeb ligikaudselt pH=4.6 juures ja kasvu pidurdumine kultiveerimise lõpus toimub kas glükoosi otsalõppemise tõttu madalamatel glükoosi algkontsentratsioonidel (2 g L^{-1}) või tänu kasvu inhibeerivate piimhappe koguste tootmisele ja kasvukeskkonna pH kahanemisele kuni väärtuseni pH=4.0 suuremate glükoosi algkontsentratsioonide korral (10 and 20 g L^{-1}). *L. lactis*-e kasvu uuriti seejärel tahkes 1%-ses agari geelis ning pH ja piimhappe kontsentratsiooni muutused ajas koos kolooniate tiheduse ja geomeetriaga olid faktorid, mis mõjutasid erineva suurusega kolooniate kasvu eripära. Andmed, mis saadi kasutades mikrokalorimeetriat ja biokeemilisi määramismeetodeid koos mikroskopeerimise andmetega, võimaldasid analüüsida ja kvantitatiivselt kirjeldada kolooniate kasvuprotsesse ning konstrueerida tüüpilise *L. lactis*-e koloonia kasvukõvera 1%-ses agari geelis.

Piimhappebakteri *Streptococcus thermophilus* ST12 kasvu uuriti kiiritamata ja gammakiirgusega kiiritatud piimapulbrist (lõssipulbrist) taastatud piimas. Nimetatud bakterite kasvu uurimisel tehti katsed kahel erineval inkubeerimis-temperatuuril 35 ºC ja 40 ºC nii vedelas kui ka rennetiga kalgendatud piimas, muutes inokuleerimistihedust vahemikus 10^1 - 10^6 cfu mL^{-1} kümnekordse sammuga. Saagiskoefitsiendi Y_Q väärtuseks määrati *Streptococcus thermophilus* ST12 jaoks lõssipulbrist taastatud piimas $Y_Q = (4.45\pm0.15)*10^{-9}$ J cfu^{-1}. Bakterite

kahefaasilist kasvu täheldati mõlemal inkubeerimistemperatuuril. Võimsuse-aja kõverad, ning suhkrute tarbimise profiilid erinesid tunduvalt kiiritamata ja kiiritatud proovides ja kaheastmelist kasvu ei täheldatud 40 ºC juures. Bakterite kasvukiirus kalgendatud piimas hakkas langema alates teatud kindlast bakterite arvust proovis ja kolooniates. Bakterite kasvu erinevus rennetiga ja rennetita piimades oli kõige selgemalt nähtav väiksematel inokuleerimistihedustel madalamate kasvukiiruste juures. Mikrokalorimeetrilist meetodit kasutati edukalt probiootiliste bakterite *Enterococcus faecium* NCIMB10145 kasvu uurimisel kompleksses ekstrudeeritud loomasööda maatriksis.

Kokkuvõtteks on välja arendatud bakterite kasvu iseärasusi põhjalikult kirjeldada võimaldav süsteem, mis põhineb erinevatel eksperimentaalsetel meetoditel, mille keskseks lüliks on mikrokalorimeetria. On näidatud, et kalorimeetriline meetod on käepärane, täpne ja kergesti teostatav *on-line* meetod bakterite kasvu uurimiseks mitte ainult läbipaistvates keskkondades, vaid ka mitteläbipaistvates vedelates ning kalgendatud piimades ja ekstrudeeritud toidutoodetes.

LIST OF PUBLICATIONS

This thesis is based on the following original publications, which are referred to by Roman numerals throughout the text:

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

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

III I. Stulova, **N. Kabanova**, T. Kriščiunaite, A. Taivosalo, T.-M. Laht, R. Vilu. Fermentation of reconstituted milk by *Streptococcus thermophilus*: effect of irradiation on skim milk powder (*accepted*). *Int. Dairy J.* 2013.

IV A. Kazarjan, **N. Kabanova**, R. Vilu. Microcalorimetric study of extruded dog food containing probiotic microorganisms. *Adv. in Microbiol*. 2012, 2, 436-440.

THE AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I The author performed all the experimental preparations and the analyses by IMC, HPLC, spectrophotometer, *outplating*, microscope. She interpreted the obtained results and wrote the manuscript.

II The author performed all the experimental preparations and the analyses by IMC, HPLC, microscope. She interpreted the obtained results and wrote the manuscript.

III The author participated in sample preparation and the analyses by IMC and HPLC. She participated in data interpreting and discussion of the results. She participated in preparation of the manuscript.

IV The author performed sample preparation and the analyses by IMC. She performed data analyses and participated in data interpreting and discussion of the results. She participated in preparation of the manuscript.

ADDITIONAL PUBLICATIONS:

V **N. Kabanova**, A. Kazarjan, I. Stulova, R. Vilu. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth. *Thermochim. Acta* 2009, 498, 87-92.

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

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

LIST OF CONFERENCE PRESENTATIONS

I. Kabanova, N., Stulova, I., Vilu, R. **The Use of Microcalorimetry in Studies of Solid State Fermentation Processes**. Oral Presentation at *1st Congress of Baltic Microbiologists, Riga, Latvia, October 2012*

II. Kabanova, N. **Applications of microcalorimetry and metabolic flux analysis in the dairy microbiology.** Oral presentation at *Valio Ltd. meeting, Helsinki, Finland, September 2012*

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

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

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

VI. 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**. Poster presentation at *22nd International ICFMH Symposium Food Micro 2010, Copenhagen, Denmark, August 2010*

VII. 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.** Poster presentation at *Recent Advances in Fermentation Technology (RAFT VIII*), *San Diego, CA, USA, November 2009*

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my supervisor Professor Raivo Vilu. He was the one who directed me to the exciting world of microcalorimetry. He has been a strong and supportive adviser to me for already eight years, but he has always given me great freedom to pursue independent work and thus to gain and grow. I would like to thank him for his patience, motivation and enthusiasm. His guidance and discussions helped me at all times during this research and writing of this dissertation.

I am also grateful to my colleagues in CCFFT for their understanding, advices and support during my PhD studies. I would like to address special thanks to Tiina, for her support, insightful comments and constructive critics. They were more helpful for me than she may think. Irina – I appreciate your continuous help during the experiments, positive thinking, lightness and chocolate candies to boot up my brain :). Marianna – thank you for your patience, moral support and inspiring activity. Girls, it was fUntastic to work with you.

Special thanks to my family and friends, who have never really understood what exactly I was doing during those long days in the laboratory and why should I go to the lab late in the evenings and on days off, but always supported me and showed sincere interest in my PhD work. Thank You for that, You have always cheered me up, made my life colourful and never doubted in me.

I am very grateful to my official opponents, Lars Wadsö and Vlad T. Popa, for accepting the request to review this work.

I wish to thank the CCFFT for the possibility to combine my studies and work at the same time. I also wish to thank the institutions that have provided financial support for this work, including EU Regional Development Fund, project EU29994, the Estonian Ministry of Education research grant SF0140090s08, as well as, SA Archimedes and doctoral schools in Faculty of Chemical and Materials Technology at TUT.

ACRONYMS

AC	ProBiotic LIVE Adult Chicken & Rice
ANOVA	analysis of variance
AS	ProBiotic LIVE Adult Salmon & Rice
a_w	water activity
cfu	colony-forming unit
CRM	carbohydrate-restricted medium
*Fischer´*s LSD	*Fisher*'s least significant difference test
GLC	glucose
HPLC	high-performance liquid chromatography
IMC	isothermal microcalorimetry
INRA	French National Institute for Agricultural Research
irrRSM	reconstituted skim milk prepared from irradiated skim milk powder
irrLHSMP	gamma-irradiated low heat skim milk powder
kGy	kiloGrey
LA	lactic acid
LAB	lactic acid bacteria
LHSMP	low heat skim milk powder
N	number of bacteria
N_0	inoculation rate
N_{exp}	number of bacteria produced during the exponential phase
N_{tot}	number of bacteria produced at the end of growth
OD	optical density
P_{max}	maximum heat flow
PCA	plate count agar
Q_{exp}	amount of heat produced during the exponential growth phase
Q_{tot}	amount of heat produced during the whole cultivation period
R_{COL}	the radius of a colony
R_{GLC}	the radius of an area from which glucose was scavenged for the growth of a colony
RSM	reconstituted skim milk
SC	ProBiotic LIVE Senior Chicken & Rice
SSF	solid state fermentations
subsp.	subspecies
TAM III	Thermal Activity Monitor III
t_d	doubling time
Y_Q	heat yield coefficient
Y_{XS}	yield coefficient
μ_{max}	maximum specific growth rate
μ	specific growth rate
λ	lag phase

1 LITERATURE OVERVIEW

1.1 Description of bacterial growth

1.1.1 Bacterial growth in liquid cultures

Bacterial growth takes place as a result of the growth and division of one bacterium into two daughter cells. The biomass growth rate is proportional to the biomass amount. The microbial growth is described by the first order differential equation:

$$dX/dt = \mu X \quad (1)$$

in which dX/dt is the biomass growth rate, μ is the specific growth rate and X describes the biomass amount.

Equation (1) can be integrated:

$$X = X_o e^{\mu t} \quad (2)$$

in which X is the biomass amount at time t, X_0 is the initial bacterial number and t is time.

The dependence of the growth rate on the concentration of limiting substrate is described by a *Monod* equation:

$$\mu = \frac{\mu_{max} S}{S + K_S} \quad (3)$$

in which S is the concentration of the limiting substrate, μ_{max} (h^{-1}) is the maximum specific growth rate and K_S is the substrate specific constant, which describes the bacterial affinity to the limiting substrate. K_S is the *Monod* constant, which corresponds to the concentration at which the biomass growth rate is one-half of its maximum value. This can be seen in the *Monod* equation by setting S equal to K_S. If the substrate concentration is higher than the K_S value ($S >> K_S$), then bacteria in the exponential growth phase are growing at the maximal possible growth rate ($\mu = \mu_{max}$). Taking into account the *Monod* equation, the typical bacterial growth curve can be constructed (See Figure 1).

The specific growth rate μ depends, in addition to the limiting substrate concentration, on the process conditions (e.g., pH and temperature), the concentrations of non-limiting substrates and/or the metabolites formed. μ is positive in the case of microbial growth and negative in the case of microbial inactivation and the prevailing death of cells (Van Impe et al., 2005).

Microbial growth in a single species batch culture, whether in a bioreactor or in a (liquid) food product, normally passes through three phases: first a lag phase, during which the microbial cells adapt to their new environment, followed by an exponential growth phase, during which the cells multiply exponentially, and finally a stationary phase, during which the maximum population density is reached (as shown in Figure 1).

During the lag phase of the bacterial growth cycle, the synthesis of RNA,

enzymes and other molecules occurs. The lag phase duration is influenced by the inoculation rate (Robinson et al., 2001; Prats et al., 2006), pH (Francois et al., 2005; Ölmez and Aran 2005), inoculation temperature (Swinnen et al., 2004; Francois et al., 2005; Swinnen et al., 2005; Prats et al., 2006; Ölmez and Aran 2005), limiting substrate concentration (Ölmez and Aran 2005), the "physiological state" of the bacteria and the difference between the pre- and incubation environmental conditions (Swinnen et al. 2004).

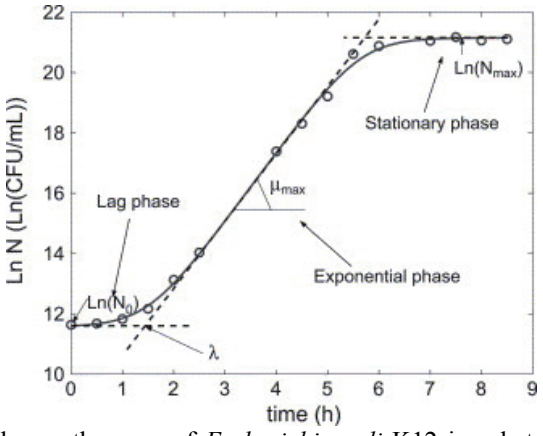


Figure 1. A typical growth curve of *Escherichia coli* K12 in a batch monoculture (by kind permission of Elsevier[1])

The lag phase is followed by the exponential growth phase, during which the cells multiply exponentially. The exponential phase (sometimes called the log phase or the logarithmic phase) is a period characterized by cell doubling. The number of cells produced during the exponential phase per time unit is proportional to the number of cells present. If growth is not limited, doubling will continue at a constant rate. For this type of exponential growth, plotting the natural logarithm of cell number against time results in a straight line. The slope of the growth curve is determined by the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth (i.e. the slope of the line in the figure) depends on the growth conditions. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes. In order to determine what causes the end of exponential growth – substrate concentration or formation of toxic products – the initial concentration of the substrate can be changed. If the increase in initial substrate concentration causes an increase in maximal bacterial concentration, then the limiting factor is the availability of the substrate. If the increase in the initial substrate concentration (C-source, N-

[1]Reprinted from *Journal of Food Microbiology*, Vol. 100, van Impe JF, Poschet F, Geeraerd AH, Vereecken KM. Towards a novel class of predictive microbial growth models, Pages No. 97-105, Copyright (2005), with permission from Elsevier.

source, vitamins etc.) does not influence the final bacterial density, then the reason is most probably the formation of toxic products. The growth rate diminishes in the stationary phase and the density of the bacterial culture reaches its maximal value (Van Impe et al., 2005).

The bacterial growth curve can be described by four parameters:

- $y_0 = \ln(X_0)$, where X_0 is the initial bacterial number (cfu mL^{-1}),
- λ, lag phase duration (h),
- $\mu_{max} = \ln(2)/t_d$, where μ_{max} is the maximum specific growth rate (h^{-1}) and t_d is the minimal doubling time (h)

1.1.2 Bacterial growth in solid state matrices

In transparent liquid cultures, every bacterium grows essentially in the same environmental conditions, and this significantly simplifies the study and characterization of the growth processes. Bacterial growth in solid states is a much more complicated phenomenon: solid matrices force bacteria to grow in colonies and thus the diffusion of substrates and products, possible accumulation of toxic products in the colonies etc. lead to growth curves (laws) different than in broth. It has been reported in the literature (Wilson et al., 2002) that bacteria grow more slowly in structured systems than they do in broth. It has been proposed that growth in gelled systems is associated with additional stress on the organism. It has also been shown that the growth lag in solid media is longer than in broth (Malakar et al., 2002a).

1.1.2.1 Colonial growth

As mentioned above, the restricted movement of individual cells or groups of cells in solid matrices forces them to grow in separate individual colonies (Malakar et al., 2002a; Mitchell and Wimpenny, 1997; Wimpenny et al., 1995). As a rule, each colony is formed from one bacterium, and the number of colonies is defined by the size of the inoculum. The size of the inoculum also determines the spatial distribution of colonies and the distances between colonies (Malakar et al., 2003; Jeanson et al., 2011). According to Malakar et al. (2002a), the growth of bacteria in agar gel, at inoculation rates higher than 100 cfu mL^{-1}, is similar to that in liquid cultures, as diffusion in solid media at short distances is fast enough. However, at low inoculation rates – less than 100 cfu mL^{-1} - colonies are separated by long distances, they grow bigger and they practically do not interact "directly" through diffusion processes, but diffusion of growth substrates and side products (e.g. lactate) may become growth rate limiting factors.

The specific growth rate of bacteria in liquid cultures depends directly on the concentrations of the limiting substrate and the metabolites produced in the media (see *Section 1.1.1*), but in the case of solid state fermentations (SSF) it may also depend on the rates of the diffusion processes (Hutkins and Nannen, 1993; Malakar et al., 2002b; Papagianni et al., 2007; Theys et al., 2008). As bacterial colonies increase in size, the microgradients of substrates, products,

and pH occur around and within the colonies (Malakar et al., 2000; Malakar et al., 2002a; Malakar et al., 2003). It has been shown experimentally that, during the growth of colonies of lactic acid bacteria (LAB), lactic acid (LA) first accumulates in the centres of the colonies and afterwards diffuses into the environment, lowering the pH of the media and leading to a decrease in the growth rate and interaction of colonies, thus inhibiting bacterial growth, glucose (GLC) consumption rate etc. (Andersen et al., 2009; Malakar et al., 2002a; Malakar et al., 2003; Mercade et al., 2000; Theys et al., 2008). The highest value of the maximum specific growth rate in the case of *L.lactis* is usually observed if pH values are in the interval 5.5-6.6 and the critical pH value has been found to be 4.0. pH values lower than 5.5 negatively influence the rate of glucose consumption by the bacteria (Andersen et al., 2009; Mercade et al., 2000).

It has been shown that the colonies grow exponentially until a certain size and then they grow linearly, with the diameter of a colony still increasing even if the number of viable cells in the colony remains constant (Wimpenny et al., 1995). It was assumed that the cells in the centre of the colonies die due to inhibition by toxic products and low pH, or by starvation. In contrast, cells that are located on the surface of the colonies appear not to be affected by inhibition reactions and thus can divide, leading to the growth of the size of the colonies (Wimpenny et al., 1995).

The solid matrices not only force cells to grow in colonies but also determine the shapes of the colonies, as well as peculiarities in the packing of the cells in them. It has been observed that at a concentration of agar around 0.65% (w v^{-1}) the snowball-shaped small colonies observed at lower agar concentrations become small-lobed to spherical and, with a further increase in agar concentration, colonies become ellipsoid in shape (Mitchell and Wimpenny, 1997; Wimpenny et al., 1995). On the other hand, colonies growing in gelatine (protein gel) mostly have a spherical shape. At lower gelatine concentrations (0.5%), colonies are not formed and bacteria grow the same way as in liquids. If the concentration of gelatine is increased by 1.5-3%, the colonies become rather big, creating a diffuse colony type. Starting with a 5% gelatine concentration in the medium, the borders of the colonies are clear, and the colonies become compact and spherical (Antwi et al. 2006).

The (mechanical) stress exerted on growing colonies by structured systems is comparable with the stress by such environmental parameters as temperature, water activity (a_w) and pH: bacteria growing under stress change their growth characteristics (Antwi et al., 2006; Wilson et al., 2002).

1.1.2.2 Inoculation rate and the distances between colonies

The distances between colonies depend on the inoculation rate. With an increase in inoculation rate, the distances between the colonies decrease. In the case of lower inoculation rates, the colonies are situated far from each other and thus the influence on the growth of one by another is minimal – bacteria grow as

individual colonies.

For a detailed interpretation of colonial growth in structured gel, an idealised matrix model can be introduced. The distribution of the colonies in an idealised matrix is homogeneous throughout the sample. The idealised gel matrix is made up of identical cubes which contain a spherical colony in the centre. Each of the colonies has a radius of R_{col}, and half of the distance between the colonies is R_{bnd} (Figure 2). R_{bnd} is fixed by the initial bacterial number, which means that at higher inoculation rates R_{bnd} is smaller as smaller cubes present and vice versa. The distance R_{bnd} is then one-half of the cube root of the volume of each cube, where X_0 (cfu mL^{-1}) is the initial inoculation density (see Equation (4)) (Malakar et al, 2002a).

$$R_{bnd} = \frac{1}{2}\sqrt[3]{\frac{10^{-6}}{X_0}} \text{ (meters)} \quad (4)$$

Each colony starts its growth from one cell and, in time, the radius of the colony, R_{col}, can be calculated from the number of cells present. It can be assumed that all of the colonies behave similarly and that the total number of cells in a system is the sum of the numbers of cells in every individual colony.

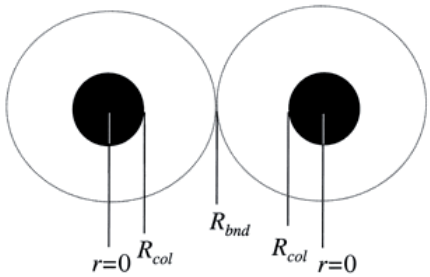


Figure 2. Each colony is in its own space, which is divided into two regions – a zone of colony growth (R_{col}) and a zone of living space (R_{bnd}) - which depends on the initial inoculation density (adapted from Malakar et al, 2002a).

1.1.2.3 Packaging density of bacteria in the colonies

The number of bacteria in the colony should be calculated by taking into account the packaging density of bacteria. It was proposed by Kepler that both in the case of cubic and hexagonal close packing, the densest possible sphere packing, the maximum density is slightly greater than 74% (see Equation (5)). This assertion is known as the *Kepler conjecture*.

$$\frac{\pi}{\sqrt{18}} = 0.74048 \quad (5)$$

The *Kepler conjecture* is a mathematical conjecture about sphere packing in three-dimensional Euclidean space. It says that no arrangement of equally sized spheres filling a space has a greater average density than that of the cubic close-packing and hexagonal close-packing arrangements (Hales, 1994; Hsiang, 1993).

1.1.2.4 Diffusion processes and gradients around the colonies

With colony growth, the gradients of the redox potential, pH, substrate and inhibitor concentrations etc. occur in agar gel around the colonies. These gradients can influence the growth of bacteria in individual colonies. Microelectrodes, together with other methods, can be used to measure some of the mentioned gradients (Wimpenny et al., 1995).

Diffusion processes play an important role during bacterial growth in structured media. Not only the diffusion through the matrix, but also the diffusion within the colony is important. With the diffusion processes, the substrate is transported towards the colony and the products are transported away from the colony. The analysis of the diffusion processes is convenient to observe on the basis of the growth of an individual colony in the case of spherical symmetry. Initially, the bacteria grow exponentially if the growth is not limited by the substrate concentration. The exponential growth ends when the substrate gradient gets smaller and diffusion starts to limit the growth.

According to the literature (Schantz and Lauffer, 1962), diffusion in agar gel is similar to free diffusion in water. Salts, sugars, amino acids and other low-molecular-weight substances give corrected values for their diffusion coefficients in line with published values for free diffusion. The same has been found to be true for proteins under conditions where the interactions with the gel do not occur. The diffusion coefficient of glucose in agar gel is $0.59*10^{5}$ $cm^{2}\,sec^{-1}$ measured at 20°C.

1.1.2.5 Agar gel - characteristics

Agar is widely used in microbiological research, as well as in food production. Commercially available agar includes small amounts of oligomers, proteins and electro-negative groups, which can influence the diffusion of charged molecules. Agar consists of a mixture of linear galactanes (agarose and agaropectine – see Figure 3). Agarose (molecular weight ~120000 Da) is a linear polymer that consists of units of (1→3)-β-D-galactopyranose-(1→4)-3,6-unhydro-α-L-galactopyranose.

In solutions, the agarose tertiary structure is considered a double helix. Agarose can form the grid of differently bonded helices into randomly forming pores. Agaropectin is a poor or non-gelling charged polysaccharide with low molecular weight. The structure of agaropectin resembles agarose; the difference is that a part of the galactosyl units is sulphated in agaropectin (Lahaye and Rochas, 1991). An increase in agar concentration causes a decrease in gel pore size. During bacterial growth in agar gel, colonies increase in size, which can lead to a splitting of gel matrices (Wimpenny et al., 1995; Mitchell and Wimpenny 1997).

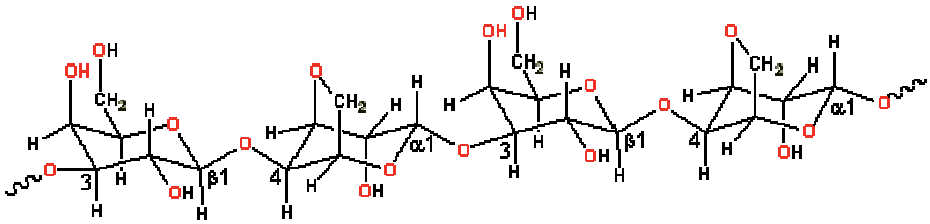


Figure 3. A fragment of the agar chain

1.1.3 Bacterial growth in milk

Skim milk powder is among the most abundantly produced and important dairy products. It is used in a multitude of food applications, many of which require the powder to be dissolved back into an aqueous solution (Martin et al., 2007). Milk reconstituted from this powder is frequently used for investigations of cheese-making processes in pilot plants (Mohamed et al., 1982; Peters, 1960; Wang et al., 2012; White & Ryan, 1983), as well as for studying the activity of starter bacteria in laboratory practice (Christopherson & Zottola, 1989), and for the propagation of mother starter cultures (Horrall et al., 1950). It is known that the milk used to grow mother and batch starters must be of the highest quality for consistently high starter activity. In the early days of the dairy industry, raw milk was used for these purposes at dairies. Considering that raw milk quality variations can affect the uniformity of starter activity, raw milk was replaced by milk reconstituted from non-fat dry milk powder of selected producers to improve product quality and ensure better control of the production processes (Whitehead et al., 1993). Most manufacturers use skim milk powder, which is specially screened to ensure that it is free of antibiotics as the standard medium for growing starter bacteria. The powder is reconstituted to milk containing 12% solids and heated at 85-95°C for 30-60 mins. (Ranken et al., 1997). The same reconstituted media is used as the standard for monitoring the acidification activity of LAB.

1.1.3.1 Irradiation of skim milk powder

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 et al., 2008; Farkas, 2006; Grolichová, Dvořák, & Musilová, 2004; Żegota & Małolepszy, 2008). 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 specifically shown that irradiation prevents the spoiling of dairy products (Bandekar et al., 1998; Ham et al., 2009b; Konteles et al., 2009). A level of 5 kGy is sufficient to decrease the total microflora in milk powder to the acceptable level (Żegota & Małolepszy, 2008). On the other hand, it has been shown that irradiation treatment causes physical-chemical changes in foods (Cieśla et al., 2004). This leads to conformational changes in proteins (De la Hoz & Netto, 2008; Ham et al. 2009a), modifications of amino acids (Bhattacharya et

al., 2000), a breakdown of the protein backbone (Davies, 2012), which influences the nutritional value and sensory properties of some irradiated foods (Grolichová et al. 2004; Żegota et al., 2008). It is known that ionizing radiation can lead to fragmentation, aggregation, conformational and net electrical charge changes, oxidation of amino acids and rupture of covalent bonds (Cho et al., 2010; 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: superoxide anion radicals (O_2^-), hydroxyl radicals ($OH^\bullet$) (Thibessard et al., 2001) and non-radical hydrogen peroxide (H_2O_2) (Davies, 1987). 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 et al. (2009a) that α_{s1}-casein and β_{A1}-casein are degraded, whereas α_{s0}-, β_B-, and β_{A3}-caseins are formed by gamma-irradiation. Irradiation treatment may also affect the buffering capacity of milk, which mainly depends on the composition and distribution of minerals and proteins between aqueous and solid phases (Salaün et al., 2005).

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

The acidification activity of milk is a very important characteristic of the quality of dairy starters. The methods used for the quantification of the acidification activity of starter bacteria are usually based on measuring pH changes or on the determination of the accumulation of lactic acid (Zanatta & Basso, 1992).

1.2 Calorimetric method

Due to their irreversible nature, life processes invariably and continuously dissipate Gibbs energy. As this is virtually always coupled with the continuous production and exchange of heat, calorimeters can be readily used to monitor life processes. It has been shown that the generation of heat by microbial cultures can be used for the *on-line* monitoring of the growth and metabolism of cells (Yi et al., 2000; Gustafsson, 1991; Vandenhove, 1998; Lamprecht, 2003; Wadsö and Galindo, 2009). Calorimetry is especially helpful in studies of growth in opaque and solid media, for example where agar, gelatine etc. are used as jellifying agents (Mitchell et al., 2004), but it can also be successfully applied in studies of

spoilage processes and shelf-life determination (Wadsö and Galindo, 2009; Riva et al., 2001; Alklint et al., 2005; Wadso and Sjoholm, 2005; von Stockar and van der Wieler, 1997) - anywhere where the possibilities of optical and other physical methods are limited due to the properties of non-transparent media (Antwi et al., 2007; Wilson et al., 2002).

As heat effects are non-specific, measurements of heat evolution should be combined, for richer interpretation, with the results of parallel experiments using such other methods as measurements of concentrations of substrates and products, e.g. *outplating*, for the determination of the number of bacteria, etc. (Boe and Lovrien, 1990; Menert et al., 2001). These parallel experiments make it possible to add special, new meanings and interpretations to the results of calorimetric measurements. Taking these circumstances into account, a "rich use" of the calorimetric method is possible after power-time curves have been complemented with the data of the parallel experiments mentioned above. Knowledge of necessary conversion, yield etc. coefficients makes it possible to derive a number of characteristics of the growth of bacteria from power-time curves.

1.2.1 Microcalorimetric method in studies of bacterial growth

Microcalorimetry has been used for the experimental study of bacterial growth for the last 50 years. The first experiments were carried out with *Streptococcus faecalis* and it was shown that the rate of heat production is coupled with the rate of biomass growth (Forrest and Walker, 1963). Later, the growth of other bacteria was studied: the growth of *Escherichia coli* in anaerobic conditions in the case of low substrate and high inoculation concentrations (Boe and Lovrien, 1990), the growth of *Lactobacillus helveticus* in different environmental conditions (Liu et al., 1999) etc. Further studies have elaborated the coupling of kinetic and thermodynamic processes (Beezer, 2001; Rong et al., 2007; Nan et al., 2001; Maskow and Babel, 2003; Schäffer et al., 2004; Higuera-Guisset et al., 2005; Peitzsch et al., 2008). It has been shown that calorimetry can provide information about different metabolic changes, such as a shift from one substrate/type of catabolism to another, and the occurrence of limitations and inhibitions (Yi et al., 2000; von Stockar and van der Wieler, 1997; Winkelmann et al., 2004; Yao et al., 2008). The enthalpy balance elaborated using calorimetric measurements can reveal the formation of unknown, unexpected by-products (Maskow and Babel, 2003; Yao et al., 2008), or can be used in studying the synthesis of intermediate products of metabolism, and their effect on bacterial growth (Wang et al., 2005). Calorimetric power-time curves can also be used as imprints of (complicated) microbial processes, as the shape and the number of phases of the curves depend on the composition of the bacterial consortia (Vandenhove, 1998), and reflect complicated patterns of multi-stage growth processes.

1.2.1.1 Heat production

The exponential growth of a single species batch culture of bacteria is described by the following equation:

$$dX/dt = \mu X \quad (6),$$

where X and μ are the concentration of biomass and the specific growth rate, respectively. μ is generally determined by the measurement of biomass, i.e. the plot of $\ln X$ vs time t. Calorimetry provides an alternative for the determination of specific growth rate. If the stoichiometry of the heat evolution during the growth reaction is constant, then the rate and the amount of biomass formed, (dX/dt) and (X-X_0), are proportional to the rate and the amount of heat production, (dQ/dt) and Q, respectively. μ can be determined from calorimetric measurements as a plot of $\ln Q$ vs. time t.

The biomass growth rate is proportional to the heat production rate (Y_Q is the proportional factor or heat yield coefficient):

$$dX/dt = Y_Q * dQ/dt \quad (7)$$

Using the law of biomass exponential growth (see Equation (1)), the relation between μ and dQ/dt can be obtained:

$$\mu X = Y_Q * dQ/dt \qquad (8)$$

Using Equation (3), the following relation can be obtained:

$$\mu * X_0 * e^{\mu t} = Y_Q * dQ/dt \qquad (9)$$

$$dQ/dt = 1/Y_Q * \mu * X_0 * e^{\mu t} \qquad (10)$$

after taking the logarithm:

$$\ln(dQ/dt) = C + \mu t \qquad (11)$$

Equation (11) is a simple formula for the calculation of specific growth rate (Menert et al., 2001). This formula was used for the calculation of maximal specific growth rate in the present work.

1.2.2 Advantages of IMC

Colonial growth of bacteria in solid matrices has been previously described in numerous papers (Malakar et al., 2000; Malakar et al., 2002a; McKay et al., 1997; Mitchell et al., 2004; Van Impe et al., 2004; Wimpenny et al., 1995). The experimental methods used for the study of SSF - measurements of OD, *outplating*, direct microscopy measurements, chromatography etc. - are quite cumbersome and not very informative regarding the peculiarities of growth. Currently more and more attention has been focused on the use of microcalorimetry in studying bacterial growth in opaque liquids, on surfaces and in solids under a wide range of conditions (Wadsö and Galindo, 2009; Braissant et al., 2010b).

The calorimetric method has great advantages (Vandenhove, 1998) in studying solid-state fermentation processes (Beezer, 2001; Braissant et al.,

2010a; Ma et al., 2007; Mihhalevski et al., 2011), especially if compared with the other methods mentioned (see also Boe and Lovrien, 1990; Menert et al., 2001). An extensive overview of potential applications of microcalorimetry has been well presented by Braissant et al. (2010b), Gustafsson (1991), Lamprecht (2003), Vandenhove (1998) and Wadsö (2009). However, general experimental strategies for the detailed study of SSF that make it possible to monitor and describe the growth of individual (average) colonies have not been developed.

Calorimetric power-time curves provide a lot more information about the peculiarities of bacterial growth and its metabolism (Lago et al., 2011; Stulova et al., 2011), which are difficult to obtain using conventional methods: multiauxic growth, the switch from one substrate to another etc. Calorimetry also provides an efficient alternative for the precise determination of specific growth rates (Liu et al., 1999). Moreover, the calorimetric method makes it possible to detect concentrations of bacteria in the range of 10^4-10^5 cells, which cannot be detected using spectrophotometry, the generally used method (Braissant et al., 2010b). Despite the fact that power-time curves provide a lot of information on growth, it is very useful to collect some additional data using other methods in parallel (Chen et al., 2008).

The calorimetric method has definite advantages in comparison with other methods used in microbiology: (i) a microcalorimeter measures the heat produced by the growth of a bacterial culture, which depends not only on the biomass amount, but also on the amount of metabolised substrate and on the efficiency of carbon source utilization; (ii) a microcalorimeter provides more information for the calculation of the energy balance and the efficiency of metabolism; (iii) the growth of a bacterial culture can be continuously recorded with a microcalorimeter and without growth disturbance; (iv) microcalorimetric measurements are highly reproducible; (v) it is possible to analyse coloured and turbid samples, which means that the technique does not require optical transparency of samples and is invariant irrespective of their physical form; (vi) no specific sample pre-treatment or preparation is needed, as the samples are housed within an ampoule and monitored *in situ* (Gaisford et al., 2009; Vandenhove, 1998).

Summarizing, it should be noted that microcalorimetry, when used in combination with other relevant measurements, is a very powerful instrument in studying quantitative detailed peculiarities of solid-state fermentations and growth in opaque media.

1.2.3 Thermal activity monitor - TAM III

The TAM III (Figure 4) is designed to continuously monitor a wide variety of processes and complex systems over a temperature range of 15–150°C. The TAM III maintains outstanding sensitivity because of the excellent stability of the thermostat (< ± 100 μK over 24h), even over long periods of time. A detection limit of 0.5μW and a baseline stability (over a period of 24 h) of ±0.2 μW are characteristic of the instrument. The TAM III is operated in static

ampoule mode (batch process). The construction and functioning of the TAM III calorimeter has been previously described by Wadsö (2002). A heat flow calorimeter works by channelling the heat produced or consumed by a reaction in the sample through heat flow sensors comprised of thermoelectric modules. When a temperature gradient is imposed (or formed) across the thermoelectric module, a voltage is created in accordance with the *Seebeck* effect. This voltage is proportional to the heat flow through the thermoelectric module and hence proportional to the rate of heat production or consumption by the sample. One side of the thermoelectric module is in contact with the sample and the other is kept isothermal by a heat sink which is in contact with the TAM III thermostat.

The thermostat is a liquid-based system, utilizing a mineral oil to quickly dissipate heat and minimize temperature gradients in the system. Efficient circulation of the liquid also permits precise temperature changes to be made. Temperature is controlled by a unique, patented regulation system. The average temperature fluctuation of the TAM III is better than ±10 μK over a range of 15 to 150 °C. The drift over 24 hours is within ±100 μK. The unmatched stability of the TAM III thermostat contributes to a perfect environment for isothermal and temperature scanning measurements (minicalorimeter). The thermostat is controlled by the software dedicated to the TAM III: TAM Assistant™. This is the classical mode for microcalorimetric experiments. The liquid thermostat is maintained at a constant temperature for the duration of the experiment. Any heat generated or absorbed by the sample due to a chemical or physical process is continuously measured. Isothermal measurements give quantitative and continuous data reflecting the rate of the process under study.

Figure 4. Isothermal microcalorimeter TAM III (*TA Instruments*, Delaware, US)

The TAM multicalorimeter contains six minicalorimeters (Figure 4). The minicalorimeter is a 3 or 4 ml microcalorimeter with a special design to reduce the space occupied by the calorimeter inside the thermostat. The reference is positioned below the sample ampoule. It is intended for use with the TAM III thermostat to increase the sample throughput combining with high sensitivity (TA Instruments).

2 AIMS OF THIS DISSERTATION

The general aim of the present study was to develop a practical microcalorimetric method for the studies of bacterial growth in liquid cultures and during solid-state fermentation processes. Specifically, the aims were as follows:

- to develop a method of serial dilutions for the studies of bacterial growth using a microcalorimeter together with other analytical methods on the basis of the growth of *Lactococcus lactis* IL1403 in transparent liquid and solid media
- to compare the details of the growth of bacteria in liquid and solid cultures and to investigate the growth of individual bacterial colonies of different sizes
- to evaluate the developed microcalorimetric method in the description of bacterial growth in opaque liquid and solid media (both irradiated and not irradiated) on the basis of the growth of *Streptococcus thermophilus* ST12 in reconstituted skim milk at different incubation temperatures and inoculation rates
- to evaluate the use of the microcalorimetric method for the determination and analysis of the probiotic ingredient in solid matrices of extruded food products

3 EXPERIMENTAL

3.1 Bacterial cultures studied

3.1.1 *Lactococcus lactis*

Lactococcus lactis is a spherical-shaped, non-motile, non-pathogenic, gram-positive, facultative anaerobic lactic acid bacterium closely related to the genus *Streptococcus. L. lactis* is most commonly used for the industrial production of fermented dairy products, such as milk, cheese and yoghurt. Because of its industrial importance, it is the best-characterized lactic acid bacterium.

The metabolic network of *L. lactis* can function in aerobic and anaerobic conditions. In anaerobic conditions, glycolysis breaks down extracellular carbohydrates to pyruvate, then converts pyruvate to lactic acid. The lactic acid that is produced during the growth of *L. lactis* lowers extracellular pH, thus negatively influencing the growth (growth rate) of the bacteria. *L. lactis* grows optimally at pH values around 6.5, and growth arrest occurs only at around pH 4. Although *L. lactis* is able to survive at low pH, the glycolysis is strongly affected at pH values below 5, showing a reduced rate of glucose consumption (Andersen et al., 2009).

3.1.2 *Streptococcus thermophilus*

Streptococcus thermophilus is a thermophilic spherical-shaped gram-positive, non-pathogenic, non-motile, non-sporulating, facultative anaerobic bacterium with an optimal growth rate at 45°C. It is the second most important dairy starter after *Lactococcus lactis* and is used for commercial purposes, which includes the production of milk, cheese and other dairy products (Hols et al., 2005; Thomas & Crow, 1984). It is a member of the thermophilic LAB and has traditionally been used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus* for the manufacture of yoghurt and hard "cooked" cheeses, respectively (e.g. Emmental, Gruyere and Grana) (Delorme, 2008).

3.1.3 *Enterococcus faecium*

Enterococcus faecium is a gram-positive, non-motile spherical-shaped bacterial, which can exist in pairs or chains of cells. The normal habitat includes the gastrointestinal tract of a multitude of animals but it can also be found in the oral cavity and vaginal tract (Huycke et al., 1998). The microbe can survive for long periods of time in soil, sewage and inside hospitals on a variety of surfaces (van Wamel et al., 2007). It can grow in temperatures ranging from 10 to 45°C, in basic or acidic environments, and in environments which are isotonic or hypertonic (Huycke et al., 1998). *E. faecium* can be used as a probiotic to out-compete deleterious bacteria in the gastrointestinal tract (Kang and Lee, 2005).

The studied strain *Enterococcus faecium* NCIMB10415 is registered in the catalogue of the "EU Community register of feed additives pursuant to Regulation (EC) No 1831/2003 - under a class of zootechnical additives", under the number "E1707" (for dogs).

3.2 Inoculum preparation

3.2.1 *Lactococcus lactis* IL1403

The cultivated strain *L. lactis* subsp. *lactis* IL1403 was provided by Dr. Ogier from INRA (French National Institute for Agricultural Research, Jouy-en-Josas, France). Frozen storage cultures of *L. lactis* IL1403 were thawed and pre-grown on Petri dishes with M17 Agar (LAB M, UK) for 24 h at 35°C. One colony from a pre-grown Petri dish was used as the inoculum for a 10 mL culture in liquid M17 Broth (FLUKA) at 35°C. 1% of bacterial suspension grown overnight (exactly 24 hours) was used as the inoculum for the next liquid 10 mL M17 broth culture and grown for 12-14 h, which allowed the *L. lactis* IL1403 bacteria to reach the middle of the exponential growth phase. The number of bacteria was determined by plating on M17 Agar and incubating for three days at 35°C, and the average number of bacteria in the mid-exponential culture was determined as $(4.15\pm0.15)*10^8$ cfu mL^{-1}.

3.2.2 *Streptococcus thermophilus* ST12

A strain of *St. thermophilus* ST12, a gram-positive facultative anaerobe, was provided by Chr. Hansen (Denmark). Frozen cultures of *St. thermophilus* ST12 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 the inoculum for a 10 mL culture in sterilized reconstituted skim milk (RSM) and left for 16 h to grow at 40°C. 1% of the pre-grown culture was used as the inoculum for the next 10 mL of RSM, and left for 12 h to grow. The preceding procedure was repeated one more time and the obtained bacterial suspension was further used for inoculation. The number of bacteria was determined by plating on M17 Agar and incubating for three days at 40°C. The final average number of bacteria in the inocula was $(1.15\pm0.19)*10^9$ cfu mL^{-1}.

3.2.3 Dilution procedure

The inoculum needed for the experiments was prepared by diluting samples of the cultures that were pre-grown according to the procedure described above (see *Sections 3.2.1 Lactococcus lactis* IL1403 and *3.3.2 Streptococcus thermophilus* ST12) in peptone water. The calculated inoculum concentrations in calorimetric and in parallel samples were varied from 10^0 to 10^6 cfu mL^{-1} with 10-fold increments depending on the experiment and bacterial culture.

It was important to have the correct desired number of colonies in solid samples as special attention was being paid to study the growth of individual colonies. From the practical point of view, it is quite difficult to get up to about three colonies, and not more, in a sample (inoculation rate 10^0 cfu mL^{-1}). These colonies are separated from each other sufficiently and thus grow as independent individual colonies. The dilution procedures were developed and carefully implemented to assure that serial dilutions led to the desired inoculation rates. The exact following of the procedures of the preparation of bacterial cultures described above, which ensured high reproducibility at lower inoculation rates,

validated by the direct counting of the colonies in the samples after the experiments in the case of transparent agar gels, allowed us to be confident that the number of bacteria in samples was as assumed at inoculation rates higher than 10^2 cfu mL^{-1}. Moreover, following the dilution procedure also allowed us to be confident of the final inoculum concentration in the opaque reconstituted skim milk samples.

3.3 Growth experiments

3.3.1 Medium composition for *L. lactis* cultivation

A CRM (*CRM – Carbohydrate-restricted medium*) growth medium of the following composition was used: casitone (Difco) 20 g L^{-1}, Tween 80 1.08 g L^{-1}, sodium chloride (MERCK) 25 g L^{-1}, magnesium sulfate 0.58 g L^{-1}, manganese sulfate 0.3 g L^{-1}, ferric sulfate 0.06 g L^{-1}, B-vitamin solution (SIGMA) 10 ml L^{-1}, and MES (2-[N-Morpholino]ethanesulfonic acid, SIGMA) 8.5 g L^{-1}.

3.3.2 Bacterial growth in liquid media

A CRM broth for growth media was prepared in screw-capped glass bottles for all of the studied initial glucose concentrations (GLC 2, 3, 4, 5, 6, 7, 10 and 20 g L^{-1}). All solutions were sterilized by autoclaving at 121°C for 15 min. A definite amount (1%, v v^{-1}) of the inoculum was added to the bottles with media solutions at 25°C to get the desired inoculation rate, and after that the suspensions were intensively stirred. 2 mL of the culture from each bottle were transferred into 3 mL autoclaved microcalorimetric glass ampoules and calorimetric experiments were run at +35°C. Bottles with residual bacterial suspensions were placed into a thermostat at +35°C. Glucose and lactic acid concentrations, pH and OD were determined in 4 mL samples; *outplating* was carried out using 100 μL samples at regular time intervals determined by the bacterial calorimetric growth curves. Sample solutions for the HPLC measurements were prepared by fixing with isopropanol in a proportion of 1:1.

3.3.3 Bacterial growth in solid media

CRM Agar for growth media was prepared in screw-capped glass bottles with the addition of all studied concentrations of glucose (GLC 2, 3, 4, 5, 6, 7, 8, 10 and 20 g L^{-1}) and agar (1%, w v^{-1}). All solutions were sterilized by autoclaving at 121°C for 15 min. After the addition of a definite amount of the inoculum (1%, v v^{-1}) to the flasks with media solutions at 45°C, suspensions were intensively stirred and then 3 mL from each flask was transferred into the autoclaved microcalorimetric glass ampoules. The filled ampoules were put into the microcalorimeter and experiments were run at 35°C. After filling the calorimetric ampoules, the remaining solutions were divided into 2 mL samples in plastic tubes and placed into the thermostat at 35°C – these were the parallels to the calorimetric experiments. Glucose and lactic acid concentrations and pH were determined at time intervals considered appropriate by bacterial calorimetric growth curves. Sample solutions for the HPLC measurements were

prepared by homogenizing the solid media in Falcon tubes with an appropriate amount of MilliQ water in a proportion of 1:2, and afterwards 500 µl of the probe was fixed with isopropanol in a proportion of 1:1.

3.3.4 Bacterial growth in reconstituted milk powder

LHSMP was irradiated in plastic Stomacher bags at a dose of 10 kGy using the dosimetric system GEX WinDose (Centennial, CO, USA). For the growth experiments, both low-heat skim milk powder (LHSMP) and irradiated low-heat skim milk powder (iLHSMP) were reconstituted in distilled water with thorough mixing for 1 h to yield a final concentration of 10% (w v^{-1}) milk solids, and heated at 90˚C for 30 min. $CaCl_2$ was added to the low-heat reconstituted skim milk (LHRSM) and irradiated low-heat reconstituted skim milk (iLHRSM) before mixing and heating. Chymosin (CHY-MAX Powder Extra) was obtained from Chr. Hansen (Hørsholm, Denmark). 0.2 g chymosin was dissolved in 10 ml MilliQ water and kept at 4°C. 100 µl of chymosin solution was added per 100 mL of inoculated milk in the experiments with solid casein gels.

2 mL of the culture of all of the milk samples from each bottle were transferred into 3 mL autoclaved microcalorimetric glass ampoules and calorimetric experiments were run at the chosen incubation temperature (40°C or 35°C). After filling the calorimetric ampoules, the remaining solutions were divided into 1 mL samples in eppendorf centrifuge tubes and placed into the thermostat at the appropriate incubation temperature – these were the parallels to the calorimetric experiments. Glucose and lactic acid concentrations and pH were determined every hour for the first 9 hours of growth. Sample solutions for the HPLC measurements were prepared by fixing with isopropanol in a proportion of 1:1.

3.3.5 Bacterial growth in dry pet food kibbles

The experiments were performed with dry dog food in the form of extruded kibbles under the brand name ProBiotic LIVE (Bacterfield S.A., Luxembourg), which according to the producer contains viable probiotic bacteria *Enterococcus faecium* (NCIMB10415). The concentration of bacteria as declared on the packaging is 10^6 cfu per gram of dog food on average. Three commercially available dog food products differentiated by dog age (adult and senior) and by taste (salmon and chicken formulations) were used in the experiments: ProBiotic LIVE Adult Chicken & Rice (AC), ProBiotic LIVE Adult Salmon & Rice (AS) and ProBiotic LIVE Senior Chicken & Rice (SC). All dog food samples used in the experiments were initially received in hermetically closed separate 1.5 kg bags, with expiry date/lot. nr. 23.11.2012. All the bags were aseptically opened immediately before the experiments. For the experiments, dry pet food kibbles of different types were ground into fine particles and afterwards weighed in sterile plastic tubes. Samples were aseptically transferred into 3 mL autoclaved microcalorimetric glass ampoules. Depending on the experimental conditions, sterile MilliQ water, adjusted to pH=2 (dog stomach equivalent pH (Akimoto et

al., 2000)) or pH=7 (neutral value), was added to the ground dry pet food in a mass ratio of 1:1. All the solutions had been previously sterilized by autoclaving at 121^{o}C for 15 min.

In order to determine the initial bacterial concentration in the dog food kibbles, outplating on PCA (*Scharlau, Spain*) was carried out for all three pet food formulations. All experiments with pet food kibbles were performed at the fixed temperature of incubation of 37^{o}C (the equivalent of a dog's body temperature (Carlson and Griffin, 1992).

3.4 Analytical methods

3.4.1 Instrumental

A TAM III (24-channels, *TA Instruments, Delaware, USA*), a type of heat conduction multi-channel microcalorimeter, was used to monitor the growth of cells. HPLC Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 x 7.8 mm to measure glucose and lactate concentrations (solvent–sulfuric acid solution (2.2 mL per 1 L MilliQ water), flow rate 0.6 mL min^{-1}, and an experimental temperature of 35°C). Optical density (for bacterial growth in a liquid state) was observed at 540 nm using a spectrophotometer (PHOTOMETR KFK-3, USSR). pH was detected with a pH meter S20 Seven Easy Mettler Toledo with an InLab 413 Mettler Toledo electrode. The dimensions of the colonies were measured using a Zeiss Axiovert 200M microscope with a Zeiss AxioCam MRc5 camera and Zeiss AxioVs40 V45.0.0 software.

3.4.1.1 Analysis of power-time curves – the output of the microcalorimeter

The curves that are the output of a microcalorimeter are called calorimetric thermograms or power-time curves. The power-time curve is a derivation of a standard bacterial growth curve in batch cultures. Calorimetric power-time curves (see Figure 5) can be divided into three growth phases (Figure 5(a)), essentially the same way as the bacterial growth curves, but with some reservations. Firstly, there is the lag phase, which determines the adjustment period during which bacterial cells adapt to the new environment and start to divide exponentially. The length of the lag phase was determined as shown in Figure 5(b). The calculation procedure was 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, besides by the physiology of the cell, 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 μW), 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. It should be noted that this is also valid if some other physical method is used: OD etc. (Swinnen et al., 2004).

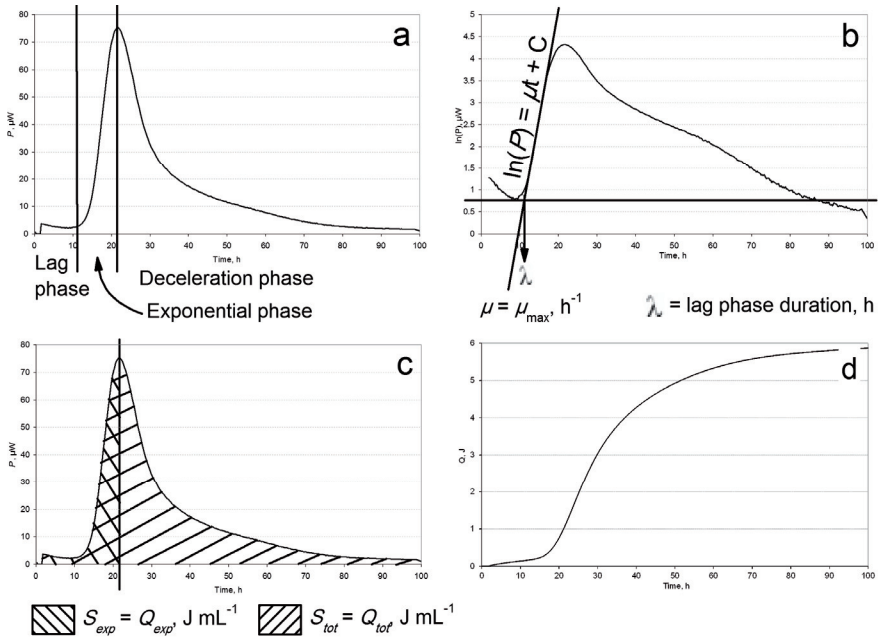


Figure 5. The explanation of the processing of calorimetric power-time curves; (a) the division of calorimetric power-time curves into three phases: lag phase, exponential growth phase, and deceleration phase; (b) determination of the maximum growth rate (μ_{max}, h^{-1}) and the lag phase duration (λ, 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

The second phase of the power-time curve, following the lag phase, corresponds to the exponential growth phase, during which bacteria grow with the maximal growth rate μ_{max} possible in the experimental conditions under study (see Figure 5). Taking into account that in the exponential growth phase the relationship between biomass concentration and specific growth rate may be described by first order kinetics and assuming that the rate of biomass formation is proportional to the rate of heat production, the maximum specific growth rate (μ_{max}, h^{-1}) is measured as shown in Figure 5(b). The heat fluxes are proportional to the growth of cells, and the heat flows are equivalent to the growth rate values. The end of the exponential phase of the bacterial growth was defined in our data analysis by the peak of the power-time curves (see Figure 5(a)). According to the results obtained by *plate count*, the end of the exponential growth occurred at some time after the peak of the calorimetric curve but, according to the power-time curve, inhibitory processes and the decrease in the growth rate had obviously started already (see also Figure 5(d)). Therefore,

considering the exponential growth phase as ending at the peak value of the power-time curves is justified. The complex post-exponential growth phase that follows the exponential growth phase was called the deceleration phase in our studies, and it ended, in principle, with the full stop of the growth of bacteria, and the experiment (see Figure 5(a)).

The total heat produced during the whole process of growth (Q_{tot}, J mL^{-1}) and the heat produced during the exponential growth phase (Q_{exp}, J mL^{-1} - see Figure 5(a, c)), as well as the maximum growth rate (μ_{max}, h) and lag-phase duration (λ, h) (Figure 5(b)), were determined using a *TAM Assistant* program (v 0.9.1012.40, *SciTech Software AB, Thermometric AB*) and *Microsoft Excel*.

All calorimetric experiments were performed in three parallels and the obtained power-time curves were normalized per millilitre in the case of the bacterial growth in the liquids, in the solid agar gels and in all the milk samples, as well as per gram in the experiments performed with the pet food. The averaged power-time curves of the three parallel runs were used for further analysis.

4 RESULTS AND DISCUSSION

4.1 The study of the growth of *Lactococcus lactis* in broth (Publication I and II)

4.1.1 Determination of heat yield coefficient Y_Q

The heat yield coefficient Y_Q (J cfu^{-1}) determines the amount of heat that is produced per one bacterium synthesized. It was calculated on the basis of the data of the heat produced at the end of the exponential growth phase (Q_{exp}, J mL^{-1}) and based on the results of the *plate counts* carried out in parallel with the calorimetric measurements. For the calculation of the heat yield coefficient, the data were collected in different initial substrate concentrations (2, 5, 10, 20 g L^{-1}) and inoculation rates (10^2 - 10^6 cfu mL^{-1}). There was no significant influence of either initial bacterial number or initial glucose concentration on the value of Y_Q. The value of Y_Q was determined to be $Y_Q = (2.58 \pm 0.44) * 10^{-9}$ J cfu^{-1}. The Y_Q value was used for the description of the exponential phase of the liquid culture and also for the solid-state growth.

4.1.2 Analysis of power-time curves of bacterial growth in liquids

The growth of *L. lactis* was monitored in broth at different inoculation rates (10^2, 10^3, 10^4, 10^5, 10^6 cfu mL^{-1}) and glucose concentrations (2, 10 and 20 g L^{-1}). Changing the inoculation rate provided an opportunity to study the growth termination processes, together with the buffer capacity of the media.

The data obtained at glucose concentrations of 2 g L^{-1} and 10 g L^{-1} at different inoculation rates (10^2 – 10^6 cfu mL^{-1}) are shown in Figure 6. The same series of power-time curves were obtained for the 20 g L^{-1} initial concentration of glucose but, as they were similar to the curves obtained at 10 g L^{-1} glucose concentration, they are not separately brought out.

As seen in Figure 6, the length of the lag phase of the growth curves was notably shortened in increasing the inoculum. A separate study showed that in the case of *L. lactis* the level of sensitivity of the instrument corresponds to the growth of approximately 10^5 cells per mL. This means that the growth of cells at inoculation rates higher than 10^5 cfu mL^{-1} is recorded from the beginning of the growth, after a "genuine" lag phase, and at lower inoculation rates bacterial growth is recorded after the number of bacteria has reached 10^5 cfu mL^{-1} after growth of different lengths. In agreement with this, the experimentally determined length of the lag phase should differ by 3.33 times generation times ($2^{3.33} = 10$) at different inoculation rates lower than 10^5 cfu mL^{-1}. Indeed, this was observed in our experiments (see Figure 6) (the generation times in liquid cultures at high inoculation rates were about 1 hour).

The maximum growth rates μ_{max} (Figure 7) slightly decreased from 0.56 h^{-1} at the inoculation rate of 10^6 cfu mL^{-1} to 0.44 h^{-1} at the inoculation rate of 10^2 cfu mL^{-1}. The maximum values of the observed growth rates of the bacteria in the exponential phase at high inoculation rates coincided with the μ_{max} value measured for these bacteria in liquid cultures using other (conventional)

methods, such as *outplating* and optical density measurements. The decrease of the μ_{max} values at lower inoculations rates can be explained by the fact that in these cases we observed the growth of cultures with the same number of cells as in the case of high inoculation rates. However, as the cells had increased from the initial low numbers, they had synthesized some amount of growth inhibiting product – lactate – to the media of low buffering capacity, which led to the inhibition of the growth rate.

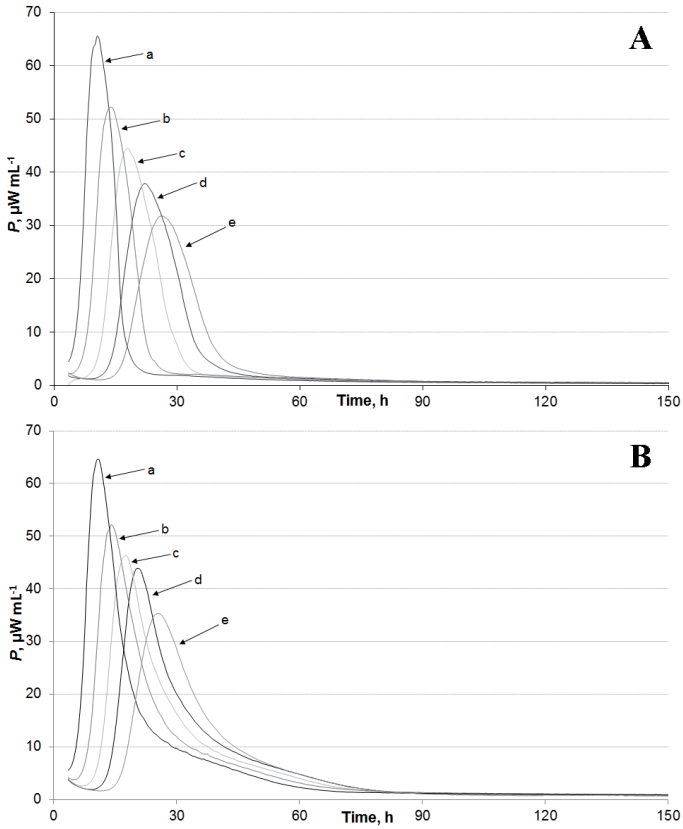


Figure 6. Power-time curves describing bacterial growth in broth at different inoculation rates and at initial glucose concentration 2 g L^{-1} (A) and 10 g L^{-1} (B): a – inoculation rate – 10^6 cfu mL^{-1}; b – 10^5 cfu mL^{-1}; c – 10^4 cfu mL^{-1}; d – 10^3 cfu mL^{-1}; e – 10^2 cfu mL^{-1}

The maximum specific growth rate μ_{max} (h^{-1}) did not notably depend on the initial glucose concentration (Figure 7). This result strongly indicates that the bacteria grew at μ_{max} possible for current environmental conditions during the rapid (exponential) growth phase.

Besides μ_{max}, the exponential growth phase is characterized by the amount of heat produced by the growing cells, which is proportional to the number of bacteria grown. It was observed that the heat produced during the exponential growth phase was practically the same at a value of 0.80±0.05 J mL^{-1}, which

corresponds to $3.10*10^8$ cells at different inoculation rates and different glucose concentrations studied. This indicates that there was no substrate (glucose) limitation in the exponential phase and the termination of the exponential growth was caused by accumulating lactate either through direct inhibition or more probably through the change of the pH of the media. Essentially the same conclusion was drawn from the HPLC and pH measurements.

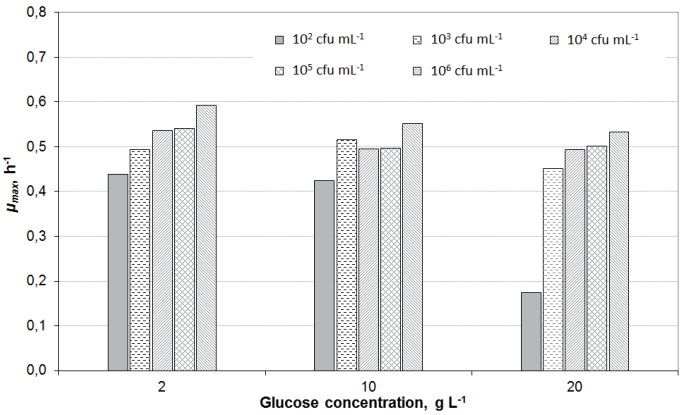


Figure 7. The maximum specific growth rates at different initial glucose concentrations (2, 10, 20 g L^{-1}) and various inoculation rates from 10^2 cfu mL^{-1} to 10^6 cfu mL^{-1} determined for bacterial growth in broth

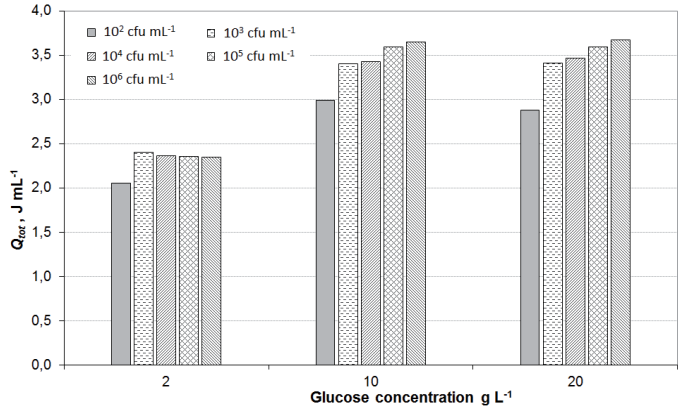


Figure 8. The heat produced during the total bacterial growth in broth at different initial glucose concentrations (2, 10, 20 g L^{-1}) and various inoculation rates, from 10^2 cfu mL^{-1} to 10^6 cfu mL^{-1}

The total heat amounts produced by a bacterial culture at the end of growth, Q_{tot}, do not greatly depend on inoculation rate, but notably depend on initial glucose concentration (Figure 8). The heat produced at the 2 g L^{-1} initial glucose concentration is equal at inoculation rates higher that 10^2 cfu mL^{-1}. This means that the growth has stopped because all the available substrate is totally

consumed during the growth. At higher initial glucose concentrations, there is enough of the substrate available in the media, which leads to higher biomass yields and thus higher heat production.

4.1.2.1 Study of deceleration phase

In order to describe the deceleration growth phase, the growth of *L. lactis* was studied in lower substrate concentrations, from 2 g L^{-1} up to 10 g L^{-1} with a stepwise increase of 1 g L^{-1} at the 10^2 cfu mL^{-1} inoculation rate. The calorimetric power-time curves obtained in all of the glucose concentrations studied in broth are presented in Figure 9. In order to analyse the power-time curves, additional measurements of concentrations of glucose (GLC, g L^{-1}) and lactic acid (LA, g L^{-1}), pH and bacterial number, as well as the optical density (OD), were carried out for three chosen glucose concentrations (2, 5 and 10 g L^{-1}), and the data obtained are presented in Figure 10. The data of additional measurements were splined (Pollock, 1993).

The power-time curves obtained in liquids in the lowest glucose concentration studied – 2 g L^{-1} – resemble symmetric (*Gaussian)* curves (Figure 9). The deceleration phases of the growth curves were more prolonged in higher glucose concentrations than 2 g L^{-1} and calorimetric curves were practically identical in the range of 4-10 g L^{-1} of glucose. In the 3 g L^{-1} the curve coincided with those of the higher glucose concentrations until the 40th hour of fermentation, after which a quite abrupt decrease in the growth rate was observed.

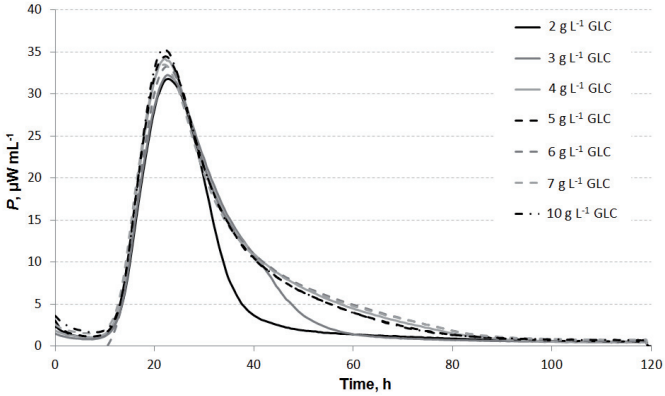


Figure 9. Calorimetric power-time curves of *L. lactis* in liquid media with different glucose concentrations (2, 3, 5, 6, 7 and 10 g L^{-1}) at an inoculation rate of 10^2 cfu mL^{-1}

As seen in Figure 10, the end of the exponential growth phase measured on the power-time curves coincided with the time when the transition phase between the exponential and stationary growth phases started, according to the growth curve obtained from *plate count* and OD results. This fact means that it is experimentally justified to take the peak of the calorimetric power-time curve as

the end of the exponential growth phase. In fact, on the basis of the calorimetric curves, the time when the growth rate of the culture started to deviate from the maximum values could be detected more precisely in comparison with conventional methods.

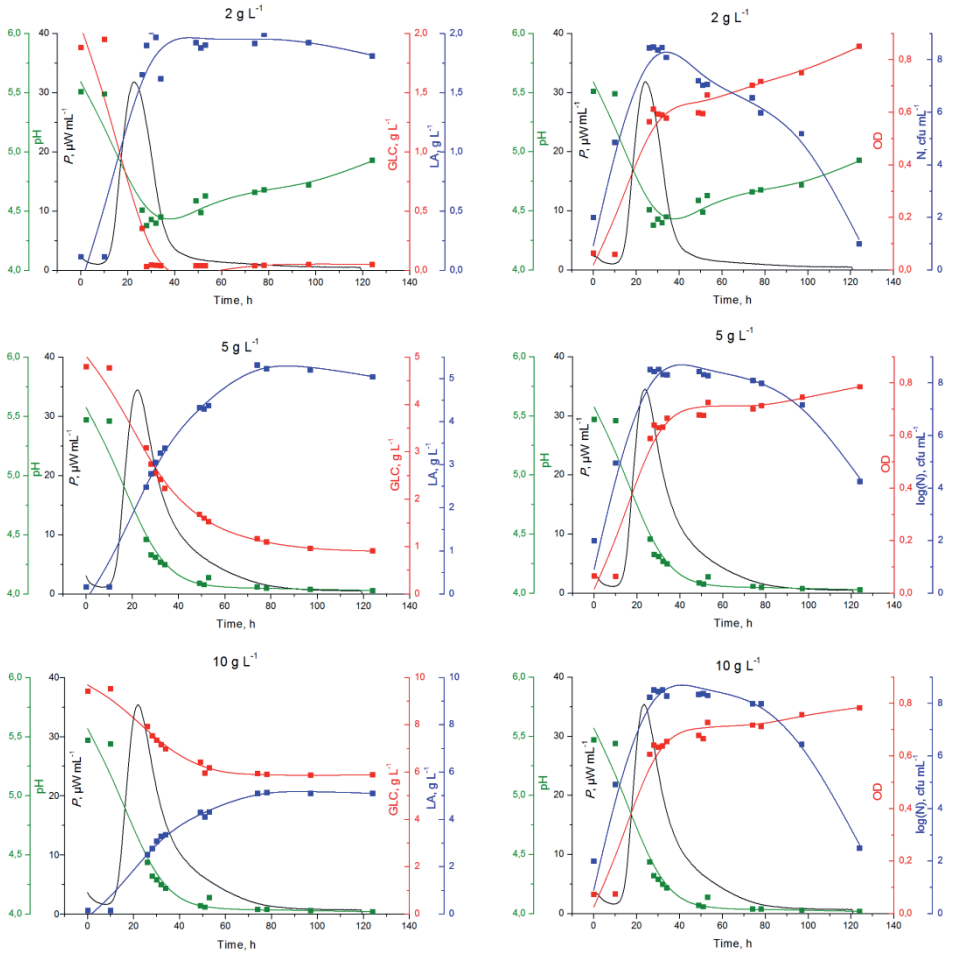


Figure 10. Calorimetric power–time curves together with additional measurements of the pH change, lactic acid (LA) production and glucose (GLC) consumption curves, changes in bacterial number (N) and the curves of optical density (OD) describing the growth of bacteria at different initial glucose concentrations of 2, 5 and 10 g L^{-1} in liquid cultures

According to Figure 10, the pH value was about 4.5-4.6 at the end of the exponential growth in the glucose concentrations studied. This means that the reason for the stopping of the exponential growth was probably the change of the pH of the media at a definite level on account of the production of lactic acid.

The heat amount produced by the end of the growth in liquid cultures at higher initial glucose concentrations (4-10 g L^{-1}) was around 2.8±0.3 J mL^{-1},

which corresponds to the number of bacteria of $1.09*10^9$ cfu mL^{-1} (calculated using the heat yield coefficient Y_Q). A comparison of the biomasses grown during the complete cultivation experiments and during the exponential growth phase showed that about 70% of the total was synthesized during the post-exponential growth phase. At low initial glucose concentrations, at 2 g L^{-1} and 3 g L^{-1}, the total heat amounts produced by the cultures were lower in comparison with higher initial glucose concentrations. As seen in Figure 10, the profiles of consumed glucose, produced lactate and pH were also different at the lower initial glucose concentrations (2 g L^{-1}) in comparison with the higher ones (5 and 10 g L^{-1}).

The growth of the liquid culture was eventually terminated due to the exhaustion of the glucose in the media at 2 g L^{-1} and 3 g L^{-1}. To understand the mechanism of the termination of growth in cultures with higher than 3 g L^{-1} of initial glucose, it should be noted that the final concentrations of lactic acid in the cultures did not exceed 5 g L^{-1}, which is quite low: usually at least two times higher concentrations are required to notably inhibit the growth of LABs. On the other hand, the pH values were in the range of 4.0-4.2 at the end of cultivations, which could well be the reason for the termination of the growth of the cultures.

4.2 The study of the growth of *Lactococcus lactis* in solid agar gels (Publication I and II)

4.2.1 Analysis of the power-time curves of the solid-state growth of bacteria

The growth of *L. lactis* was monitored in agar gels at different inoculation rates (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu mL^{-1}) and glucose concentrations (2, 10, 20 g L^{-1}). The obtained calorimetric power-time curves of the growth of the bacteria in solid matrices are presented in Figure 11.

A longer lag phase, by approximately 1.4 hours, was observed in the solid-state cultures in comparison with the liquid cultures. This can be explained by the differences in preparation of the cultures. Inoculating the bacteria into media containing agar, the temperature of the media should be high enough (higher than 40ºC) for good mixing, and this is stressful for microorganisms (Malakar, 2002a), leading to the prolongation of the lag phase. There is no additional heat stress in the case of liquid cultures: inoculation of bacteria in broth takes place at room temperature.

Maximum growth rates μ_{max} were determined from the power-time curves as described in *Section 3.4.1.1*. In Figure 12 the intervals in which the maximum growth rate was constant are marked with bold black lines. Even at lower inoculation rates (10^1 and 10^0 cfu mL^{-1}), the linear correlation coefficients were equal to R^2 = 0,999 for up to 12 hours, indicating that bacteria were growing exponentially.

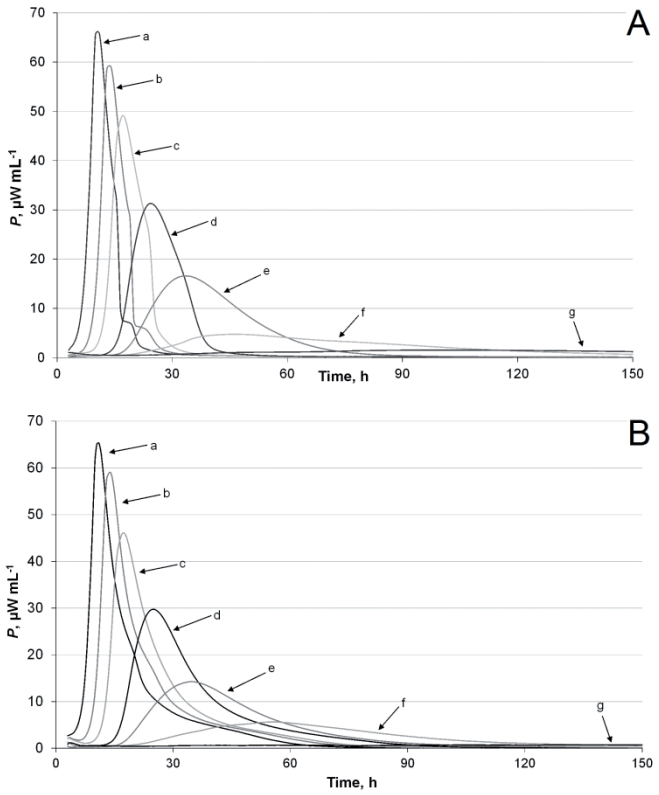


Figure 11. Bacterial growth monitored by calorimetric power-time curves in agar gels with different inoculation rates in two different glucose concentrations: (A) 2 g L^{-1} and (B) 10 g L^{-1} in solid agar (1%) gels: a – 10^6 cfu mL^{-1}; b – 10^5 cfu mL^{-1}; c – 10^4 cfu mL^{-1}; d – 10^3 cfu mL^{-1}; e – 10^2 cfu mL^{-1}; f – 10^1 cfu mL^{-1}; g – 10^0 cfu mL^{-1}

The values of the maximum growth rates measured from the power-time curves presented in Figure 11, and also in curves not presented in the text, are given in Figure 13. The values of the maximum growth rates μ_{max} notably depended on the inoculation rates, but did not depend on the concentration of glucose in the media. It is also clear that in the case of inoculation rates of 10^4-10^6 cfu mL^{-1} the μ_{max} values of about 0.65 h^{-1} were slightly higher, but in the same range as the μ_{max} value in broth (Figure 7). It could be concluded that no diffusion limitation of the growth by glucose was observed in the growth of small colonies in 1% agar gels. Small colonies possibly containing one bacterium at the beginning grew with the maximum growth rate μ_{max} characterizing the bacteria in excess of glucose. The lower recorded growth rates μ_{max} of the colonies at lower inoculation rates are explained by the fact that in these cases we monitored the growth of the bigger colonies growing in conditions of a higher concentration of the lactate synthesized within the colonies and to the bulk media during the latent period of the measurements of

the heat flow. A comparison of the decrease in the growth rates in liquid culture and in the solid state at the same low inoculation rates (lower than 10^3 cfu mL^{-1}) showed that the decrease was more pronounced in the latter case. This can be explained by assuming that lactate accumulated within the colonies and thus inhibited the bacterial growth more efficiently than lactate equally distributed in the broth in the liquid cultures.

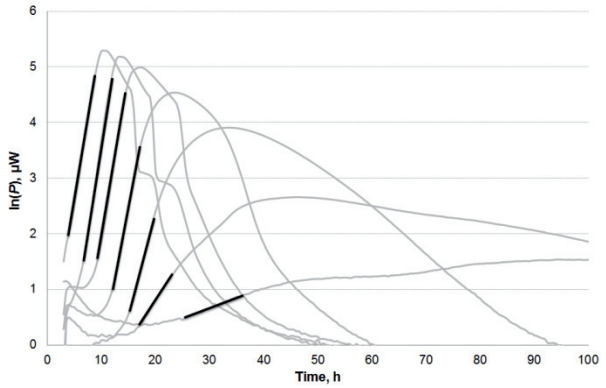


Figure 12. The determination of maximum specific growth rate μ_{max} (h^{-1}) from the calorimetric power-time curves in solid agar gels at different inoculation rates, from 10^0 to 10^6 cfu mL^{-1} with 10-fold increments. The bold solid lines show the time interval of the curve in which the specific growth rate was maximum and constant during the exponential growth phase

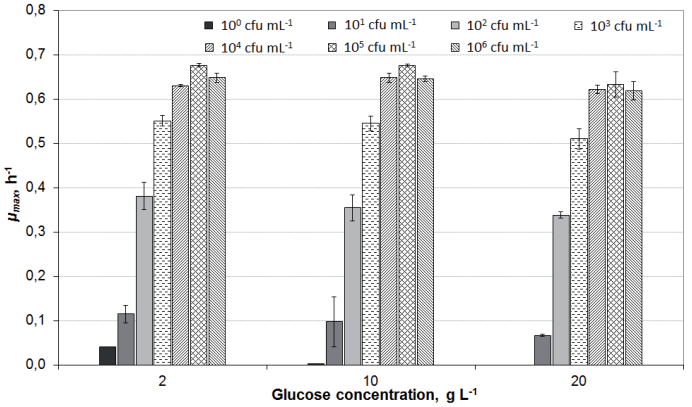


Figure 13. The maximum specific growth rates at different initial glucose concentrations (2, 10, 20 g L^{-1}) and various inoculation rates, from 10^0 cfu mL^{-1} to 10^6 cfu mL^{-1} in bacterial growth in agar gel

A microcalorimeter measures the heat evolution of a sample, and this data can be recalculated for the growth of a culture, and eventually for the growth of an average colony, taking into account the sensitivity of the instrument and the inoculation rate. Changing the inoculation rate provides an opportunity to study

the growth of colonies of different sizes. The scheme of calculation of the numbers of bacteria at different inoculation rates is illustrated in a schematic drawing in Figure 14. It was possible to calculate the heats that produced during the growth, the numbers of bacteria in the sample and colonies etc. on the basis of the figure, and understand how "lower inoculation rate effects" and "colony-effects" led to the differences in the power-time curves in solid-state growth and growth in liquid cultures. The total numbers of bacteria in the sample were calculated using microscopy (see *Section 4.2.2*). Microscopy was found to be more suitable than power-time curves for the determination of the total numbers of cells in average colonies at the end of the growth. However, these issues require further and more detailed investigations.

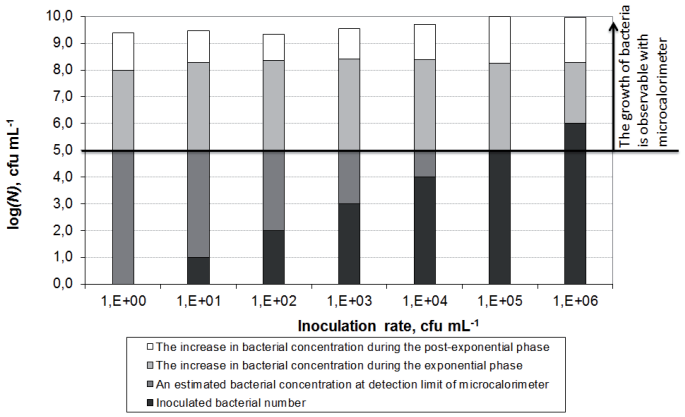


Figure 14. The numbers of bacteria of different "origin" (inoculum, exponentially grown, and grown during the post-exponential growth phase) in samples, with cultures at different inoculation rates. The number of the bacteria grown in a sample was calculated by subtracting from the total number of bacteria, or from the number of bacteria at the end of the exponential phase, the number of bacteria inoculated into the sample at the beginning of the experiment. The number of bacteria in an average colony was calculated by dividing the number of bacteria grown in a sample by the inoculation rate. The detection limit of the instrument and the number of bacteria needed to grow until power-time curves could be measured are also presented

A snapshot of the solid culture at the end of the exponential growth phase calculated on the basis of the experimental results obtained is presented in Table 1. Taking into account the inoculation rates, it was possible to calculate the distances of the colonies in idealised grids (third column). The numbers of bacteria in average colonies at the end of the exponential growth phase, calculated using Y_Q in different glucose concentrations and inoculation rates, are presented in the fifth column of Table 1. The radii of average (spherical) colonies at the end of exponential growth were calculated using *Kepler`s* conjecture. It should be noted that the colonies were lenticular rather than spherical in the agar gels, but for bigger intuitive transparency simplified calculations using spherical shapes of colonies were carried out.

Table 1. Calculated parameters describing the growth of the bacterial colonies at the end of the exponential growth phase at different initial glucose concentrations (2, 10, 20 g L^{-1}) and inoculation rates N_0 (from 10^0 cfu mL^{-1} to 10^6 cfu mL^{-1}): yield coefficient Y_{XS} (g g^{-1}), amounts of produced heat during the exponential phase - Q_{exp} (J mL^{-1}), and the number of bacteria N_{exp} (cfu col^{-1}) formed in an average colony with the radius R_{COL} (μm); R_{GLC} (μm) is the radius of an area from which glucose was scavenged for the growth of an average colony

N_0, cfu mL^{-1}	GLC g L^{-1}	Distance between colonies, μm	Q_{exp}, J mL^{-1}	N_{exp} (1), cfu col^{-1}	R_{COL} (2) μm	R_{GLC} (3) μm	$Y_{XS}*10^{-2}$ g g^{-1}
10^0	2	10000	0.13	$4.9*10^7$	185	5149	1.36
	10		0.36	$1.4*10^8$	263	4281	1.36
10^1	2	4642	0.27	$1.0*10^7$	110	3063	1.36
	10		0.35	$1.4*10^7$	142	1963	1.36
	20		0.30	$1.2*10^7$	150	1475	1.36
10^2	2	2154	0.62	$2.4*10^6$	68	1876	1.37
	10		0.60	$2.3*10^6$	67	1086	1.36
	20		0.57	$2.2*10^6$	66	845	1.36
10^3	2	1000	0.66	$2.6*10^5$	32	876	1.44
	10		0.63	$2.6*10^5$	32	510	1.45
	20		0.66	$2.5*10^5$	32	381	1.85
10^4	2	464	0.63	$2.4*10^4$	15	409	1.34
	10		0.61	$2.3*10^4$	14	238	1.31
	20		0.61	$2.4*10^4$	14	174	1.69
10^5	2	215	0.45	$1.7*10^3$	6	190	0.95
	10		0.46	$1.8*10^3$	6	109	1.02
	20		0.46	$1.8*10^3$	6	77	1.45
10^6	2	100	0.50	$1.9*10^2$	3	88	1.05
	10		0.48	$1.9*10^2$	3	54	0.92
	20		0.49	$1.9*10^2$	3	37	1.40

(1) The number of bacteria was calculated using the data on the heat produced during the exponential growth, and the Y_Q value determined

(2) Radius of an average spherical model colony calculated from an average volume of the colony, calculated using *Kepler's* conjecture of bacterial packing in colonies, the sizes of which were measured using a microscope

(3) Radius of an average spherical area from which glucose was used by an average colony – the radius was calculated from the volumes of glucose consumed by one colony determined using HPLC analysis

Radii of the average spheres from which glucose was scavenged by average colonies were calculated using the HPLC data on glucose consumption. As also seen in Table 1, the spheres of influence of average colonies did not overlap heavily, which is in good agreement with the conclusion reached on the basis of other data that during the exponential growth phase colonies would grow essentially separately.

Using the data on heat amounts produced Q_{exp} and glucose consumed, the values of yield coefficient Y_{XS} (g g^{-1}) were calculated for the growth during the exponential phase, under conditions where there were no strong inhibitory effects influencing the growth. First, the bacterial number per ampoule was calculated on the basis of the heat amount produced during the exponential phase using heat yield coefficient Y_Q. To calculate the biomass in the sample, the number of bacteria was multiplied by the dry weight of one cell. The mass of a cell (an average cell) was obtained two different ways. Data on the volume and the density of the cell were used in the first method. According to the literature (Kokkinos et al., 1998), the size of *L. lactis* subsp. *lactis* is around 0.35 μm^3 and multiplying it by the cell density (ρ = 1 g cm^{-3}) the dry weight of the cell would be (1.05±0.49)*10^{-13} g. The mass of an average cell was also experimentally measured in chemostat cultures of *L. lactis*. The concentration of the bacteria was determined using *plate count*; the dry weight of the biomass was determined using gravimetry. The average mass of the cells was calculated to be (2.08±0.54)*10^{-13} g, which matches well with the result of the first method. According to the data in Table 1, the Y_{XS} was constant irrespective of initial substrate concentration at inoculation rates lower than 10^3 cfu mL^{-1}. With further inoculation rate increase in the interval of 10^3-10^6 cfu mL^{-1} a decrease in Y_{XS} was observed, which means that the bacteria in smaller colonies spilt more energy in comparison with bigger colonies: at higher inoculation rates in the case of faster growing cells more substrate was consumed to synthesize the same amount of biomass. The opposite trend was observed in liquid continuous culture in Lahtvee et al. (2011). This can be explained by assuming that *L. lactis* exercises different metabolic strategies in broth and solid-state media.

While the heats produced (number of bacteria grown) during the exponential growth phase were practically the same at all glucose concentrations (Table 1), the total heats produced during the complete growth processes were notably different (Figure 15). The total amount of heat produced during the complete growth was in the range of 1.5-2.8 J mL^{-1} in practically all cases, indicating that most of the biomass, about 3-5 times more than by the end of the exponential growth phase, was synthesized during the post-exponential growth phase. However, there were notable differences between the glucose concentration 2 g L^{-1} and other glucose concentrations studied. In the initial glucose concentration of 2 g L^{-1} the total heat produced was 1.8 J mL^{-1} at all inoculation rates, except for the lowest: 10^0 and 10^1 cfu mL^{-1}. This supports the conclusion reached for liquid cultures according to which the number of bacteria at the end of the growth was limited by the amount of glucose present in the media, which is also supported by the HPLC measurements. The higher initial concentrations of glucose were also able to support the growth of notably higher numbers of cells - 2*10^9-4*10^9 bacteria per mL - producing higher amounts of heat. According to the HPLC data, the energy metabolism at higher initial glucose concentrations was changed to heterofermentative (Publication II, Table 2). The mechanisms leading to the change of the metabolism of the bacteria from

homofermentative to more efficient heterofermentative mechanisms ensuring the prolongation of the production of heat, and the prolongation of post-exponential growth, however, require further investigation. An uncoupling of energy production is the most probable factor involved in determining the peculiarities observed, as proposed earlier by Belaich et al., 1972, Senez, 1962 and Russel and Cook, 1995.

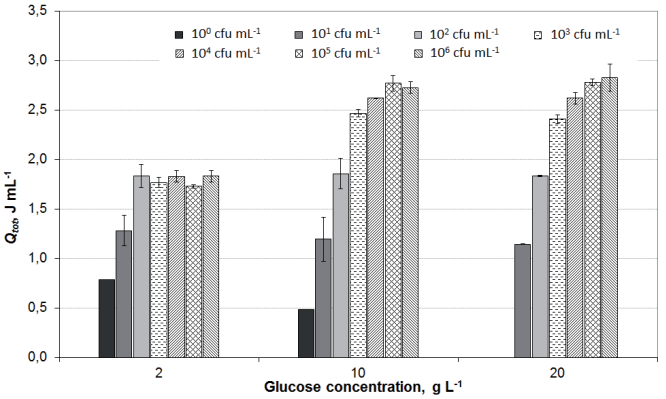


Figure 15. Total heat produced during bacterial growth in 1% agar gels at different initial glucose concentrations (2, 10, 20 g L^{-1}) and various inoculation rates, from 10^0 cfu mL^{-1} to 10^6 cfu mL^{-1}

4.2.1.1 The study of the deceleration phase

The calorimetric power-time curves of the growth of *L. lactis* in lower substrate concentrations, from 2 g L^{-1} up to 10 g L^{-1}, with stepwise increases by 1 g L^{-1} in the case of the 10^2 cfu mL^{-1} inoculation rate are presented in Figure 16. In order to analyse the calorimetric power-time curves, additional measurements of concentrations of glucose and lactic acid, as well as pH, were carried out for three chosen glucose concentrations (2, 5 and 10 g L^{-1}) and the data obtained are presented in Figure 17. The data from additional measurements were splined (Pollock, 1993).

The power-time curves obtained in liquids (Figure 9) and in solids (Figure 17) are quite similar in shape, but some notable differences can nevertheless be observed. In all cases, also at 2 g L^{-1} glucose concentrations the post-exponential parts of the solid state curves were prolonged. The 2 g L^{-1} curve was not Gaussian; however, the growth of the culture was stopped earlier than in the case of other glucose concentrations, from 3 g L^{-1} and higher. This and the fact that the maximum heat flows P_{max} (W mL^{-1}) were remarkably lower in the solid-state matrices than in broth were the clear differences characterizing growth in the two cases studied.

The heat produced in solid-state matrices was 1.5 times lower than in broth, and thus also the number of bacteria grown during the exponential growth was smaller: 0.55±0.02 J mL^{-1} and $2.12*10^8$ cfu mL^{-1}, respectively (Publication II, Table 1). However, this apparent contradiction was solved by noting that in solid media the pH value of 4.6 (pH was the factor terminating the exponential growth

in both media) was reached with a smaller amount of lactate (LA ~1.0 g L^{-1}) produced than in the liquid media (~2.2 g L^{-1}) (Figure 10 and Figure 17). One of the possible explanations for the lower buffering capacity of solid matrices is that part of the free water was bound to the gel in the solid media, and thus less lactic acid than in the broth was needed to acidify the environment and bring the pH down. Another possible assumption is that the homofermentative metabolism was replaced by a heterofermentative metabolism of *L. lactis* in the solid-state matrices with the formation of formate and acetate, stronger acids than lactate.

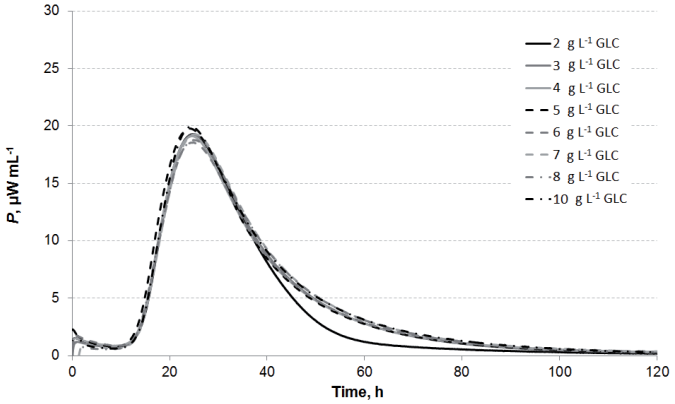


Figure 16. Calorimetric power-time curves of *L. lactis* in media with different glucose concentrations (GLC, g L^{-1}) in solid-state matrices at an inoculation rate of 10^2 cfu mL^{-1}

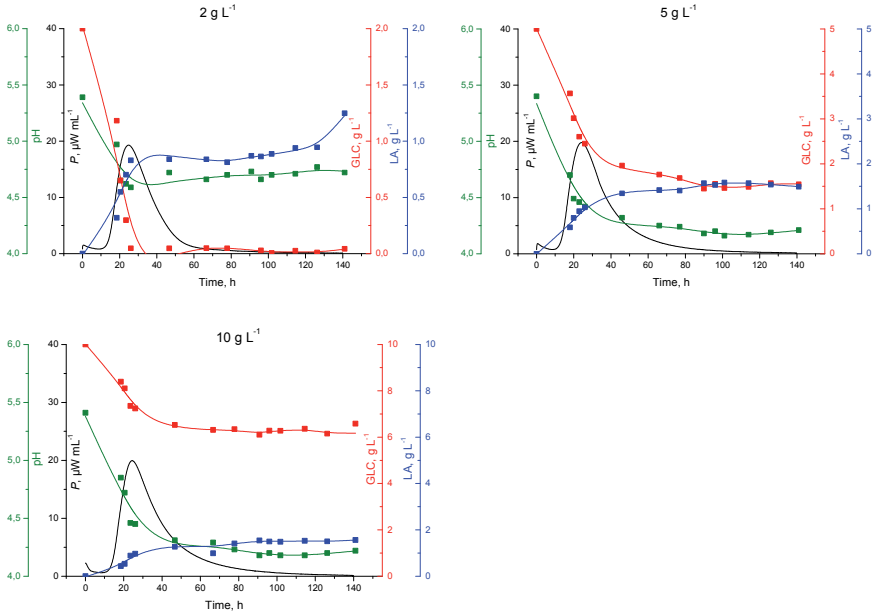


Figure 17. Calorimetric power–time curves, pH change, lactic acid production and glucose consumption curves describing the growth of bacteria at different initial glucose concentrations of 2, 5 and 10 g L^{-1} in solid agar gels

However, it should be noted that no extra peaks in addition to lactic acid on the HPLC curves were detected. Therefore, the assumption that the smaller buffering capacity of agar gel was the reason for the smaller amount of biomass formed in the solid-state matrices seems to be more attractive than the other assumption.

The total heat effects in the bacterial growth in the solid-state matrices were on average 1.92±0.18 J mL^{-1} and the number of bacteria grown in the solid cultures calculated using the heat yield coefficient was $(7.41\pm0.38)*10^8$ cfu mL^{-1}. The total number of bacteria at the end of the growth in solid agar gels could also be calculated on the basis of microscopic data, using *Kepler's* conjecture. The number of bacteria at the end of the growth in the solid state was $(1.1\pm0.19)*10^9$ cfu mL^{-1} forming colonies with radii of 111±6.69 μm. The number of the bacteria determined based on microscopy was 1.5 times higher than the number calculated from the power-time curves. This difference can be explained most probably by the less dense packing of bacterial cells in the colonies than assumed in the *Kepler's* conjecture, which is the densest possible packing of spheres. But a reasonable correspondence of the calorimetric and microscopy data should be considered encouraging in attempts to determine datasets that fit with each other on the growth of bacteria in solid-state matrices.

The profiles of consumed glucose, produced lactate and pH were also different at lower initial glucose concentrations (2 g L^{-1}) in comparison with the higher ones (5 and 10 g L^{-1}), as in the bacterial growth in broth. In the 2 g L^{-1} glucose concentration, the substrate was completely consumed by the end of the growth. In the 5 g L^{-1} glucose concentration, more than half of the total glucose available initially (3.4 g L^{-1}) was consumed and 35% of the initial glucose was eventually consumed at 10 g L^{-1} in the solid-state cultures. In the higher than 3 g L^{-1} of initial glucose concentration, the same growth inhibiting factors as in the liquid cultures should be mentioned: the production of growth inhibiting amounts of lactic acid led to extremely low environmental pH values. The fact that the growth of the cultures at high initial glucose concentrations (5 and 10 g L^{-1}) was terminated at pH=4.2 in solid-state cultures and at pH=4.0 in liquid showed that this was most probably due to the fact that even the lower amounts of lactic acid produced by the bacteria within the colonies was sufficient to stop the growth. It is necessary here to assume that the diffusion of lactic acid inside the colonies was a notably slower process than in water, which allowed higher local accumulation of lactic acid and the inhibition of growth at bulk pH higher (pH=4.2) than in the broth (pH=4.0), where there were no diffusion limitations. This conclusion is in agreement with the data in the literature (Andersen et al., 2009; Mercade et al., 2000).

For homofermentative bacteria such as *L. lactis*, it is common to produce lactic acid from consumed glucose in a ratio of 1:1 (gram per gram). During the growth of bacteria in the solid-state matrices, the metabolism of the bacteria was most probably changed to more efficient heterofermentative mechanisms, as the concentrations of the produced lactic acid made up approximately half of the

consumed glucose amounts. According to the literature (Papagianni et al., 2007), homofermentative metabolism in anaerobic cultures occurs on substrates supporting rapid growth in which a significant amount of glucose is in the medium and a mixed acid fermentation occurs when growth rates are low. We observed the shift in the change in growth from the liquid to the solid-state culture.

4.2.2 The sizes and shapes of the colonies determined by microscopy

The lenticular shape of the colonies could be visually well observed starting at 10^4 cfu mL^{-1} and with a further decrease in the colony number in the samples (Publication II, Figure 9). The shape of the colonies was barely observable at higher than 10^4 cfu mL^{-1} inoculation rates using 10-fold magnification but, taking into account the structure of the agar gel, it could be assumed that the colonies were also lenticular in these conditions. According to Pernodet (1997), the sizes of agar gel pores are around 350 - 400 nm in agar concentrations of 1% (w v^{-1}). When bacteria started to multiply in the solid media, they formed colonies in the pores of the agar gel overcoming the resistance of the media by breaking the bonds between the chains of polysaccharides and thus "enlarging" the pores. It could be assumed that the colonies grew lenticular-shaped as a result of the agar structure as it was easier to break the bonds between the chains and form lenticular colonies than to stretch the inter-chain bonding, and form spherical colonies.

The size of the average colony varied from 17 μm at the highest inoculation rate of 10^6 cfu mL^{-1} to 2200 μm at the inoculation rate of 10^0 cfu mL^{-1}. The sizes of the colonies, determined by microscopy, were used for the calculation of the numbers of cells in the colonies at the end of the growth (Figure 14). The standard deviation of colony sizes was high at low inoculation rates of 10^0-10^1 cfu mL^{-1} and vice versa. A limited number of colonies (less than 10-20) could have been orientated in solid samples differently, and this led to the differences in the sizes and the distances between the colonies, which in turn also influenced the size of the colonies. At higher inoculation rates, the colonies were evenly distributed throughout the matrix in the sample and thus their dimensions were less variable. The same circumstances determined the variability of the power-time curves. In higher inoculation rates (number of colonies higher than 10^2), the differences "averaged out" and in these conditions the reproducibility of the microcalorimetric method was (very) high: $\sigma = 0.37$ μW.

4.2.3 The model of growth of an average individual colony of *L. lactis* in agarose gel

A model of the changes in the specific growth rate of individual (average) colonies of different sizes using data on maximum specific growth rates μ_{max} (Figure 13) and on the numbers of bacteria, both at the beginning and at the end of the exponential growth phase, in different growth conditions (Figure 14) was

developed (see Figure 18). The average colony grew exponentially until the number of bacteria reached approximately 10^5, and the radius of the average colony was 46 µm. The growth rate of bigger colonies, containing 10^5-10^8 cells, decreased due to the accumulation of lactic acid, leading to the lowering of pH. The growth of bacteria inside the colonies was restricted and the size of the colony increased due to the division of the bacteria on the surface of the colony (McKay et al., 1997). The power-time curves of the growth of bacteria in big colonies at the inoculation rates of 10^0 and 10^1 cfu mL^{-1} were observed in the microcalorimeter under conditions where the number of bacteria in a colony reached 10^4 -10^5 and lactate started inhibiting the growth. This explains the lower growth rates and heat productions at 10^0 and 10^1 cfu mL^{-1} in comparison with higher inoculation rates (Figure 14 and Figure 16).

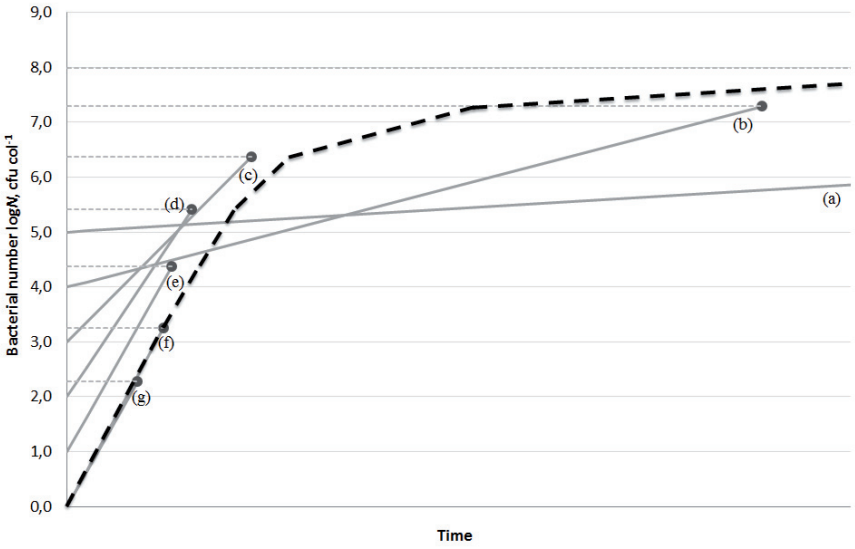


Figure 18. The growth of an averaged colony of *L. lactis* in agar gel constructed on the basis of the data obtained from the analysis of the calorimetric power-time curves. Solid lines show the μ_{max} values and the ranges of colony sizes where these values were measured: a – 10^0 cfu mL^{-1}; b – 10^1 cfu mL^{-1} c – 10^2 cfu mL^{-1}; d – 10^3 cfu mL^{-1}; e – 10^4 cfu mL^{-1}; f – 10^5 cfu mL^{-1}; g – 10^6 cfu mL^{-1}. The broken line is a constructed growth curve of an average colony of *L. lactis* in solid agar gel

Similar results were obtained by Malakar et al. (2002a), who showed that mass transfer limitations can be significant in the development of bacterial colonies in solid systems if the colonies contain about 10^5 or more cells. The growth of smaller colonies, containing less than 10^5 cells, in solid matrices at higher inoculation rates than 10^2 cfu mL^{-1} is similar to the growth in broth cultures: small colonies have sizes at which mass transfer limitations are not significant.

4.3 The study of the growth of *Streptococcus thermophilus* in skim milk (Publication III and unpublished data)

The developed microcalorimetric method was applied for the studies of the growth of *Streptococcus thermophilus* ST12 in different environmental conditions. During the experiments, the growth of bacteria was monitored at two different temperatures: the more appropriate for the growth of thermophilic bacteria 40°C, and the lower temperature of 35°C. Additionally, the low heat skim milk powder (LHSMP) was irradiated at a dose of 10 kGy and the growth of ST12 was then observed both in milk reconstituted from irradiated and non-irradiated low heat skim milk powders (irrRSM and RSM, respectively). All of the experiments were performed both in liquid and in renneted skim milk samples at different inoculation rates, from 10^1 cfu mL^{-1} up to 10^6 cfu mL^{-1} with 10-fold increments. The influence of all the mentioned parameters on the growth of *Streptococcus thermophilus* ST12, using the microcalorimetric method, is described in the following four subtopics.

4.3.1 Effect of the incubation temperature

The growth of *Streptococcus thermophilus* ST12 was studied at two different incubation temperatures – 40°C and 35°C – at an inoculation rate of 10^5 cfu mL^{-1} (Figure 19). The higher monitored temperature – 40°C – is more appropriate for the growth of studied thermophilic bacteria and the lower one – 35°C – could obviously negatively influence the growth of *St. thermophilus*. The power-time curves 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 was in agreement with the results reported in the literature for *Lactococcus lactis* (Juillard et al., 1995; Letort et al., 2002) 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 *St. thermophilus* was studied in the cited papers; however, Juillard et al. (1995) found that the transport of oligopeptides in these two bacteria is probably similar.

The power-time curves in RSM at both incubation temperatures retained the two-stage growth pattern, but at a lower incubation temperature both of the growth stages were shifted further in time. The maximum specific growth rates were lower in the 35°C incubation temperature in comparison with the results obtained at 40°C during both exponential phases. During the first exponential growth phase and the second exponential growth phase at 35°C, the μ_{max} reached values of 1.28±0.0 h^{-1} and 0.90±0.01 h^{-1} respectively. At the more suitable incubation temperature for the growth of *St. thermophilus* of 40°C, the μ_{max} were higher at the values of 1.85±0.05 h^{-1} and 1.18±0.00 h^{-1} respectively, during both of the previously mentioned exponential phases. According to the results of the

heat production obtained by the processing of the calorimetric growth curves, the slower growing bacterial cultures produced more heat and thus more biomass (2.27±0.02 J mL^{-1} and (5.10±0.02)*10^8 cfu mL^{-1} respectively). At the higher incubation temperature, the heat effect during the exponential phase was 2.10±0.01 J mL^{-1}, which corresponds to (4.73±0.01)*10^8 cfu mL^{-1}. The biomass amount was calculated on the basis of the heat yield coefficient Y_Q determined for the growth of *St. thermophilus* ST12 in RSM. Y_Q (J cfu^{-1}) was calculated by dividing the heat amounts produced by the end of the exponential growth phase with the number of bacteria formed by that time. The number of bacteria was determined by *outplating*. The heat yield coefficient was determined during a separate experiment at 40°C at different inoculation rates. Y_Q was equal to (4.45±0.15) *10^{-9} J cfu^{-1} for the growing *St. thermophilus* ST12 bacteria in the skim milk samples. The differences in total heat amounts produced during the bacterial growth at both incubation temperatures were not significant.

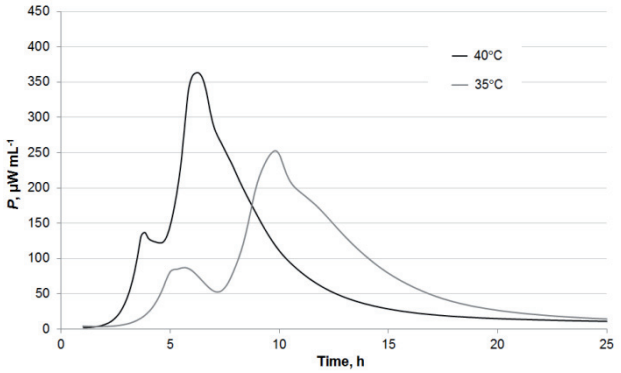


Figure 19. Calorimetric power-time curves of *St. thermophilus* ST12 in RSM at two different growth temperatures – 35°C and 40°C – at an inoculation rate of 10^5 cfu mL^{-1}

4.3.2 Effect of irradiation

The growth of *St. thermophilus* ST12 was studied in irrLHSMP at two different incubation temperatures, 40°C and 35°C, at an inoculation rate of 10^5 cfu mL^{-1} and the results were compared with those obtained in RSM (see *Section 4.3.1*) The irrLHSMP was yellowish in colour and had a pronounced off-flavour compared with LHSMP.

The power-time curves were notably different in the case of ST12 growth in RSM and irrRSM (Figure 19 and Figure 20, respectively). According to our data, 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 at 40°C. The calorimetric power-time curves of ST12 were found to be affected by the incubation temperature, and the multiphase growth curve was observed in irrRSM at 35°C.

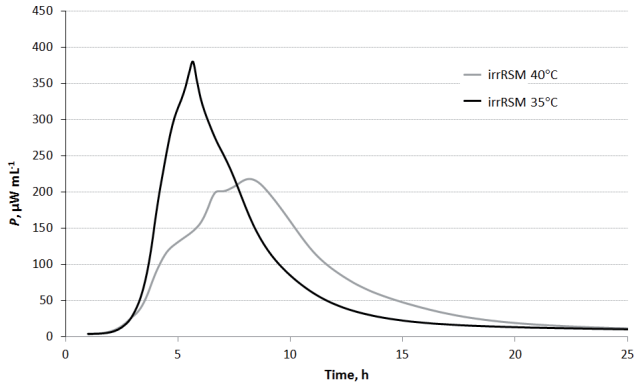


Figure 20. Calorimetric power-time curves of *Streptococcus thermophilus* ST12 in irradiated RSM at two different growth temperatures – 35°C and 40°C – at an inoculation rate of 10^5 cfu mL^{-1}

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 (Table 2). The same tendency was true for the lower 35°C incubation temperature. The heat amounts produced both at the end of exponential growth and at the end of the total growth were higher at the lower incubation temperature studied in both RSM and irrRSM (see Table 2). These results lead to the conclusion that slower growing bacterial culture at lower than the appropriate growth temperatures are connected with biomass production.

Notable differences were observed in the acidification profiles of the reconstituted milk samples studied depending on irradiation (Figure 21). The same tendency of the pH changes also occurred at the lower incubation temperature of 35°C. As shown in Figure 21, the calorimetric power-time curves provided a lot more information about the peculiarities of the growth of bacterial culture in comparison with the pH curves obtained, revealing, for example, the diauxic growth of ST12 in RSM. At the same time, the pH curves provided additional information useful for the interpretation of the calorimetric growth curves. 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). The total free amino acids (Stulova et al. 2013) were substantially different in RSM and irrRSM; this could indicate that initially the samples also differed in terms of buffering capacity. The pH of the RSM and irrRSM milk samples decreased slowly up to 4 h of fermentation and then more rapidly in the irrRSM samples. However, the pH was higher than 6.0 during the whole exponential growth phase, which was a clear indication that acidification was not the reason for the termination of the exponential growth. A notable 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, these values were also clearly higher than the pH usually observed at the end of the growth of the *St. thermophilus:* 4.0-4.2 (Zanatta & Basso, 1992).

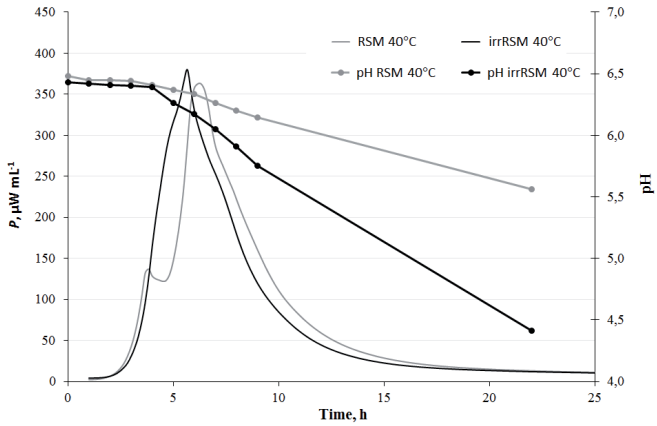


Figure 21. Calorimetric power-time curves and the pH curves of *St. thermophilus* ST12 in non-irradiated and irradiated RSM at 40˚C, at an inoculation rate of 10^5 cfu mL^{-1}

Table 2. Calculated parameters describing the growth of the *St. thermophilus* in RSM and irrRSM at two different incubation temperatures, 40°C and 35°C, at an inoculation rate of 10^5 cfu mL^{-1}

Milk	Temp.	1^{st} μ_{max}, h^{-1}	2^{st} μ_{max}, h^{-1}	Q_{exp}, J mL^{-1}	N_{exp}, $*10^8$ cfu mL^{-1}	Q_{tot}, J mL^{-1}
RSM	40°C	1.85	1.18	2.10	4.73	7.21
	35°C	1.28	0.90	2.27	5.10	7.55
irrRSM	40°C	1.68	-	2.09	4.70	6.94
	35°C	1.27	0.38	2.77	6.23	7.30

- no second peak

4.3.3 Effect of gel structure

The calorimetric power-time curves of the growth of ST12 in liquid, as well as renneted RSM and irrRSM, at an inoculation rate of 10^5 cfu ml^{-1} at both incubation temperatures (40°C and 35°C) are presented in Figure 22. The data that was obtained by processing the calorimetric curves is presented in Table 3. There were notable differences in the profiles of the power-time curves of all the renneted RSM samples. The power-time curves in the solid state samples obtained the two-stage growth pattern even in the case of irrRSM+rennet at 40°C. It is interesting to note that the μ_{max} decreased in all renneted RSM samples, starting from a certain number of the bacteria in the samples (this is marked with a circle in Figure 22) – the point on the calorimetric curves at which the growth curve obtained in liquid RSM samples started to diverge from the growth curve obtained in the samples of RSM with an addition of rennet. The number of bacteria (calculated with Y_Q) in the described point was higher when the less negative treatment was applied to the samples: lower than appropriate incubation temperature, and the irradiation of skim milk powder.

The numbers of bacteria in non-irradiated RSM at 40°C, irradiated RSM at 40°C, non-irradiated RSM at 35°C and irradiated RSM at 35°C were $7.43*10^7$ cfu mL^{-1}, $4.30*10^7$ cfu mL^{-1}, $3.95*10^7$ cfu mL^{-1} and $1.96*10^7$ cfu mL^{-1} respectively. The negative effect on the growth of *St. thermophilus* in samples with an addition of rennet was more pronounced when decreasing the temperature, in comparison with the irradiation effect.

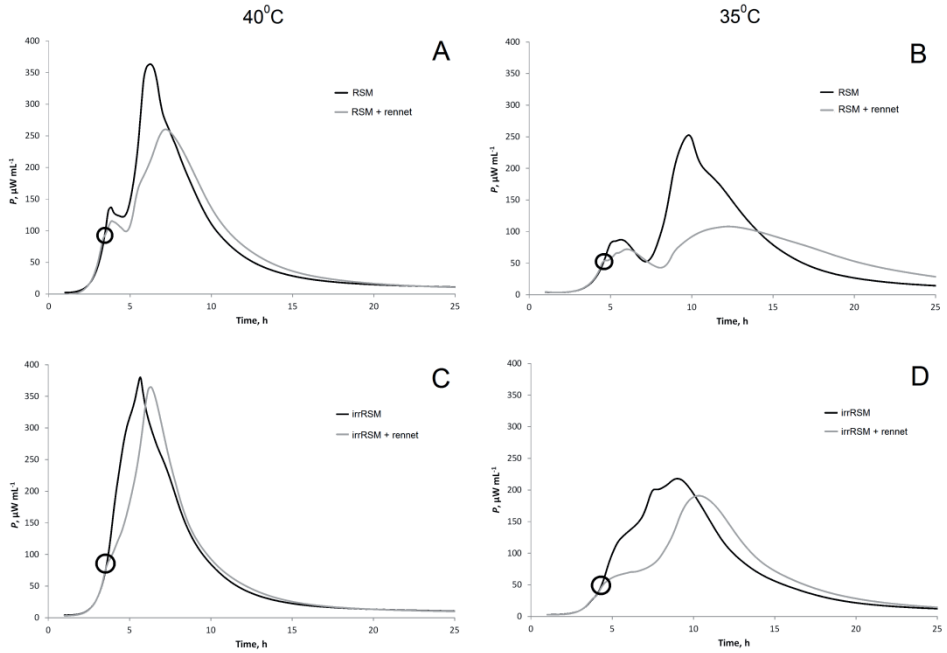


Figure 22. Power-time curves describing the growth of *St. thermophilus* ST12 at a 10^5 cfu mL^{-1} inoculation rate and under different environmental conditions: (A) non-irradiated RSM at 40°C; (B) non-irradiated RSM at 35°C; (C) irradiated RSM at 40°C; (B) irradiated RSM at 35°C

Table 3. Calculated parameters describing the growth of the *St. thermophilus* in RSM and irrRSM with an addition of rennet at two different incubation temperatures, 40°C and 35°C, at an inoculation rate of 10^5 cfu mL^{-1}

Milk	Temp.	1^{st} μ_{max}, h^{-1}	2^{st} μ_{max}, h^{-1}	Q_{exp}, J mL^{-1}	N_{exp}, $*10^8$ cfu mL^{-1}	Q_{tot}, J mL^{-1}
RSM + rennet	40°C	2.11	1.06	2.36	5.30	6.92
	35°C	1.27	0.81	2.22	4.99	6.74
irrRSM + rennet	40°C	1.73	0.56	2.07	4.66	6.76
	35°C	1.40	0.44	2.36	5.30	6.88

Comparing Table 2 and Table 3, it is clear that the formation of the gelled structure negatively influenced the total heat production, whereas the maximum growth rate and the heat production during both exponential growth phases were not greatly influenced. However, the lag phase between the first and the second exponential growth phases was more pronounced and lengthy in the solid RSM samples, which leads to the conclusion that the adaptation of bacterial culture to a new substrate, or in other words a switch from one substrate to another, is more complicated during the solid state growth. Diffusion limitations may provide one possible reason for this.

4.3.4 Effect of the inoculation rate

The calorimetric power-time curves of ST12 were obtained in RSM and irrRSM at different inoculation rates in the range of 10^6 to 10^1 cfu mL^{-1} (Figure 23).

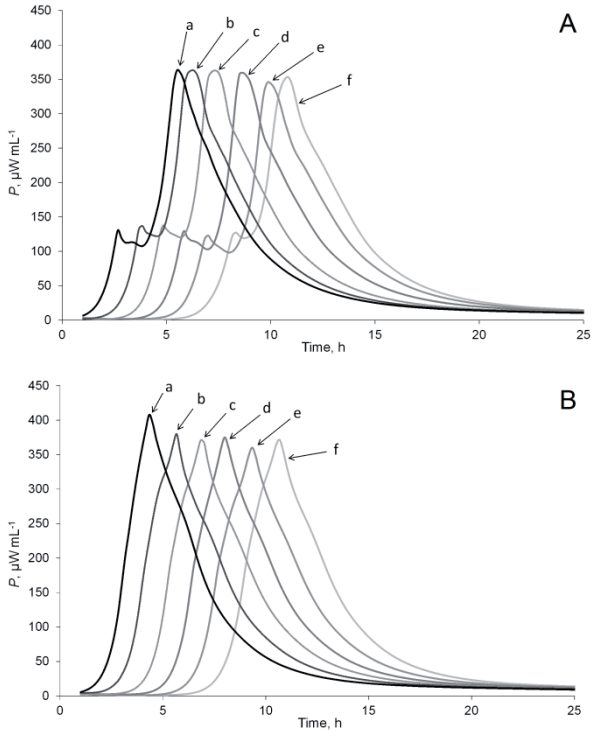


Figure 23. Calorimetric power-time curves of *St. thermophilus* ST12 in non-irradiated RSM (A) and irradiated RSM (B) at 40°C at different inoculation rates: a) 10^6 cfu mL^{-1}; b) 10^5 cfu mL^{-1}; c) 10^4 cfu mL^{-1}; d) 10^3 cfu mL^{-1}; e) 10^2 cfu mL^{-1} and f) 10^1 cfu mL^{-1}

The diauxic growth of ST12 at an inoculation rate of 10^5 cfu mL^{-1} observed in RSM and the disappearance of diauxie in irrRSM were seen at all inoculation rates studied. The power-time curves were strikingly similar at all inoculation rates studied. This was not the case in our earlier studies with LAB in

transparent media (Publication II), and this cannot be considered a trivial difference. It can be explained if we assume that the reconstituted milk had higher buffering capacity in comparison with the solid state matrices of agar.

As expected from the similarity of the power-time curves, and as shown in the data presented in Table 4, 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 differed in the two media, with significantly lower values in irrRSM than in RSM.

The amount of heat produced during the exponential phases at inoculation rates from 10^6 to 10^2 cfu mL^{-1} was roughly the same in RSM and irrRSM, which means that the numbers of bacteria grown during the fast growth phases should have been rather similar in both media studied. However, these numbers were statistically different, according to the data in Table 4. The amount of heat produced during the whole studied growth period of the bacteria was unrelated to the inoculation rate and was almost the same in RSM and irrRSM. 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 $Y_Q = (4.45±0.15)*10^{-9}$ J cfu^{-1} the total numbers of bacteria (N_{tot}) grown during the whole process were calculated from Q_{tot} and were $(1.62±0.02)*10^9$ and $(1.57±0.02)*10^9$ cfu mL^{-1} in RSM and irrRSM, respectively.

To elucidate the mechanisms terminating the exponential growth and the growth of the cultures, additional measurements of carbohydrates and organic acids were carried out (Figure 24). According to the results obtained, practically all of the lactose was hydrolysed by the end of the exponential phases in RSM, except for at the inoculation rate of 10^1 cfu mL^{-1}. Glucose and galactose are formed from lactose during fermentation, glucose is consumed and lactate is synthesized simultaneously by bacteria. The mentioned balance was not maintained during the bacterial growth in our experiments: there were less than the expected amounts of galactose present. The error levels are rather small, indicating that the deviations from the expected amounts of galactose were not caused by measurement errors. The noticed imbalance can be explained by the fact that not all of the possible products involved were measured – polysaccharides in particular - and also not all growth by-products were determined. 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. The patterns of the 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 hydrolysed during the active growth phase of the bacteria in irrRSM, less than 15 mmol L^{-1} of the glucose and of galactose was utilized by the bacteria, and about 10 mmol L^{-1} of lactic acid was formed. These data indicate that irradiation of the skim milk powder led to the deterioration of the lactose hydrolysis processes by the bacteria. As the bacteria were obviously not irradiated, the deterioration may be explained by the changes in the state of the lactose (Adachi, 1962) in irrRSM.

Table 4. *St. thermophilus* ST12 growth parameters in RSM and in irrRSM at 40˚C. Means[a] of maximum specific growth rate (μ_{max}, h^{-1}) in the first and second exponential growth phases, the heat produced during the exponential phase (Q_{exp}, J mL^{-1}), the number of bacteria at the end of the exponential growth phase (N_{exp}, cfu mL^{-1}), the total heat produced during the whole fermentation (Q_{tot}, J mL^{-1}) and the lag phase duration (λ, h) obtained from the microcalorimetric curves at different inoculation rates N_0 (cfu mL^{-1})

Milk	N_0, 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}	λ, h
		1st exp. ph.	2nd exp. ph				
RSM	10^6	1.88^{aA}	1.05^{aA}	2.00^{aA}	7.12^{aA}	4.50^{aA}	0.02^{aA}
	10^5	1.85^{aA}	1.18^{bA}	2.10^{bA}	7.21^{aA}	4.73^{bA}	0.98^{bA}
	10^4	1.91^{aA}	1.17^{bA}	2.11^{bA}	7.12^{aA}	4.74^{bA}	2.08^{cA}
	10^3	1.85^{aA}	1.14^{bA}	1.97^{aA}	7.24^{aA}	4.42^{aA}	3.05^{dA}
	10^2	1.83^{aA}	1.13^{bA}	2.00^{aA}	7.23^{aA}	4.50^{aA}	4.07^{eA}
	10^1	1.61^{bA}	1.01^{cA}	2.26^{cA}	7.36^{aA}	5.08^{cA}	4.98^{fA}
irrRSM	10^6	1.72^{abB}	-	1.82^{aB}	6.83^{aA}	4.09^{aB}	0.13^{aA}
	10^5	1.68^{abB}	-	2.09^{bcA}	6.94^{aA}	4.71^{bcA}	1.00^{bA}
	10^4	1.78^{aB}	-	2.10^{bcA}	6.96^{aB}	4.71^{bcA}	2.29^{cB}
	10^3	1.72^{abB}	-	2.05^{cA}	6.98^{aB}	4.60^{bA}	3.38^{dB}
	10^2	1.66^{bB}	-	2.17^{bdB}	7.00^{aA}	4.88^{cB}	4.43^{eA}
	10^1	1.46^{cAB}	-	2.25^{dA}	7.14^{aA}	5.05^{cA}	5.47^{fB}

[a] Different superscript lower case letters (a, b, c, d, e, f) mark significant differences at $p<0.05$ (ANOVA, followed by Fischer´s LSD test) between the data at different inoculation rates. Different superscript capital letters (A, B) denote significant differences between the RSM and irrRSM samples at the same inoculation rates

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

It should be noted that the heat effect (Q_{exp}) and numbers of grown bacteria (N_{exp}) were 10-20% higher at the inoculation rate of 10^1 than those at the other inoculation rates. 10-15% lower values of μ_{max} at the lowest inoculation rate of 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. The lactic acid concentration in the cultures at 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 in 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 reached that only the highest lactic acid concentrations at the lowest inoculation rate used (10^1 cfu mL^{-1}) influenced the growth characteristics of the bacteria in the range of 10^5 – 10^9 bacteria per mL.

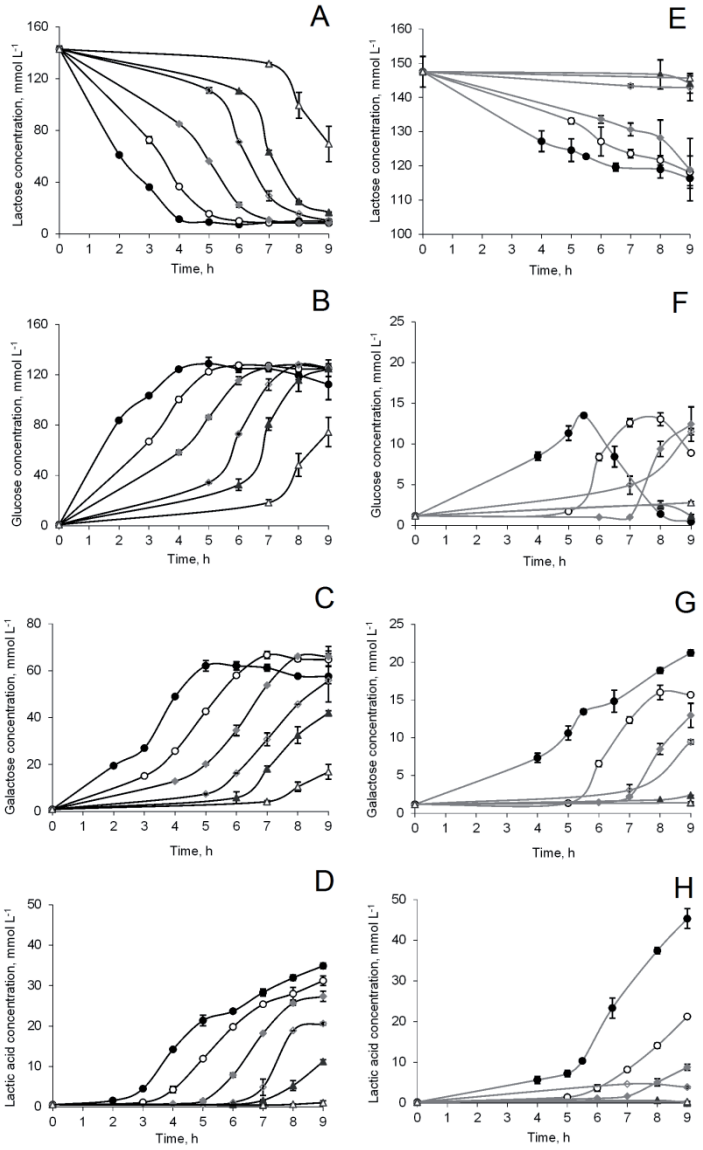


Figure 24. Comparison of lactose (A, E), glucose (B, F), galactose (C, G) and lactic acid (D, H) profiles during the growth of *St. thermophilus* ST12 in RSM (A, B, C, D) and irrRSM (E, F, G, H) at different inoculation rates (cfu mL^{-1}): 10^6 (●), 10^5 (○), 10^4 (◆), 10^3 (◇), 10^2 (▲), 10^1 (△)

The low inoculation rate effects can be explained assuming that the diffusion processes of the substrates, and the bacteria in the milk were most probably not as intensive as in the other liquids studied (solutions of gelatine and agar). The same reasoning can be used to explain the lower μ_{max} values in irrRSM than in RSM. The effects of irradiation described in Section 4.3.2 could have led to the somewhat slower diffusion processes in the irrRSM in comparison with RSM.

The growth of *St. thermophilus* ST12 was also monitored in renneted RSM and irrRSM at different inoculation rates in the range of 10^6 to 10^1 cfu mL^{-1} with 10-fold increments. The experiments were performed at different incubation temperatures: 35°C and 40°C. The calorimetric power-time curves obtained during the experiments are presented in Figure 25. The power-time curves recorded were processed and the numerical results are shown in Table 5.

The calorimetric curves obtained during the experiments with the renneted samples (Figure 25) differed a lot from the curves obtained in the RSM and irrRSM samples without an addition of rennet (Figure 23). The peaks of the power-time curves mostly became lower and more prolonged with the inoculation rate decrease from 10^6 to 10^1 cfu mL^{-1}. A similar tendency was observed during our previous studies of agar gels (Publication II). The results (Table 5) showed that the values of P_{max} (the maximum power value obtained at the highest peak of the calorimetric curve) measured in renneted RSM and irrRSM at different inoculation rates differed significantly from each other ($p<0.05$) at both of the incubation temperatures studied. This was not the case with our previous study (Publication III), where the P_{max} of the power-time curves of ST12 in RSM at different inoculation rates were similar. However, the only difference was observed in non-irradiated renneted RSM at 35°C, where the height of the peaks was almost the same regardless of the inoculation rate. The exact reason for this phenomenon can not be explained at the moment, but the answer should be searched for, taking into account the non-specific for the growth of *St. thermophilus* incubation temperature.

The calorimetric power-time curves of growth of the ST12 in renneted RSM and irrRSM at 40°C are presented in Figure 25 (A) and (C), respectively. According to our data, the first exponential growth phase of ST12 in renneted RSM was shorter and the calculated μ_{max} was higher than in the second phase. As shown 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 10^3 cfu mL^{-1}, but different in different media. The μ_{max1} of the bacterial culture at 10^2 – 10^1 cfu mL^{-1} was considerably lower. The maximum specific growth rate of the second exponential phase decreased with the inoculation rate decrease in all of the conditions studied. The same tendency in the shapes of power-time curves and the changes in maximum specific growth rates depending on initial bacterial number were observed during the fermentation of ST12 in renneted RSM and irrRSM at 35°C (Figure 25 (B) and (D)).

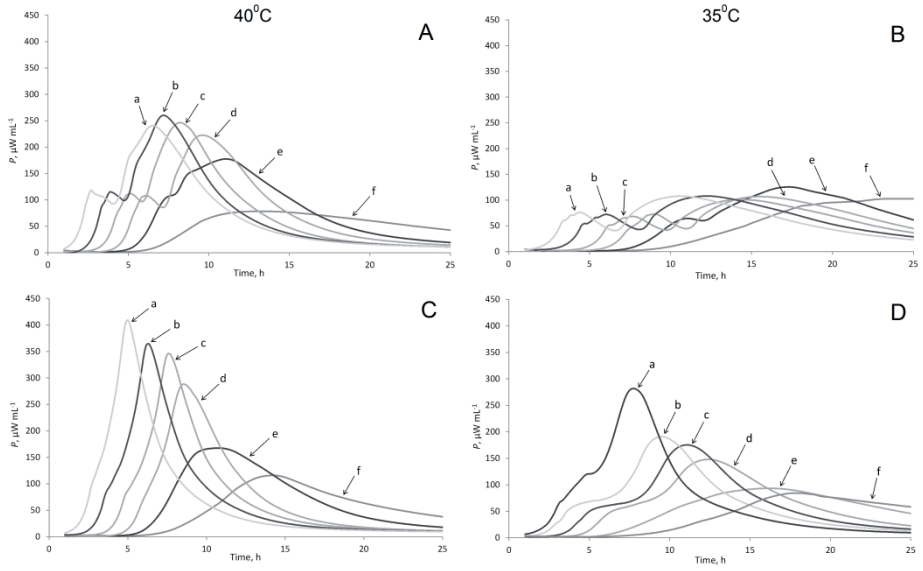


Figure 25. Calorimetric power-time curves of *St. thermophilus* ST12 in renneted milk curd at different inoculation rates (a) 10^6 cfu mL^{-1}; b) 10^5 cfu mL^{-1}; c) 10^4 cfu mL^{-1}; d) 10^3 cfu mL^{-1}; e) 10^2 cfu mL^{-1} and f) 10^1 cfu mL^{-1}) and different environmental conditions: (A) non-irradiated RSM at 40°C; (B) non-irradiated RSM at 35°C; (C) irradiated RSM at 40°C; (D) irradiated RSM at 35°C

The lower recorded growth rates μ_{max} can be explained by taking into account the fact that in the renneted samples bacteria grew in separate colonies, as in the agar gels. At lower inoculation rates, we monitored the growth of the bigger colonies growing in conditions of higher concentration of the lactate synthesized locally within the colonies and to the bulk media during the latent period of measurements of the heat flow (taking into account the level of sensitivity of the instrument – 10^5 cfu mL^{-1} – Publication II). Lactate accumulated within the colonies and thus inhibited the bacterial growth more efficiently than lactate equally distributed in liquid cultures. Besides the local accumulation of toxic products that led to local pH decrease, the diffusion limitations of the substrate through the matrix and within the colony could have negatively influenced the growth rate. A slightly longer lag phase (λ) was observed in renneted RSM samples in comparison with liquids, and the longest lag phase was observed at the lowest inoculation rate of 10^1 cfu mL^{-1} in both media studied. Fermentation at a lower temperature led to the prolongation of the lag phase of the growth (Table 5). There was no strict dependence of heat produced during the exponential growth phase on inoculation rate (except the lowest one studied, 10^1 cfu mL^{-1}) and the incubation temperature in renneted milk samples. The number of bacteria produced by the end of the exponential phase could be calculated on the basis of Q_{exp} (J mL^{-1}) using Y_Q. By dividing the number of bacteria obtained by the inoculation rate, the number of bacteria per colony was calculated.

Table 5. Parameters describing *St. thermophilus* ST12 growth in renneted RSM and in renneted irrRSM at different incubation temperatures, 40°C and 35°C: means[a] of the maximum specific growth rate (μ_{max}, h^{-1}), lag phase duration (λ), maximum heat flow (P_{max}, µW), the heat produced during the exponential phase (Q_{exp}, J mL^{-1}), and the total heat produced (Q_{tot}, J mL^{-1}) at different inoculation rates N_0 (cfu mL^{-1})

	Milk	N_0, cfu mL^{-1}	μ_{max}, h^{-1} 1st exp.ph	μ_{max}, h^{-1} 2nd exp.ph	Q_{exp}, J mL^{-1}	Q_{tot}, J mL^{-1}	P_{max}, µW	λ, h
40°C	RSM	10^6	1.89^{abA}	1.00^{aA}	2.41^{aA}	7.02^{aA}	240.7^{abA}	0.04^{aA}
		10^5	2.11^{aA}	1.06^{aA}	2.36^{aA}	6.92^{aA}	260.3^{aA}	1.15^{bA}
		10^4	1.89^{abA}	0.94^{aA}	2.26^{aA}	6.89^{aA}	247.3^{aA}	1.96^{cA}
		10^3	1.88^{abA}	0.93^{aA}	2.28^{aA}	7.12^{aA}	222.7^{bA}	2.97^{dA}
		10^2	1.67^{bA}	0.49^{bA}	2.45^{aA}	7.35^{aA}	177.9^{cA}	3.87^{eA}
		10^1	1.12^{cA}	-	1.37^{bA}	6.47^{aA}	78.8^{dA}	4.64^{fA}
	irrRSM	10^6	1.70^{aB}	0.65^{aB}	2.08^{aB}	6.88^{aAB}	409.7^{aB}	0.05^{aA}
		10^5	1.73^{aAB}	0.56^{bcB}	2.07^{aB}	6.76^{bA}	364.5^{bB}	1.06^{bA}
		10^4	1.75^{aB}	0.55^{bB}	2.06^{aA}	6.78^{bA}	345.6^{bB}	2.29^{cB}
		10^3	1.82^{aA}	0.61^{acB}	1.79^{bB}	6.58^{bB}	288.3^{cB}	3.51^{dB}
		10^2	1.42^{bB}	-	1.86^{bA}	6.62^{bA}	167.7^{dB}	4.26^{eB}
		10^1	0.84^{cB}	-	1.59^{cA}	6.06^{bA}	116.0^{eA}	4.69^{fA}
35°C	RSM	10^6	1.37^{aC}	0.88^{aC}	2.15^{aB}	6.53^{aB}	107.8^{aC}	0.34^{aB}
		10^5	1.27^{bB}	0.81^{bC}	2.22^{aA}	6.74^{aA}	108.1^{aC}	1.45^{bA}
		10^4	1.32^{cC}	0.67^{cBC}	2.28^{aA}	6.73^{aA}	101.1^{aC}	3.20^{cC}
		10^3	1.27^{bB}	0.62^{cB}	2.34^{aC}	6.91^{aAC}	107.01^{aC}	4.36^{dC}
		10^2	1.09^{dC}	0.32^{dB}	2.57^{aA}	7.84^{bA}	125.81^{aC}	5.72^{eC}
		10^1	0.68^{eC}	-	3.89^{aB}	8.09^{bB}	104.54^{aA}	6.56^{fB}
	irrRSM	10^6	1.42^{aC}	0.49^{aD}	2.74^{aC}	7.25^{aA}	281.76^{aD}	0.35^{aB}
		10^5	1.40^{aB}	0.44^{bD}	2.36^{bA}	6.88^{aA}	190.95^{bD}	1.42^{bA}
		10^4	1.41^{aD}	0.43^{bC}	2.31^{bA}	6.78^{aA}	175.36^{bD}	2.73^{cD}
		10^3	1.37^{aB}	0.34^{cC}	2.22^{bD}	6.89^{aC}	148.09^{bD}	3.94^{dD}
		10^2	1.00^{bC}	-	2.15^{bcA}	6.43^{aA}	93.40^{cD}	5.17^{eD}
		10^1	0.46^{cD}	-	1.89^{cA}	6.78^{aA}	84.49^{cA}	6.27^{fC}

[a] Different superscript lower case letters (a, b, c, d, e, f) mark significant differences at $p<0.05$ (ANOVA, followed by Fischer´s LSD test) between the data of the samples at different inoculation rates. Different superscript capital letters (A, B, C, D) denote significant differences between the inoculation rates of different environmental conditions

On the basis of the calculation method used in Publication II, the radii of the average colonies at different inoculation rates could be obtained. With the inoculation rate increase from 10^1 cfu mL^{-1} up to 10^6 cfu mL^{-1} the radii of the colonies formed decreased from an average of 206.7±33.7 µm up to 4.5±0.2 µm at the end of the exponential phase in all of the renneted samples. The radii of

the colonies did not significantly depend on the irradiation effect and incubation temperature except when there were up to 10 colonies per mL in the sample (10^1 cfu mL^{-1}). The amounts of heat produced during the total growth (Q_{tot}) at all inoculation rates were practically the same in RSM and irrRSM at both incubation temperatures, even at the lowest inoculation rate studied. This indicates that the numbers of bacteria formed during the fermentation were almost the same in both media. The radii of the colonies formed increased up to 6.7±0.1 μm at the highest inoculation rate studied (10^6 cfu mL^{-1}) and up to 308.3±12.9 μm at the lowest inocula concentration (10^1 cfu mL^{-1}).

Notable differences were observed in the acidification profiles during the growth of *St. thermophilus* in milk. The initial pH of the RSM (6.48±0.01) was slightly higher than that of the irrRSM (6.43±0.01). It should be noted that the acidification in irradiated samples depended not only on temperature, but also on the growth media used, renneted or liquid RSM. In the non-irradiated milk, the final pH of the renneted samples was the same as in the liquid samples, and a lower pH at the values of 5.67±0.08 was obtained at the higher incubation temperature. The final pH value, equal for all initial bacterial numbers studied at the lower temperature, was 6.34±0.09. A different situation was observed in irradiated RSM samples where the final pH depended on the inoculation rate, obtaining the highest value of 5.37±0.01 in the lowest inoculum at 40°C and the value of 6.20±0.01 at 35°C. With the inoculation rate increase, the final pH decreased. This phenomenon can be explained by taking into consideration the amounts of lactic acid produced during the same time of growth. At the lower incubation temperature, not only considerably lower amounts of lactate were produced, but also lower amounts of lactose were hydrolysed and lower amounts of glucose and galactose were produced in comparison with the higher incubation temperature. The profiles of glucose consumption were more significantly dependent on the inoculation rate in comparison with the curves obtained at 40°C, revealing slower substrate consumption rates as well.

The addition of rennet did not significantly influence the profiles of sugar metabolism in non-irradiated samples and they were comparable to those obtained in liquid milk samples (Figure 24). The only interesting feature is that the glucose, galactose and lactate production curves started to deviate from that obtained in liquid RSM from the moment that almost coincided with the time on the calorimetric curves when the curves obtained in liquid and solid milk samples started to “deviate” from each other also (Figure 22).

All of the results obtained in the case of bacterial growth in the solid irrRSM and RSM samples should be analysed and explained by taking into account the fact that bacteria grew in colonies in renneted milk samples and thus all the bacteria could have been in different conditions, influenced by the diffusion limitation of substrates and products within the colonies and through the matrix. The colonial growth in the opaque solid states should be more thoroughly analysed and explained in future studies.

4.4 The study of the growth of *Enterococcus faecium* in dry dog food kibbles (Publication IV)

The power-time curves of the growth of *E. faecium* NCIMB10415 in different formulations of dog food kibbles (Adult Chicken (AC), Adult Salmon (AS) and Senior Chicken (SC)) in dry samples and in moisturised samples at two different pH values are presented in Figure 26. All three formulations of ProBiotic LIVE dog food (AC, AS and SC), enriched with probiotic bacteria of *Enterococcus faecium* (NCIMB10415), had the same initial average bacterial count (2.86±0.53)*10^6 cfu g^{-1} confirmed by *outplating* of the samples on a PCA. This confirms the claim by the producer of the average *E. faecium* load of 10^6 cfu per gram in the product.

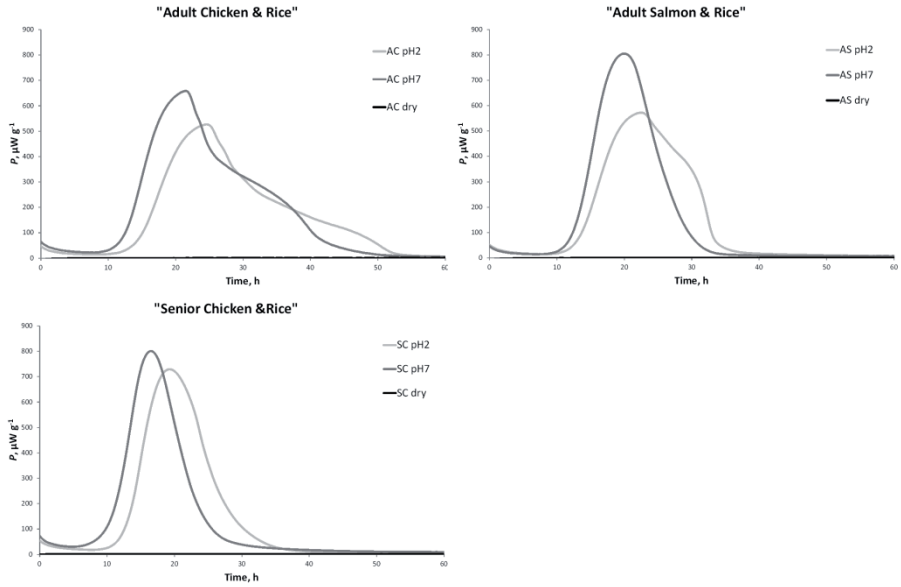


Figure 26. Power-time curves of the growth of probiotic bacteria in three different dog food formulations: dry and moisturised samples (AC, AS and SC) at two different pH values (pH=2 and pH=7)

At first sight, the calorimetric curves of the bacterial growth at the lower pH value pH=2, which is the pH level in the stomach of dogs (Akimoto et al., 2000), differ a lot in comparison with the growth curves obtained at neutral pH. The power-time curves were processed and the data on the main bacterial growth describing parameters are presented in Table 6.

According to Table 6, the maximal specific growth rate (μ_{max}, h^{-1}) of the *E. faecium* bacteria was practically the same at low and neutral pH levels (0.52±0.06 h^{-1} and 0.54±0.05 h^{-1} respectively). A slight increase in the lag phase duration in bacterial growth at pH=2 in comparison with the growth at pH=7 was observed ((4.9±0.2 hours and 3.9±0.7 hours, respectively). The prolongation of the lag phase at the lower studied pH values can be explained by the acidic

stress effect on the probiotic bacteria (Ölmez and Aran, 2005).

Table 6. Parameters describing bacterial growth of three dog food formulations (AC, AS, SC) at two different pH levels (pH=2 and pH=7), obtained from the processed power-time curves

		λ, h	μ_{max}, h^{-1}	$Q_{exp,}$ J g^{-1}	$N_{exp,}$ cfu g^{-1}	$Q_{tot,}$ J g^{-1}	$N_{tot,}$ cfu g^{-1}
pH 2	AC	4.94	0.45	14.28	$5.53*10^{9}$	37.09	$1.44*10^{10}$
	AS	5.08	0.53	13.89	$5.38*10^{9}$	32.18	$1.25*10^{10}$
	SC	4.68	0.58	12.85	$4.97*10^{9}$	32.37	$1.25*10^{10}$
pH 7	AC	3.54	0.50	17.24	$6.67*10^{9}$	41.62	$1.61*10^{10}$
	AS	4.73	0.60	15.52	$6.01*10^{9}$	34.45	$1.33*10^{10}$
	SC	3.42	0.51	13.21	$5.11*10^{9}$	31.68	$1.23*10^{10}$

The average heat produced during the exponential phase (Q_{exp}, J g^{-1}) at the neutral pH was 15.33±0.90 J g^{-1} and 13.67±0.74 J g^{-1} in the acidic pH condition. Using the Y_Q value $2.58±0.44*10^{-9}$ J cfu^{-1} determined for *Lactococcus lactis* IL1403 (Publication II), the number of bacteria grown during the exponential growth was calculated (Table 6). The estimates of biomass obtained can be considered reliable, as the values of Y_Q were practically the same in different studies: $5.53*10^{-8}$ J cfu^{-1} for soil samples (Kimura & Takahashi, 1985), and $4.22*10^{-9}$ J cfu^{-1} and $3.4*10^{-8}$ J cfu^{-1} for *Staphylococcus aureus* and *E. coli,* respectively (Bayne-Jones & Rhees, 1929). The total heat produced during the entire period of thermodynamic activity (Q_{tot}, J g^{-1}) did not depend on the environmental pH. The numbers of bacteria grown during the exponential growth phase (N_{exp}, cfu g^{-1}) and bacteria grown during the complete growth (N_{tot}, cfu g^{-1}) achieved the same levels of 10^{9} and 10^{10} cfu g^{-1}, respectively, at both studied pH values.

The *Enterococcus faecium* bacteria present in the dog food of ProBiotic LIVE formulations showed strong metabolic activity in the case of the addition of MilliQ with low acidic pH value.

5 CONCLUSIONS

A microcalorimetric standardized complex method of serial dilutions for the study of bacterial growth was developed and successfully implemented in various environmental conditions and media. The data obtained showed that microcalorimetry is a prospective and sensitive method and its use, together with other relevant measurements, is a powerful tool in studying quantitative detailed peculiarities of the growth of anaerobic bacteria in solid and opaque media, and even in such complicated matrices as extruded feed or food products.

The main results of the study are as follows:

- experimental procedures for serial dilutions were developed and implemented to assure that serial dilutions lead to the desired inoculation rates; furthermore, the growth of the colonies of different sizes was thoroughly studied
- calculation methods for the analysis of power-time curves obtained were developed and validated
- the level of sensitivity of the instrument was determined in terms of the number of bacteria produced, which turned out to be 10^5 cfu mL^{-1}, and this was used in the interpretation and description of colonial growth
- the heat yield coefficient Y_Q values were determined for the studied bacterial species and used for the calculation of bacterial numbers produced at different times
- complex experimental procedures, in addition to calorimetric power-time curves, including parallel measurements of sugars, metabolites, amino acids etc., were developed and validated in different liquid and solid-state media
- microscopy was used for the study of colony growth in transparent solid-state media, and the change in the number of bacteria in individual colonies was evaluated using *Kepler*'s conjecture as the reference mode of the packing of spherical cells; the data obtained by microscopy was in good agreement with the results of the calorimetric measurements

In detail, the conclusions from this dissertation can be divided into three sections related to the studied bacterial species:

I. Conclusions reached for the growth of *Lactococcus lactis* IL1403

a. μ_{max} does not depend on initial glucose concentration, but notably depends on inoculation rate, obtaining smaller values at lower inocula concentrations both in liquids and solids. The lower μ_{max} values at lower inoculation rates in agar gels were due to local lactate accumulation within the colonies and thus more efficient inhibition of bacterial growth.

b. The decrease in pH was the reason for the termination of the exponential growth of the bacterial cultures.

c. At low glucose concentration (2 g L^{-1}), the growth was stopped as the substrate was totally consumed. At higher than 3 g L^{-1} glucose concentrations in liquid cultures, the growth was stopped because of critically low pH (pH = 4.0) and the accumulation of lactate in the environment. In agar gels the growth was stopped at higher bulk pH = 4.2 at the end of the cultivation presumably due to the lactic acid diffusion limitations within the colonies.

d. The metabolism of the bacteria was changed to more efficient heterofermentative "mode" in solid-state matrices in comparison with the bacterial growth in broth.

e. The length of the lag phase increased with the decrease in inoculation rate and in the changing of the environment from a liquid to solid state.

f. A model of the changes in the specific rate of average colony growth from one cell to approximately10^8 cells was developed.

II. Conclusions reached for the growth of *Streptococcus thermophilus* ST12

a. Fermentation at the lower temperature and the addition of rennet, as well as the decrease in inoculation rate, led to the prolongation of the lag phase.

b. The growth of *St. thermophilus* ST12 in irrRSM resulted in differences in the acidification profile and sugar metabolism, and substantial differences in the fermentation curve patterns in comparison with the data obtained in the RSM samples.

c. In all of the renneted RSM samples, μ_{max} decreased, starting from a certain number of the bacteria in the samples, in comparison with the liquid RSM samples.

d. The power-time curves were similar at all inoculation rates studied in the liquid RSM due to the high buffer capacity of the milk, but became lower and more prolonged with the inoculation rate decrease from 10^6 to 10^1 cfu mL^{-1} in the renneted milk samples.

III. A conclusion reached for the growth of *E. faecium* NCIMB10145

a. The microcalorimetric method was successfully implemented in the observation of the growth of probiotic bacteria in extruded food and feed products.

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.

Akimoto M, Nagahata N, Furuya A, Fukushima K, Higuchi S, Suwa T. (2000) Gastric pH profiles of beagle dogs and their use as an alternative to human testing. *European Journal of Pharmaceutics and Biopharmaceutics* 49, 99-102.

Alklint C, Wadsö L, Sjöholm I, (2005) Accelerated storage and isothermal microcalorimetry as methods of predicting carrot juice shelf-life. *Journal of the Science of Food and Agriculture* 85, 281-285.

Andersen AZ, Carvalho AL, Neves AR, Santos H, Kummer U, Olsen LF. (2009) The metabolic pH response in *Lactococcus lactis*: an integrative experimental and modelling approach. *Computational Biology and Chemistry,* 33, 71-83.

Antwi M, Geeraerd AH, Vereecken KM, Jenne R, Bernaerts K, Van Impe JF. (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.

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.

Bayne-Jones S, Rhees HS. (1929) Relationship of heat pro-duction to phases of growth of bacteria. *Journal of Bacteriology* 17, 123–140.

Belaich JP, Belaich A, Simonpietri P. (1972) Uncoupling in bacterial growth: effect of pantothenate starvation on growth of *Zymomonas mobilis*. *Journal of General Microbiology* 70, 179-185.

Beezer AE, (2001) An outline of new calculation methods for the determination of both thermodynamic and kinetic parameters from isothermal heat conduction microcalorimeter. *Thermochimica Acta* 380, 205-208.

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

Boe I, Lovrien R (1990) Cell counting and carbon utilization velocities via microbial calorimetry. *Biotechnology and Bioengineering* 35, 1-7.

Braissant O, Wirz D, Goepfert B, Daniels AU, (2010a) "The heat is on": Rapid microcalorimetric detection of mycobacteria in culture. *Tuberculosis* 90, 57-59.

Braissant O, Wirz D, Goepfert B, Daniels AU. (2010b) Use of isothermal microcalorimetry to monitor microbial activities, *FEMS Microbiology Letters* 303 1-8.

Carlson DG, Griffin JM. (1992) Dog Owner's Home Veterinary Handbook

Howell, New York.

Chamba JF, 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.

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

Chen Y, Yao J, Wang F, Zhou Y, Chen H, Gai N, Chen H, Chen K, Maskow T, Ceccanti B, Trebse P, Zaray G. (2008) Toxic effect of inorganic arsenite [As(III)] on metabolic activity of *Bacillus subtilis* by combined methods. *Current Microbiology* 57, 258-263.

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.

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 KJA. (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 EA, Fross DA, 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.

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 & Technology* 17, 148–152.

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

Forrest WW, Walker DJ. (1963) Calorimetric Measurements of Energy of Maintenance of *Streptococcus faecalis*, *Biochemical and Biophysical Research Communications* 13, 217-222.

Francois K, Devlieghere F, Smet K, Standaert AR, Geeraerd AH, Van Impe JF Debevere J. (2005) Modelling the individual cell lag phase: effect of temperature and pH on the individual cell lag distribution of *Listeria monocytogenes*. *International Journal of Food Microbiology* 100, 41-53

Gaisford S, O'Neill MAA, Beezer AE. (2009) Shelf life prediction of complex food systems by quantitative interpretation of isothermal calorimetric data. In G. Kaletunç (Eds.), *Calorimetry in food processing: analysis and design of food systems* (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.

Gustafsson L. (1991). Microbiological calorimetry. *Thermochimica Acta* 193, 145-171.

Hales TC. (1994) The status of the *Kepler's* conjecture. *Mathematical Intelligencer* 16, 47-58.

Ham JS, Jeong SG, Lee SG, Han GS, Chae HS, Yoo YM, Kim DH, Lee WK, 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 JS, Jeong SG, Lee SG, Han GS, Jang A, Yoo YM, Chae HS, Kim DH, Kim HJ, Lee WK, Jo C. (2009b) Quality of irradiated plain yogurt during storage at different temperatures. *Asian - Australasian Journal of Animal Sciences* 22, 289–295.

Higuera-Guisset J, Rodriguez-Viejo J, Chacon M, Muñoz FJ, Vigues N, Mas J. (2005) Calorimetry of microbial growth using a thermopile based microreactor, *Thermochimica Acta* 427, 187-191.

Hsiang W-I. (1993) On the sphere packing problem and the proof of *Kepler's* conjecture. *Proceedings of Kaist Mathematics Workshop*, 41-52.

Hols P, Hancy F, Fontaine L, Grossiord B, Prozzi D, Leblond-Bourget N, Decaris B, Bolotin A, Delorme C, Dusko Ehrlich S, Guédon E, Monnet V, Renault P, Kleerebezem M. (2005) New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiology Reviews* 29, 435–463.

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

Hutkins RW, Nannen NL. (1993) pH Homeostasis in Lactic Acid Bacteria. *Journal of Dairy Science* 76, 2354-2365.

Huycke MM, Sahm DF, Gilmore MS. (1998) Multiple-Drug Resistant *Enterococci*: The Nature of the Problem and an Agenda for the Future. *Emerging Infectious Diseases* 4, 195-209.

Jeanson S, Chadoeuf J, Madec MN, Aly S, Floury J, Brocklehurst TF, Lortal S. (2011) Spatial distribution of bacterial colonies in a model cheese. *Applied and Environmental Microbiology* 77, 1493-1500.

Juillard V, Le Bars D, Kunji ERS, Konings WN, Gripon JC, Richard J. (1995) Oligopeptides are the main source of nitrogen for *Lactococcus lactis* growth in milk. *Applied and Environmental Microbiology* 61, 3024–3030.

Kang JH, Lee MS. (2005) Characterization of a bacteriocin produced by *Enterococcus faecium* GM-1 isolated from an infant. *Journal of Applied Microbiology* 98, 1169–1176.

Kimura T, Takahashi K. (1985) Calorimetric studies of soil microbes: quantitative relation between heat evolution during microbial degradation of glucose and changes in microbial activity in soil. *Journal of general microbiology* 131, 3083-3089.

Kokkinos A, Fasseas C, Eliopoulos E, Kalantzopoulos G. (1998) Cell size of

various lactic acid bacteria as determined by scanning electron microscope and image analysis. *Lait* 78, 491-500.

Konteles S, Sinanoglou VJ, 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.

Lago N, Legido JL, Paz Andrade MI, Arias I, Casas LM. (2011) Microcalorimetric study on the growth and metabolism of *Pseudomonas aeruginosa*. *Journal of Thermal Analysis and Calorimetry* 105, 651-655.

Lahaye M. (1991) Cyrille Rochas Chemical structure and physico-chemical properties of agar. *Hydrobiologia* 221, 137-148.

Lahtvee P-J, Adamberg K, Arike L, Nahku R, Aller K, Vilu R. (2011). Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. *Microbial Cell Factories* 10, 1-12.

Lamprecht I. (2003) Calorimetry and thermodynamics of living systems. *Thermochimica Acta* 405, 1-13.

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 J-S, Marison IW, von Stockar U. (1999) Anaerobic calorimetry of the growth of *Lactobacillus helveticus* using a highly sensitive BIO-RCI. *Journal of Thermal Analysis and Calorimetry* 56, 1191-1195.

Ma J, Qi WT, Yang LN, Yu WT, Xie YB, Wang W, Ma XJ, Xu F, Sun LX. (2007). Microcalorimetric study on the growth and metabolism of microencapsulated microbial cell culture. *Journal of Microbiological Methods* 68, 172–177.

Malakar PK, Brocklehurst TF, Mackie AR, Wilson PDG, Zwietering MH, van't Riet K. (2000) Microgradients in bacterial colonies: use of fluorescence ratio imaging, a non-invasive technique. *International Journal of Food Microbiology* 56, 71-80.

Malakar PK, Martens DE, van Breukelen W, Boom RM, Zwietering MH, van't Riet K. (2002a) Modelling the interactions of *Lactobacillus curvatus* colonies in solid medium: consequences for food quality and safety, Applied and Environmental Microbiology 68, 3432-3441.

Malakar PK, Zwietering MH, Boom RM, Brocklehurst TF, Wilson PDG, Mackie AR, van't Riet K. (2002b) Diffusion of lactic acid in a buffered gel system supporting growth of *Lactobacillus curvatus*. J*ournal of the Science of Food and Agriculture* 82, 1729-1734.

Malakar PK, Barker GC, Zwietering MH, van't Riet K. (2003) Relevance of microbial interactions to predictive microbiology. *International Journal of Food Microbiology* 84, 263-272.

Martin GJO, Williams RPW, Dunstan DE. (2007) Comparison of casein micelles in raw and reconstituted skim milk. *Journal of Dairy Science*, 90, 4543–4551.

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.

McKay AL, Peters AC, Wimpenny JWT. (1997) Determining specific growth rates in different regions of *Salmonella typhimurium* colonies. *Letters of Applied Microbiology* 24, 74-76.

Menert A, Liiders M, Kurissoo T, Vilu R. (2001) Microcalorimetric monitoring of anaerobic digestion processes. *Journal of Thermal Analysis* 64 281–291.

Mercade M, Lindley ND, Loubiere P. (2000) Metabolism of *Lactococcus lactis subsp. cremoris* MG 1363 in acid stress conditions, *International Journal of Food Microbiology*, 55, 161-165

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.

Mitchell AJ, Wimpenny JWT. (1997) The effects of agar concentration on the growth and morphology of submerged colonies of motile and non-motile bacteria. *Journal of Applied Microbiology* 83, 76-84.

Mitchell DA, von Meien OF, Krieger N, Dalsenter FDH. (2004) A review of recent developments in modelling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. *Biochemical Engineering Journal* 17, 15–26.

Mohamed MO, Morris HA, Breene WM. (1982) A comparison of stirred curd cheeses made from normal milks and reconstituted non-fat dry milk with vegetable oils. *Journal of food processing and preservation* 6, 15–30.

Nan Z-D, Xiang Y, Cheng S-Q, Zeng X-C, Zhang H-L. (2001) Thermokinetic research method for bacterial growth in conduction calorimeter. *Journal of Thermal Analysis and Calorimetry* 63, 423–431.

Papagianni M, Avramidis N, Filiousis G. (2007) Glycolysis and the regulation of glucose transport in *Lactococcus lactis* spp. *lactis* in batch and fed-batch culture. *Microbial Cell Factories* 6.

Peitzsch M, Kiesel B, Harms H, Maskow T. (2008) Real time analysis of *Escherichia coli* biofilms using calorimetry. *Chemical Engineering and Processing* 47, 1000–1006.

Pernodet N, Maaloum M, Tinland B. (1997) Pore size of agarose gels by atomic force microscopy. *Electrophoresis* 18, 55-58.

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

Pollock DSG. (1993) Smoothing with cubic splines. *Queen Mary and Westfield College*, The University of London, England

Prats C, López D, Giró A, Ferrer J, Valls J. (2006) Individual-based modelling of bacterial cultures to study the microscopic causes of the lag phase. *Journal of Theoretical Biology*, 241, 939-953.

Ranken MD, Kill RC, Baker CGJ. (1997) Food Industries Manual. (24th ed.).

UK: Blackie Academic & Professional, (Chapter 3).

Riva M, Fessas D, Schiraldi A. (2001) Isothermal calorimetry approach to evaluate the shelf-life of foods. *Thermochimica Acta* 370, 73–81.

Robinson TP, Aboaba OO, Kaloti A, Ocio MJ, Baranyi J, Mackey BM. (2001) The effect of inoculum size on the lag phase of *Listeria monocytogenes*. *International Journal of Food Microbiology* 70, 163-173.

Rong XM, Huang QY, Jiang DH, Cai P, Liang W. (2007) Isothermal Microcalorimetry: A review of applications in soil and environmental sciences. *Pedosphere* 17, 137–145.

Russel JB, Cook GM. (1995) Energetics of Bacterial Growth: Balance of Anabolic and Catabolic Reactions. *Microbiological Reviews* 59, 48-62.

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

Schantz E, Lauffer MA. (1962) Diffusion measurements in agar gel. *Biochemistry* 1, 658-663.

Schäffer B, Szakály S, Lörinczy D. (2004) Examination of the growth of probiotic culture combinations by the isoperibolic batch calorimetry. Thermochimica Acta 415, 123–126.

Senez JC. (1962) Some considerations on the energetics of bacterial growth. *Bacteriological Reviews* 26, 95-107.

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

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(II), 473–478.

Swinnen IAM, Bernaerts K, Dens EJJ, Geeraerd AH, van Impe JF. (2004) Predictive modelling of the microbial lag phase: a review. *International Journal of Food Microbiology* 94, 137-159.

Swinnen IAM, Bernaerts K, Gysemans K, van Impe JF. (2005) Quantifying microbial lag phenomena due to a sudden rise in temperature: a systematic macroscopic study, *International Journal of Food Microbiology* 100, 85-96.

TA Instruments - http://www.tainstruments.com

Theys TE, Geeraerd AH, Verhulst A, Poot K, Van Bree I, Devlieghere F, Moldenaers P, Wilson D, Brocklehurst T, Van Impe JF. (2008) Effect of pH, water activity and gel micro-structure, including oxygen profiles and rheological characterization, on the growth kinetics of *Salmonella Typhimurium. International Journal of Food Microbiology* 128, 67-77.

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 TD, Crow VL. (1984) Selection of galactose-fermenting *Streptococcus thermophilus* in lactose-limited chemostat cultures. *Applied and Envoronmental Microbiology* 48, 186–191.

van Impe JF, Poschet F, Geeraerd AH, Vereecken KM. (2005) Towards a

novel class of predictive microbial growth models. *Journal of Food Microbiology* 100, 97-105.

van Wamel WJB, Hendrickx APA, Bonten MJM. (2007) Growth condition-dependent Esp expression by *Enterococcus faecium* affects initial adherence and biofilm formation. *Infection and Immunity* 75, 924-931.

Vandenhove H. (1998) Microcalorimetric characteriazation of bacterial inocula. Chapter 5, 121–158. *Kluwer Acadimic Publishers.*

von Stockar U, van der Wieler LAM. (1997) Thermodynamics in biochemical engineering. *Journal of Biotechnology* 59, 25-37.

Wadsö I. (2002) Isothermal microcalorimetry in applied biology. *Thermochimica Acta* 394, 305-311.

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

Wang X, Liu Y, Xie B, Shi X, Zhou J, Zhang H. (2005) Effect of nisin on the growth of *Staphylococcus aureus* determined by a microcalorimetric method. *Molecular Nutrition and Food Research* 49, 350–354.

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.

White CH, Ryan JM. (1983) Defining optimal conditions for making cottage cheese from reconstituted milk powder. *Journal of food protection* 46, 686–692.

Whitehead WE, Ayres JW, Sandine WEA. (1993) Review of starter media for cheese making. *Journal of Dairy Science* 76, 2344–2353.

Wilson RDG, Brocklehurst TF, Arino S, Thuault D, Jakobsen M, Lange M, Farkas J, Wimpenny JWT, van Impe JF. (2002) Modelling microbial growth in structured foods: towards a unified approach, *International Journal of Food Microbiology* 73, 275-289.

Wimpenny JWT, Leistner L, Thomas VL, Mitchell AJ, Katsaras K, Peetz P. (1995) Submereged bacterial colonies within food and model systems: their growth, distribution and interactions. *International Journal of Food Microbiology* 28, 299-315.

Winkelmann M, Hüttl R, Wolf G. (2004) Application of batch-calorimetry for the investigation of microbial activity. *Thermochimica Acta* 415, 75-82.

Yao J, Tian L, Wang Y, Djah A, Wang F, Chen H, Su C, Zhuang R, Zhou Y, Choi MMF, Bramanti E. (2008) Microcalorimetric study the toxic effect of hexavalent chromium on microbial activity of Wuhan brown sandy soil: An in vitro approach. *Ecotoxicology and Environmental Safety* 69, 289–295.

Yi L, Than TT, Zhong C, Songsheng Q, Ping S, (2000) Microcalorimetric studies on the metabolism of *Chlorella vulgaris*. *Chemosphere* 40, 845-849.

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.

Ölmez HK, Aran N. (2005) Modeling the growth kinetics of *Bacillus cereus* as a function of temperature, pH, sodium lactate and sodium chloride concentrations. *International Journal of Food Microbiology* 98, 135-143.

PUBLICATION I

N. Kabanova, I. Stulova, R. Vilu. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at low glucose concentration in liquids and solid agar gels. *Thermochim. Acta* 2013, 559, 69-75[2]

[2] Copyright 2013, reprinted with permission from Elsevier

Thermochimica Acta 559 (2013) 69–75

Contents lists available at SciVerse ScienceDirect

Thermochimica Acta

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

Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at low glucose concentration in liquids and solid agar gels

N. Kabanova[a,b,*], I. Stulova[a,b], R. Vilu[a,b]

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

ARTICLE INFO

Article history:
Received 12 July 2012
Received in revised form
28 December 2012
Accepted 25 February 2013
Available online xxx

Keywords:
Microcalorimetry
Bacterial growth
Lactic acid bacteria
Lactococcus lactis
Colonial growth
Solid-state fermentations

ABSTRACT

Growth of *Lactococcus lactis* IL1403 in liquid cultures and solid agar gels at different low glucose concentrations starting from 2 g L^{-1} up to 10 g L^{-1} with a stepwise increase by 1 g L^{-1} was studied using thermal activity monitor TAM III. In parallel to calorimetric measurements the changes of glucose and lactic acid concentrations and pH of culture media were measured in order to obtain additional information for the interpretation of calorimetric power–time curves. The data obtained together with calculated heat yield coefficient Y_Q allowed to analyze the growth of bacteria in colonies in solid agar gels in detail. The variation of glucose concentration at low levels allows analyzing and describing growth deceleration phase and growth stopping mechanisms both in liquid cultures and in solid-state fermentations.

1. Introduction

In transparent homogenized liquid cultures every bacterium is growing essentially in the same environmental conditions, and this simplifies significantly the study and characterization of the growth processes. Bacterial growth in solid states is a much more complicated phenomenon – solid matrix forces bacteria to grow in colonies and thus diffusion of substrates and products, possible accumulation of toxic products in the colonies, etc. lead to the different growth curves (laws) and growth stopping mechanisms than in broth.

It has been reported in the literature [1] that bacteria grow more slowly in structured systems than they do in broth. It has been proposed that growth in gelled systems is associated with additional stress on the organism. It has been shown also that the growth lag in solid media is longer than in broth [2]. The highest value of the maximum specific growth rate in case of *Lactococcus lactis* has been observed when pH values are in the interval 5.5–6.6 and the critical pH value was defined to be 4.0. Lower than pH = 5.5, pH values are negatively influencing the rate of glucose consumption [3] and [4].

Colonial growth of bacteria in solid matrices has been previously described in numerous papers [2,5–10]. The experimental methods used for the study of solid-state fermentations (SSF) – measurements of OD, outplating, direct microscopy measurements, chromatography etc. are quite cumbersome and not very informative about the peculiarities of growth. Currently more and more attention is drawn to use of microcalorimetry in studying bacterial growth in opaque liquids, on the surfaces and in solids in wide range of conditions [5,11,12]. Calorimetric power–time curves give a lot more information about the peculiarities of bacterial growth and its metabolism [5,13,14], which hardly could be obtained using conventional methods – multiauxic growth, switch from one substrate to another, etc. Calorimetry provides also an efficient alternative for the precise determination of the specific growth rates [15]. Moreover, calorimetric method allows detecting lower cell concentrations of bacteria in the range of 10^4–10^5 cells, which could not be detected using spectrophotometer, the generally preferred method [5,12]. Despite the fact that power–time curves provide a lot of information on the growth, it is very useful to collect some additional data using other methods in parallel [5,16].

The objective of the present study was to investigate the growth of *L. lactis* bacteria both in solid and liquid cultures, and to compare the details of growth in both environments. The central idea of the research was to vary systematically initial glucose concentrations in order to study growth rates, peculiarities of metabolism, growth termination factors and the influence of structured matrix on bacterial growth. The growth of *L. lactis* IL1403 was studied in CRM

* Corresponding author at: Tallinn University of Technology, Department of Chemistry, Akadeemia tee 15, 12618, Tallinn, Estonia. Tel.: +372 6202831; fax: +372 6202828.
E-mail address: nataljakabanova@yahoo.com (N. Kabanova).

0040-6031/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.tca.2013.02.013

broth and CRM agar media (*CRM – carbohydrate restricted medium*) using a multi-channel (24-channels) thermal activity monitor TAM III in addition to other methods. The inoculation rate 10^2 cfu mL^{-1} was chosen, as according to Kabanova et al. [5], the growth of the colonies containing bacteria in the range of 10^3–10^6 cells could be observed using microcalorimeter. This range allows to study the growth of small colonies and big colonies, where diffusion and border effects start to influence the processes [2], in the same experiment.

2. Materials and methods

2.1. Inoculum preparation

The cultivated strain *L. lactis* subsp. *lactis* IL1403, a facultative anaerobic lactic acid bacterium, was provided by Dr Ogier from INRA (French National Institute for Agricultural Research, Jouy-en-Josas, France). Frozen storage cultures of *L. lactis* IL1403 were pregrown on M17 Agar (LAB M) for 24 h at 35 °C. One colony from a pregrown Petri dish was used as an inoculum for a 10 mL culture in liquid M17 Broth (FLUKA) at 35 °C. One milliliter of bacterial suspension grown exactly 24 h was used as inoculum for the next liquid 10 mL M17 Broth culture and grown 12–14 h which allowed bacteria to reach the middle of the exponential growth phase. The inoculum, needed for the experiments was prepared by diluting of samples of the mid-exponential culture in peptone water. Calculated inoculation rates in calorimetric and in parallel samples were 10^2 cfu mL^{-1}.

2.2. Growth experiments

CRM broth for growth media was prepared in a 200 mL screw capped glass bottles in case of 2, 5 and 10 g L^{-1} initial glucose (GLC) concentrations and in 25 mL Erlenmeyer flasks in case of other initial glucose concentrations 3, 4, 6, and 7 g L^{-1}. CRM agar was prepared for the experiments in solid states the same way as CRM broth but with addition of appropriate amount of agar powder (1%, wt wt^{-1}). All solutions were sterilized by autoclaving at 121 °C for 15 min. A definite amount (1% vol vol^{-1}) of the inoculum was added to the bottles with media solutions at 25 °C (broth) or 45 °C (agar) to get an inoculation rate of 10^2 cfu mL^{-1}, and after that the suspensions were intensively stirred. Two milliliters of the culture from each bottle were transferred into 3 mL autoclaved glass microcalorimetric ampoules in case of growth in liquid state, and 3 mL of the culture from each bottle to the glass microcalorimetric ampoules in case of solid-state fermentations, and the calorimetric experiments were run at +35 °C. All the calorimetric growth experiments were carried out in 3 parallels and power–time curves obtained were normalized. In case of experiments in broth 200 mL bottles with residual bacterial suspensions were placed into a thermostat at +35 °C. Glucose and lactic acid concentrations, pH, OD were determined in 4 mL samples, *outplating* was carried out using 100 μL samples at regular time intervals determined by bacterial calorimetric growth curves. The remaining solutions of CRM agar, after filling the calorimetric ampoules, were shared by 2 mL to sterile Falcon tubes and also placed into thermostat at +35 °C. Glucose and lactic acid concentrations and pH were determined at time intervals considered appropriate by bacterial calorimetric growth curves. The routine samples were taken in all cases at the beginning and at the end of exponential and deceleration growth phases and also during both growth phases to get representative data sets. In case of solid media sample solutions for the HPLC measurements were prepared by homogenizing the solid media in Falcon tubes with MilliQ water in proportion of 1:2. After the ampoules with solid state were removed from the microcalorimeter the sizes of the colonies were measured.

2.3. Compositions of the media used

CRM agar growth medium was prepared by adding Agar No. 1 (1%, wt wt^{-1}) to CRM broth medium of the following composition: casitone (DIFCO) 20 g L^{-1}, Tween 80 1.08 g L^{-1}, sodium chloride (MERCK) 25 g L^{-1}, magnesium sulfate 0.58 g L^{-1}, manganese sulfate 0.3 g L^{-1}, ferric sulfate 0.06 g L^{-1}, B-vitamin solution (SIGMA) 10 mL L^{-1}, MES (2-[*N*-morpholino]ethanesulfonic acid, SIGMA) 8.5 g L^{-1}.

2.4. Analytical methods

The TAM III (24-channels, *TA Instruments*), a heat conduction multi-channel microcalorimeter was used to monitor the growth of cells. The TAM III was operated in static ampoule mode (batch process). The construction and functioning of the TAM III calorimeter was previously described in Ref. [17] and the way the instrument was used during the experiments is described in Ref. [18]. Microcalorimetric power–time curves were analyzed as described in Ref. [5]. HPLC Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 mm × 7.8 mm for measuring glucose and lactate concentrations (solvent–sulfuric acid solution (2.2 mL per 1 L MilliQ water), flow rate 0.6 mL min^{-1}, experimental temperature 35 °C). Optical density (for bacterial growth in liquid state) was observed at 540 nm using a spectrophotometer (PHOTOMETR KFK-3, USSR). pH was detected with a pH meter S20 Seven Easy Mettler Toledo with an InLab 413 Mettler Toledo electrode. The dimensions of colonies were measured using a microscope Zeiss Axiovert 200 M with Zeiss AxioCam MRc5 camera and Zeiss AxioVs40 V45.0.0 software.

3. Results

The growth of *L. lactis* IL1403 at the inoculation rate of 10^2 cfu mL^{-1} was studied in broth and in solid agar gel. Initial glucose concentration was varied stepwise by 1 g L^{-1} from 2 g L^{-1} up to 10 g L^{-1}. The calorimetric power–time curves obtained in case of every glucose concentration studied in broth and in solid states are presented in Fig. 1 on graph (a) and (b) respectively.

Calorimetric power–time curves could be divided to three different growth phases [5] – lag phase, exponential and deceleration phases. The mechanisms that led to the stoppage of exponential and total growth as well as the peculiarity of the shape of the calorimetric power–time curve during the deceleration growth phase were of major interest in the current study. In order to analyze the calorimetric power–time curves additional measurements of concentrations of glucose and lactic acid as well as pH were carried out for three chosen glucose concentrations (2, 5 and 10 g L^{-1}) and the data obtained are presented in Fig. 2. Data of additional measurements was splined [19].

As seen in Fig. 1, the power–time curves obtained in liquids and in solids are quite similar in shape but some remarkable differences could be nevertheless observed. In case of the lowest glucose concentration studied – 2 g L^{-1} – the calorimetric power–time curve obtained in broth resembles a symmetric (*Gaussian) curve*. The deceleration phases of the growth curves were more prolonged in the case of higher glucose concentrations than 2 g L^{-1} – see Fig. 1. The calorimetric power–time curves were practically identical in the range 4–10 g L^{-1} of glucose. In case of 3 g L^{-1} of the initial glucose concentration the curve coincided with those of the higher glucose concentrations until the 40th hour of fermentation, after which a quite abrupt decrease of the growth rate was observed.

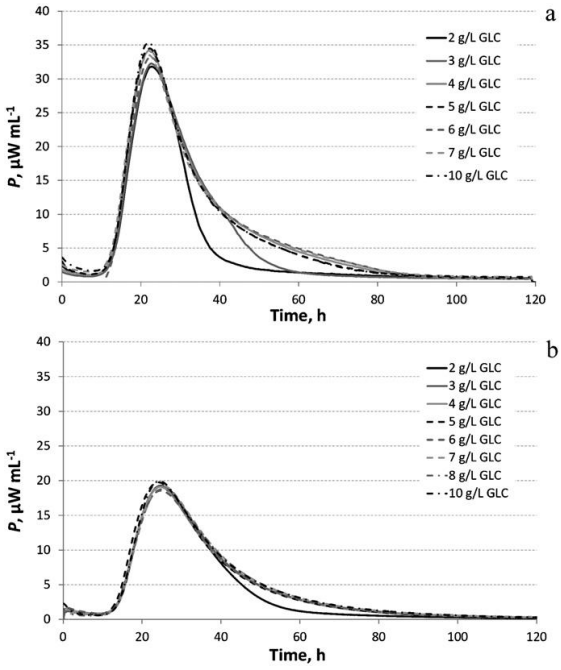


Fig. 1. Calorimetric power–time curves of *L. lactis* in media with different glucose concentrations (GLC, g L^{-1}) in broth (a) and in solid-state matrices (b) at inoculation rate 10^2 cfu/mL.

Comparing the growth curves obtained in solid matrices with those in broth (Fig. 1) it could be seen that in all cases, also at 2 g L^{-1} glucose concentration the post-exponential parts of the solid state curves were prolonged. The 2 g L^{-1} curve was not Gaussian any more, however, the growth of the culture was stopped earlier than in case of other glucose concentrations starting from 3 g L^{-1} and higher. This, and the facts that the maximum heat flows P_{max} (W mL^{-1}) were remarkably lower in the solid-state matrices than in broth, were the clear differences characterizing growth in the two cases studied.

Processing of the calorimetric power–time curves from Fig. 1, the data on heat produced during different growth phases (Q_{exp}, J mL^{-1}; Q_{tot}, J mL^{-1}) as well as the maximum specific growth rate (μ_{max}, h^{-1}) in case of broth and solid-state cultures were obtained as described in Ref. [5] and presented in Fig. 3 and Table 1. The number of bacteria grown was estimated using Y_Q value $Y_Q = (2.58 \pm 0.44) \times 10^{-9}$ J cfu^{-1}. Heat yield coefficient Y_Q was determined on the basis of the results of *plate counts* and heat effects were measured for the three different glucose concentrations 2, 5 and 10 g L^{-1}.

As seen in Figs. 1 and 3 the maximum specific growth rate μ_{max} (h^{-1}) was practically the same and did not depend on initial glucose concentration in the range studied, both, in liquids and solids, $\mu_{max} = 0.44 \pm 0.02$ h^{-1} (Fig. 3). This result is strongly indicating that the bacteria were growing with μ_{max}, and no glucose diffusion limitation was observed in agar gels during the rapid (exponential) growth phase.

The lag phases in case of solid state experiments were approximately by 1.4 h longer than in liquids – 7.93 ± 0.47 h and 9.31 ± 0.37 h in liquids and in solids respectively in case of every substrate concentration. The explanation of the difference in lag phase durations is presented in Section 4 in detail.

The amounts of heat produced during the exponential growth phase in liquid cultures were practically the same at a value of 0.82 ± 0.13 J mL^{-1} which corresponds to 3.18×10^8 cells in case of different glucose concentrations studied (Table 1). The amount of heat 0.55 ± 0.02 J mL^{-1} corresponding to 2.12×10^8 cells, 67% of the values in broth, was the similar data for solid-state cultures. These facts showed that during the exponential growth phase the same number of bacteria was produced in both cases independently of the amount of glucose available – the termination of the exponential growth was not caused by the exhaustion of glucose in the media. Accumulating lactate either through direct inhibition, or more probably through the change of the pH of the media was the reason for stopping the exponential growth. Indeed, as seen in Fig. 2 the end of exponential growth phase (the peak of the calorimetric power–time curve) coincided with the pH value about 4.6 in all cases of glucose concentrations, and in both of the media studied. The heat produced in solid-state matrices was 1.5 times lower than in broth, and thus also the number of bacteria grown during the exponential growth was smaller – 0.55 ± 0.02 J mL^{-1} and 2.12×10^8 cfu mL^{-1}, respectively (Table 1). As noted above the corresponding values in broth were 0.82 ± 0.13 J mL^{-1} and 3.18×10^8 cells. However, this apparent contradiction was solved noticing that in solid media the pH value of 4.6 was reached with less amount of lactate (LA ~1.0 g L^{-1}) produced than in liquid media (~2.2 g L^{-1}), see Fig. 2. One of the possibilities to explain lower buffering capacity of solid matrices is to take into account that part of the free water is bound to the gel in the solid media, and thus less lactic acid than in broth is needed to acidify the environment and bring the pH down. Another possible assumption is assuming that homofermentative metabolism was replaced by heterofermentative metabolism of *L. lactis* in solid-state matrices (Fig. 2) with the formation of formate and acetate, stronger acids than lactate. However, it should be noted that no extra peaks in addition to lactic acid on HPLC curves were detected. Therefore the assumption that the smaller buffering capacity of agar gel was the reason for the smaller amount of biomass formed in the solid-state matrices seems to be more attractive than the other.

The heat amount produced by the end of growth in liquid cultures was around 2.8 ± 0.3 J mL^{-1} that corresponds to the number of bacteria 1.09×10^9 cfu mL^{-1} (calculated using heat yield coefficient Y_Q). The total heat effects in case of bacterial growth in solid-state matrices were 1.5 times smaller and equalled in average to 1.92 ± 0.18 J mL^{-1} and the number of bacteria grown in the solid cultures calculated using heat yield coefficient was $(7.41 \pm 0.38) \times 10^8$ cfu mL^{-1}.

The total number of bacteria at the end of growth in solid agar gels could be also calculated on the basis of microscopic data, using *Kepler's* conjecture as described in Ref. [5]. The number of the bacteria at the end of growth in solid state was $(1.1 \pm 0.19) \times 10^9$ cfu mL^{-1} forming colonies with the radii of 111 ± 6.69 μm. The number of the bacteria determined based on microscopy was 1.5x higher than the number calculated from the power–time curves. This difference could be explained most probably by the less dense packing of bacterial cells in the colonies than assumed in the *Kepler's* conjecture, which is the densest possible packing of spheres. But a reasonable correspondence of the calorimetric and microscopy data should be considered encouraging in attempts to determine fitting with each other datasets on growth of bacteria in solid-state matrices.

Comparison of the biomasses grown during the complete cultivation experiments and during the exponential growth phase showed that about 70% out of the total was synthesized during the post-exponential growth phase in the case of both media (Fig. 3). At low initial glucose concentrations, at 2 g L^{-1} and 3 g L^{-1} in case of liquids and at 2 g L^{-1} in case of solids, the total heat amounts produced by the cultures were lower in comparison with higher initial glucose concentrations.

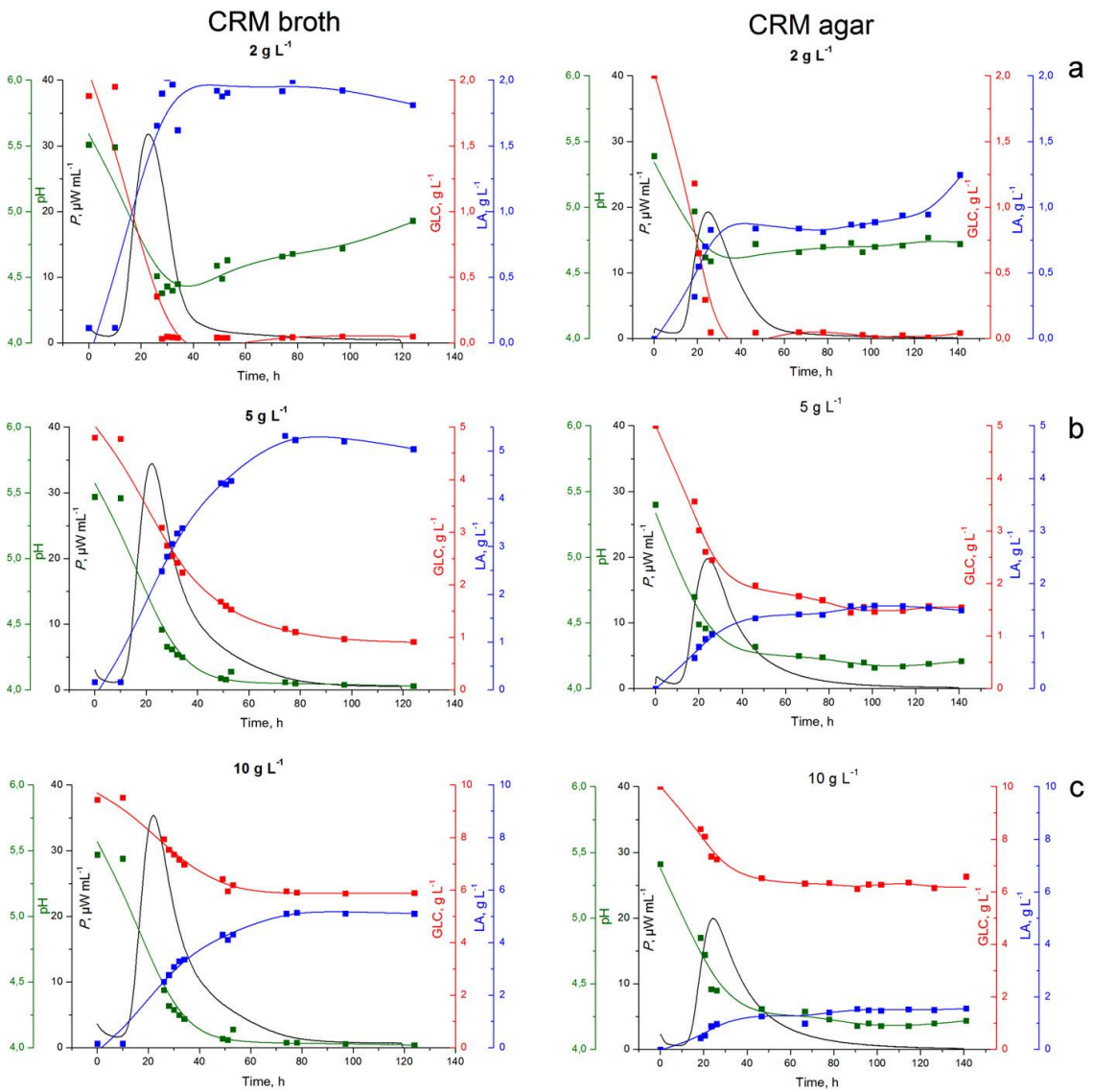


Fig. 2. Calorimetric power–time curves, pH change, lactic acid production and glucose consumption curves describing the growth of bacteria at different initial glucose concentrations 2 (a), 5 (b) and 10 (c) $g\,L^{-1}$ both in liquid cultures and in solid agar gels.

As seen in Fig. 2 the profiles of consumed glucose, produced lactate and pH were also different at lower initial glucose concentrations ($2\,g\,L^{-1}$) in comparison with the higher ones (5 and $10\,g\,L^{-1}$). In case of $2\,g\,L^{-1}$ glucose concentration both in broth and in solids, the substrate was completely consumed by the end of the growth, which indicated that this was the reason for the termination of the growth. At higher initial glucose concentration the situation with glucose consumption was depending on the matrix. In case of $5\,g\,L^{-1}$ glucose concentration almost all glucose was consumed in broth – $4.1\,g\,L^{-1}$, whereas in solid-state matrix more than a half of the total glucose available initially ($3.4\,g\,L^{-1}$) was consumed. 35% and 41% of the initial glucose was eventually consumed in the case of $10\,g\,L^{-1}$ in the solid-state and liquid cultures respectively. On the basis of the results obtained a conclusion can be made that the growth of the liquid culture was eventually terminated due to the exhaustion of the glucose in the media in case of $2\,g\,L^{-1}$ and $3\,g\,L^{-1}$. To understand the mechanism of termination of growth in the case of cultures with higher than $3\,g\,L^{-1}$ of initial glucose we should notice that the final concentrations of lactic acid in the cultures did not exceed $5\,g\,L^{-1}$ – Fig. 2, which is quite low – usually at least 2× higher concentrations are required to inhibit the growth of LABs remarkably. On the other hand pH was in the end of cultivations in the range of 4.0–4.2, both in liquid and solid-state cultures, which could well be the reason for the termination of the growth of the cultures.

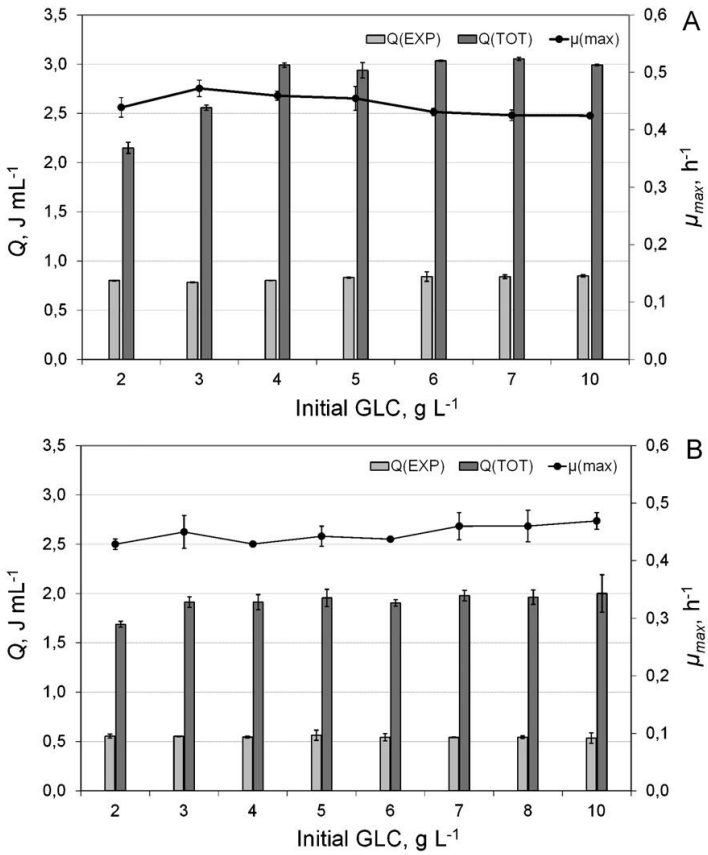


Fig. 3. Bacterial growth describing parameters in broth (a) and in solid agar gel (b): heat produced during exponential growth phase Q_{exp} (J mL^{-1}), total heat Q_{tot} (J mL^{-1}) produced during the growth of the culture and maximum specific growth rate μ_{max} (h^{-1}).

For the homofermentative bacteria as *L. lactis*, it is common to produce lactic acid from consumed glucose in ratio 1:1 (gram per gram). According to HPLC data, the energy metabolism of *L. lactis* cells was homofermentative in the whole range of glucose concentrations in the case of broth cultures (Fig. 4a). During the growth of bacteria in solid-state matrices the metabolism of the bacteria, however, was changed most probably to more efficient heterofermentative mechanisms as the concentrations of produced lactic acid made approximately half of the consumed glucose amounts (see also Fig. 4b). However, it should be stressed that we were not able to identify and quantify the growth by-products in this case. Identification of the missing by-products is one of the most interesting tasks for the future.

4. Discussion

According to the literature [1] and [6] gelled (solid-state) systems exert additional stress on the cells, which would grow

Table 1
Calculated parameters describing growth of the bacteria in broth and in agar gels (marked with *) during the exponential growth phase at different initial glucose concentrations (from 2 g L^{-1} to 10 g L^{-1}): amounts of heat produced during the exponential phase – Q_{exp} and Q^*_{exp} (J mL^{-1}) and corresponding number of bacteria N_{exp} and N^*_{exp} (cfu mL^{-1}) formed. Additionally in case of bacterial growth in agar the number of bacteria at the end of growth (N^*_{tot}, cfu col^{-1}) and the radii of the colonies at the end of exponential (R^*_{exp}, μm) and total growth (R^*_{tot}, μm) using Y_Q were calculated.

GLC g L^{-1}	Q_{exp}, J mL^{-1}	N_{exp}, cfu mL^{-1}	Q^*_{exp}, J mL^{-1}	N^*_{exp}, cfu mL^{-1}	N^*_{tot}, cfu mL^{-1}	R^*_{exp}, μm	R^*_{tot}, μm
2	0.801	3.10×10^8	0.555	2.15×10^8	8.25×10^8	65	102
3	0.783	3.03×10^8	0.553	2.14×10^8	1.22×10^9	62	116
4	0.803	3.11×10^8	0.546	2.11×10^8	9.86×10^8	65	116
5	0.832	3.22×10^8	0.564	2.18×10^8	1.19×10^9	66	108
6	0.842	3.26×10^8	0.543	2.10×10^8	1.20×10^9	65	120
7	0.841	3.25×10^8	0.542	2.10×10^8	1.20×10^9	65	115
8	–	–	0.544	2.10×10^8	9.26×10^8	65	106
10	0.850	3.29×10^8	0.535	2.07×10^8	9.09×10^8	64	105

–, Data is absent.

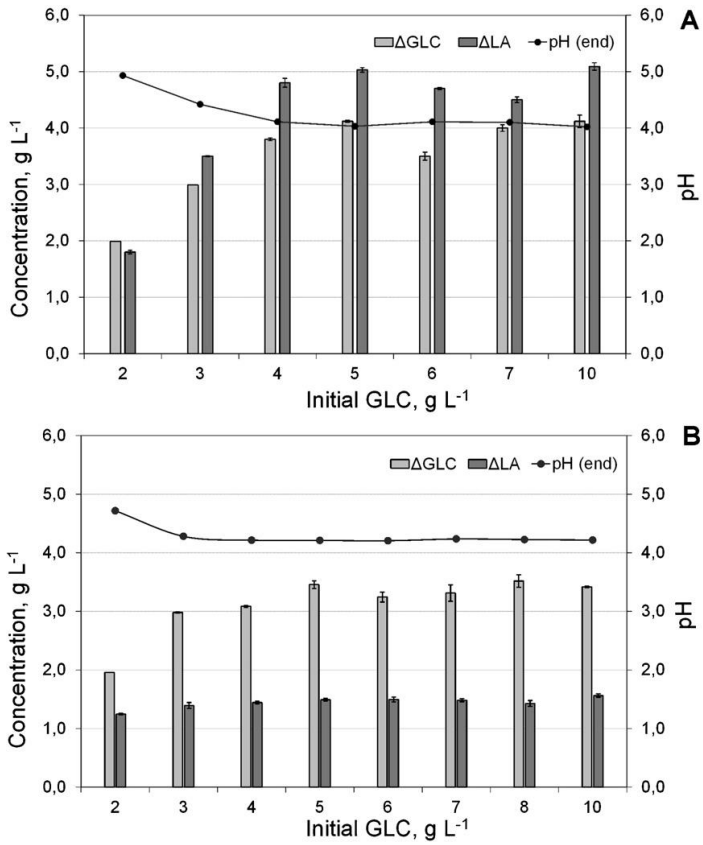


Fig. 4. The amounts of glucose (GLC, $g\,L^{-1}$) consumed and the amounts of lactic acid (LA, $g\,L^{-1}$) produced during the total bacterial growth at different initial glucose concentration studied in broth (a) and solid agar gels (b). The pH values at the end of the growth are presented as well.

therefore more slowly in solid-state matrices than in the liquid cultures. The results obtained by us showed unequivocally that the maximum specific growth rates of *L. lactis* (μ_{max}, h^{-1}) were practically the same in solid-state and liquid cultures, i.e. the bacteria were growing presumably with the maximum possible growth rates in both media. There was no diffusion limitation of glucose in agar gels. Indeed the experimental data obtained in the literature have shown that the diffusion coefficients of glucose (and lactic acid) in agar gels were practically the same as in water [20].

A shorter by 1.4 h lag phase was observed in liquid cultures in comparison with the solid-state cultures. This could be explained by the differences in preparation of the cultures. Inoculating the bacteria into media containing agar the temperature of the media should be high enough (higher than 40 °C) for good mixing, and it is stressful for microorganisms [2] leading to the prolongation of the lag phase. There is no additional heat stress in the case of liquid cultures – inoculation of bacteria in broth takes place at room temperature.

One of the most interesting observations made in this work was certainly the fact that the amounts of heat produced (the numbers of bacteria grown) during the exponential growth were practically the same in case of different initial glucose concentrations in both of the media studied, but if the average amount of heat produced in broth was $0.82 \pm 0.13\,J\,mL^{-1}$ (3.18×10^8 cells), then it was $0.55 \pm 0.02\,J\,mL^{-1}$ (2.12×10^8 cfu mL^{-1}) in solid-state matrices. These facts clearly indicated that exhaustion of the limiting substrate glucose was not the reason for the termination of the exponential growth. At the end of the exponential phase (maximal heat flow) the environmental pH was around 4.6, and the lactic acid concentrations were different in different cases. It was very interesting to notice that lactic acid needed to reach pH = 4.6 was remarkably different in the case of liquid and solid-state cultures – the buffering capacity of the solid-state matrices were about two times lower than that of the broth.

Systematic changing of the initial glucose concentration in the range 2–10 $g\,L^{-1}$ allowed to identify factors terminating the growth of the cultures in different conditions, and study some interesting differences of growth processes in liquid and solid-state media. It was shown that in the case of initial glucose concentrations 2 $g\,L^{-1}$ the growth of the liquid and solid-state cultures were stopped due to the exhaustion of glucose in the media. It was also shown that at initial glucose concentrations 4–10 $g\,L^{-1}$ the growth of the cultures was terminated by low pH (pH = 4.0 in case of liquid, and pH = 4.2 in case of solid-state cultures). There were two interesting peculiarities observed, however. In the case of 3 $g\,L^{-1}$ of glucose an interesting feature of the post-exponential power–time curve was observed in case of broth cultures. The curve followed the course of the curves of higher glucose concentrations up to the 40th hour of cultivation, and then there was a rather abrupt drop of the heat production (growth). In our opinion this showed unequivocally that the growth of the culture in broth at 3 $g\,L^{-1}$ was stopped due to the exhaustion of glucose. This conclusion was also supported by

the data on change of the glucose concentration in the media (see Fig. 2). It should be noticed that at 3 g L^{-1} of glucose in solid-state media the analogous drop was not observed, as in this case due to the decrease of the buffering capacity of agar gel the glucose was exhausted in the end of the growth of the culture earlier than pH = 4.2 was reached at higher glucose concentrations.

The fact that the growth of the cultures at high initial glucose concentrations (5 and 10 g L^{-1}) was terminated at pH = 4.0 in case of liquid, and pH = 4.2 in case of solid-state cultures showed that this was most probably due to the fact that even lower amounts of lactic acid produced by the bacteria in colonies was sufficient for the stopping of the growth. It is necessary here to assume that diffusion of lactic acid inside the colonies was remarkably slower process than in water, which allowed higher local accumulation of lactic acid and inhibition of growth at bulk pH higher (4.2) than in broth (4.0) where there were no diffusion limitations. This conclusion is in agreement with the data in the literature [3] and [4]. According to Ref. [2], if the number of the bacteria in the colony is higher than 10^5 cells then mass transfer limitations become significant for the growth processes. The number of bacteria at the end of exponential growth curve in our experiments was 3.21×10^6 cfu col^{-1} and increased further approximately 4 times. The bacteria that were in the center of the colony did not get enough of substrate and the diffusion of produced lactic acid away from the colony was presumably also complicated. The big colonies were expanding only at the expense of the peripheral growth.

The energy metabolism of the *L. lactis* cells was homofermentative in the whole range of initial glucose concentrations in case of liquid cultures (Fig. 4). However, the amounts of produced lactic acid were two times lower than the amounts of glucose consumed in solid-state cultures. According to the literature [21], homofermentative metabolism in anaerobic cultures occur on substrates supporting rapid growth in which significant amount of glucose is in the medium and a mixed acid fermentation occurs when growth rates are low. We observed the shift in the case of change of growth from liquid to solid-state culture. As said, we were not in a position to quantify and identify the growth by-products in the solid-state fermentations. This is one of the clear tasks for the future.

5. Conclusions

It was shown in the present study that the maximal specific growth rate was practically the same in broth and in solid agar gels independently of initial glucose concentration attesting that diffusion limitation was not involved in the determination of the growth rate of *L. lactis* in solid agar gels. The number of bacteria grown by the end of exponential growth phase was 1.5 times higher in liquid cultures in comparison with the growth in solid agar gels. This was explained by the lower buffering capacity of the solid-state agar gels – about 1.5 times less lactic acid was needed to reach the same pH as in broth. In case of liquid cultures the growth was stopped in the end of the cultivations because of critically low pH (pH = 4.0) and accumulation of lactate in the environment. The total growth in agar gels was stopped due to lactic acid diffusion limitations within the colony at higher environmental (bulk) pH = 4.2 at the end of growth. During the growth of bacteria in solid-state matrices the metabolism of the bacteria was changed to more efficient heterofermentative mechanisms in comparison with the bacterial growth in broth, where *L. lactis* had homofermantative metabolism. Last but not least the methods developed by us and the data obtained form a consistent system for study of quantitative peculiarities of anaerobic growth processes in liquid and solid-state bacterial cultures. Even the data obtained using microscopy were integrated and shown fitting into the detailed quantitative description of growth of colonies.

Acknowledgements

This investigation was financially supported through the grant ETF9192 of Estonian Science Foundation, through the grant SF0140090s08 of Estonian Ministry of Education and Research, through the project 3.2.0701.11-0018 and by the European Regional Development Fund project EU29994.

References

[1] R.D.G. Wilson, T.F. Brocklehurst, S. Arino, D. Thuault, M. Jakobsen, M. Lange, J. Farkas, J.W.T. Wimpenny, J.F. Van Impe, Modelling microbial growth in structured foods: towards a unified approach, Int. J. Food Microbiol. 73 (2002) 275–289.
[2] P.K. Malakar, D.E. Martens, W. Van Breukelen, R.M. Boom, M.H. Zwietering, K. van't Riet, Modeling the interactions of *Lactobacillus curvatus* colonies in solid medium: consequences for food quality and safety, Appl. Environ. Microbiol. 68 (2002) 3432–3441.
[3] A.Z. Andersen, A.L. Carvalho, A.R. Neves, H. Santos, U. Kummer, L.F. Olsen, The metabolic pH response in *Lactococcus lactis*: an integrative experimental and modelling approach, Comput. Biol. Chem. 33 (2009) 71–83.
[4] M. Mercade, N.D. Lindley, P. Loubiere, Metabolism of *Lactococcus lactis* subsp. *cremoris* MG 1363 in acid stress conditions, Int. J. Food Microbiol. 55 (2000) 161–165.
[5] N. Kabanova, I. Stulova, R. Vilu, Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels, Food Microbiol. 29 (2012) 67–79.
[6] P.K. Malakar, T.F. Brocklehurst, A.R. Mackie, P.D.G. Wilson, M.H. Zwietering, K. van't Riet, Microgradients in bacterial colonies: use of fluorescence ratio imaging, a non invasive technique, Int. J. Food Microbiol. 56 (2000) 71–80.
[7] A.L. McKay, A.C. Peters, J.W.T. Wimpenny, Determining specific growth rates in different regions of *Salmonella typhimurium* colonies, Lett. Appl. Microbiol. 24 (1997) 74–76.
[8] D.A. Mitchell, O.F. von Meien, N. Krieger, F.D.H. Dalsenter, A review of recent developments in modelling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation, Biochem. Eng. J. 17 (2004) 15–26.
[9] J.F. Van Impe, F. Poschet, A.H. Geeraerd, K.M. Vereecken, Towards a novel class of predictive microbial growth models, J. Food Microbiol. 9 (2004) 7–10, 5.
[10] J.W.T. Wimpenny, L. Leistner, V.L. Thomas, A.J. Mitchell, K. Katsaras, P. Peetz, Submereged bacterial colonies within food and model systems: their growth, distribution and interactions, Int. J. Food Microbiol. 28 (1995) 299–315.
[11] L. Wadsö, F.G. Galindo, Isothermal calorimetry for biological applications in food science and technology, Food Control 20 (2009) 956–961.
[12] O. Braissant, D. Wirz, B. Goepfert, A.U. Daniels, Use of isothermal microcalorimetry to monitor microbial activities, FEMS Microbiol. Lett. 303 (2010) 1–8.
[13] N. Lago, J.L. Legido, M.I. Paz Andrade, I. Arias, L.M. Casas, Microcalorimetric study on the growth and metabolism of *Pseudomonas aeruginosa*, J. Therm. Anal. Calorim. 105 (2011) 651–655.
[14] I. Stulova, N. Kabanova, T. Kriščiunaite, T.-M. Laht, R. Vilu, The effect of milk heat treatment on the growth characteristics of lactic acid bacteria, Agron. Res. 9 (II) (2011) 473–478.
[15] J.-S. Liu, I.W. Marison, U. von Stockar, Anaerobic calorimetry of the growth of *Lactobacillus helveticus* using a highly sensitive BIO-RCI, J. Therm. Anal. Calorim. 56 (1999) 1191–1195.
[16] Y. Chen, J. Yao, F. Wang, Y. Zhou, H. Chen, N. Gai, H. Chen, K. Chen, T. Maskow, B. Ceccanti, P. Trebse, G. Zaray, Toxic effect of inorganic arsenite [As(III)] on metabolic activity of *Bacillus subtilis* by combined methods, Curr. Microbiol. 57 (2008) 258–263.
[17] I. Wadsö, Isothermal microcalorimetry in applied biology, Thermochim. Acta 30 (2002) 5–31, 1.
[18] N. Kabanova, A. Kazarjan, I. Stulova, R. Vilu, Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth, Thermochim. Acta 496 (2009) 87–92.
[19] D.S.G. Pollock, Smoothing with Cubic Splines, Queen Mary and Westfield College, The University of London, England, 1993.
[20] E. Schantz, M.A. Lauffer, Diffusion measurements in agar gel, Biochemistry 1 (4) (1962) 658–663.
[21] M. Papagianni, N. Avramidis, G. Filiousis, Glycolysis and the regulation of glucose transport in *Lactococcus lactis spp. lactis* in batch and fed-batch culture, Microb. Cell Fact. 6 (2007) 16.

PUBLICATION II

N. Kabanova, I. Stulova, R. Vilu. Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels. *Food Microbiol*. 2012, 29, 67-79[3]

[3] Copyright 2011, reprinted with permission from Elsevier

Food Microbiology 29 (2012) 67–79

Contents lists available at SciVerse ScienceDirect

Food Microbiology

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

Microcalorimetric study of the growth of bacterial colonies of *Lactococcus lactis* IL1403 in agar gels

N. Kabanova[a,b,*], I. Stulova[a,b], R. Vilu[a,b]

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

ARTICLE INFO

Article history:
Received 3 May 2011
Received in revised form
1 August 2011
Accepted 16 August 2011
Available online 26 August 2011

Keywords:
Colonial growth
LAB
Microcalorimetry
Solid-state fermentations

ABSTRACT

Growth of *Lactococcus lactis* IL1403 in solid agar gels and liquid cultures at different glucose concentrations of 2, 10 and 20 g/L and different inoculation rates from 10^0 to 10^6 cfu/mL with the 10-fold increment was studied using thermal activity monitor TAM III. In parallel to calorimetric measurements the changes of glucose and lactic acid concentrations and pH of culture media were measured in order to obtain additional information for the interpretation of calorimetric power–time curves. Maximal specific growth rates of heat evolution proportional to growth rates of biomass μ_{max} (W/h), heat produced during different growth stages Q_{TOT} (J/mL), Q_{EXP} (J/mL) and duration of lag-phases λ (h) were obtained by processing calorimetric curves. The sizes of colonies were measured also at the end of growth using microscope. The data obtained together with calculated heat yield coefficient Y_Q (J/cfu) allowed to analyze and describe quantitatively the growth of individual colonies and develop a model of multistage growth of a typical colony of *L. lactis* in 1% agar gel. Microcalorimetry used in combination with other relevant methods is a very powerful and precise tool in studying solid-state fermentations.

1. Introduction

Studies of solid-state fermentations (SSF) have become popular during the last twenty years when prolongation of shelf-life of food products and producing safe, healthy and good-tasting fermented foods for example cheese (Pelaez and Requena, 2005 and Settanni and Giancarlo, 2010) have moved to the forefront of the investigations.

Movement of individual cells or groups of cells is restricted in solid matrices and this forces them to grow as separate individual colonies (Malakar et al., 2002a; Mitchell and Wimpenny, 1997 and Wimpenny et al., 1995). As a rule, each colony is formed from one bacterium, and the number of colonies is defined by the size of inoculum. Size of inoculum is determining also the spatial distribution of colonies and the distances between colonies (Malakar et al., 2003 and Jeanson et al., 2011). According to Malakar et al. (2002a) the growth of *Lactobacillus curvatus* in agar gel, in case of the inoculation rates higher than ≥100 cfu/mL, is similar to that in liquid cultures, as diffusion in solid media at short distances is fast enough. However, at low inoculation rates – less than 100 cfu/mL colonies are separated by long distances, they grow bigger and they practically do not interact through diffusion processes, but diffusion of growth substrates and side products (e.g. lactate) may become growth rate limiting factors.

Specific growth rate of bacteria directly depends on concentrations of limiting substrate and produced metabolites in the media, but as said, in the case of SSF it may depend also on the rates of diffusion processes (Hutkins and Nannen, 1993; Malakar et al., 2002b; Papagianni et al., 2007 and Theys et al., 2008). As the bacterial colonies are increasing in size, the microgradients of substrates, products, of pH are formed around and within the colonies (Malakar et al., 2000; Malakar et al., 2002a and Malakar et al., 2003). It has been shown experimentally that during the growth of colonies of lactic acid bacteria (LAB), lactic acid (LA) is first accumulating in the centers of the colonies and afterwards diffusing into the environment lowering pH of the media and leading to the decrease of the growth rate and interaction of colonies, thus inhibiting bacterial growth, glucose (GLC) consumption rate etc. (Andersen et al., 2009; Malakar et al., 2002a; Malakar et al., 2003; Mercade et al., 2000 and Theys et al., 2008). It has been shown that the colonies are growing exponentially until a certain size and afterwards they grow linearly with the diameter

* Corresponding author. Tallinn University of Technology, Department of Chemistry, Akadeemia tee 15, 12618 Tallinn, Estonia. Tel.: +372 6202831; fax: +372 6202828.
E-mail addresses: nataljakabanova@yahoo.com (N. Kabanova), irinastulova@yahoo.com (I. Stulova), raivo@kbfi.ee (R. Vilu).

0740-0020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2011.08.018

of a colony still increasing even if the numbers of cells in the colonies remain constant (Wimpenny et al., 1995). It was assumed that the cells in the center of the colonies are dying due to inhibition by toxic products and low pH or by starvation. In contrast the cells that were located on the surface of the colonies appeared not to be affected by inhibition reactions and thus could divide leading to the growth of the size of the colonies (Wimpenny et al., 1995).

The solid matrices do not only force cells to grow in colonies but also determine the shape of the colonies, and peculiarities of packing of the cells in them. It has been observed that at the concentration of agar around 0.65% (wt/vol) the snowball shaped small colonies are becoming small lobed to spherical and with the further increase of agar concentration colonies become ellipsoid shaped (Mitchell and Wimpenny, 1997 and Wimpenny et al., 1995). The (mechanical) stress exerted on growing colonies by structured systems is comparable with the stress by environmental parameters like temperature, water activity (a_w) and pH - bacteria growing under stress are changing their growth characteristics (Antwi et al., 2006 and Wilson et al., 2002).

Summarizing, it should be noted that the growth of bacteria in solid-state matrices is a very complex phenomenon, and the experimental methods used for the study of SSF (measurements of OD, outplating, direct microscoping measurements, chromatography etc.) are quite cumbersome and frequently not very informative about the details of the growth (of the colonies).

Calorimetric method has great advantages (Vandenhove, 1998) in studying solid-state fermentation processes (Beezer, 2001; Braissant et al., 2010a; Ma et al., 2007 and Mihhalevski et al., 2011), especially if compared with the other methods mentioned (see also Boe and Lovrien, 1990 and Menert et al., 2001). An extensive overview of potential applications of microcalorimetry was well presented by Braissant et al. (2010b); Gustafsson (1991); Lamprecht (2003); Vandenhove (1998) and Wadsö and Galindo (2009). Isothermal microcalorimeter has been used in studying the metabolism of single cells (Yi et al., 2000), prediction of shelf-life of food products (Alklint et al., 2005 and von Stockar and van der Wieler, 1997), and in analysis of substrate limitation of growth (Winkelmann et al., 2004). The heat released by the microbial cultures during growth presented as power–time curves (the output of isothermal microcalorimeter) has been found to correlate quantitatively with biomass generation, changes of numbers of cells, uptake of oxygen or substrates etc. (Birou et al., 1987). Metabolic events, such as shifts from one substrate to another, change of limitations, inhibitions and overflow metabolism would cause characteristic changes in the heat evolution curves (Braissant et al., 2010b; von Stockar and van der Wieler, 1997 and Yi et al. (2000)). Specific growth rates of cells, even rates of individual metabolic reactions could be measured using power–time curves (Boe and Lovrien, 1990 and Guan et al., 2000). However, general experimental strategies for the detailed study of SSF allowing to monitor and describe growth of individual (average) colonies have not been developed.

The objective of the present study was to develop a general method for the investigation of bacterial growth, growth of the colonies in solid-state matrices using isothermal microcalorimetry in combination with other analytical methods. The central idea of the method developed is to use wide range of inoculation rates from 10^0 cfu/mL to 10^6 cfu/mL with the 10-fold increment in order to make possible investigation of the growth of individual bacterial colonies of different size. The growth of *Lactococcus lactis* in CRM agar media (CRM - *Carbohydrate restricted medium*) was studied using a multi-channel (24-channels) thermal activity monitor TAM III.

2. Materials and Methods

2.1. Inoculum preparation

The cultivated strain *L. lactis* subsp. *lactis* IL1403 was provided by Dr. Ogier from INRA (French National Institute for Agricultural Research, Jouy-en-Josas, France). Frozen storage cultures of *L. lactis* IL1403 were thawn and pregrown on Petri dishes with M17 Agar (LAB M, UK) for 24 h at 35 °C. One colony from a pregrown Petri dish was used as an inoculum for a 10 mL culture in liquid M17 Broth (FLUKA) at 35 °C. 1 mL of bacterial suspension grown overnight (exactly 24 h) was used as inoculum for the next liquid 10 mL M17 broth culture and grown 12–14 h which allowed the *L. lactis* IL1403 bacteria to reach the middle of the exponential growth phase. The number of bacteria was determined by plating on M17 Agar and incubating for 3 days at 35 °C, and average number of bacteria in the mid-exponential culture was determined $(4.15+/-0.15)*10^8$ cfu/mL. The inoculum needed for the experiments was prepared by diluting samples of the mid-exponential culture in peptone water. The calculated inoculum concentrations in calorimetric and in parallel samples were varied from 10^6 to 10^0 cfu/mL with the 10-fold increment. It was important to have the correct desired number of colonies in the samples as special attention was paid to study growth of individual colonies. From the practical point of view it was the most difficult to get up to about three colonies, and not more, in a sample (inoculation rate 10^0 cfu/mL). These colonies are separated from each other sufficiently and grow thus as independent individual colonies. True, in these cases direct visual inspection of samples after the completion of the experiments together with the analysis of power–time curves enabled to reach an unequivocal verdict on the success of the experiments. However, taking into account that the length of the experiments is typically about one month, the pure trial and error strategy was not the most feasible. Thus dilution procedures were developed and carefully implemented to assure that serial dilutions end with the desired inoculation rates. The exact following of the procedures of preparation of bacterial cultures described above which ensured high reproducibility at lower inoculation rates validated by direct counting of the colonies in the samples after the experiments, allowed to be confident that the number of bacteria in samples was also as assumed at inoculation rates higher than 10^2 cfu/mL.

2.2. Growth experiments

CRM Agar for growth media was prepared in 200 mL Erlenmeyer flasks with addition of glucose at concentrations 2, 10, 20 g/L and agar (1%, wt/vol). All solutions were sterilized by autoclaving at 121 °C for 15 min. After the addition of definite amount of the inocula to the flasks with media solutions at 45 °C (1%, v/v), suspensions were intensively stirred and then 3 mL from each flask were transferred into the autoclaved microcalorimetric ampoules. At least three ampoules were filled per sample solution. Filled ampoules were put into the microcalorimeter and experiments were run at 35 °C.

The remaining, after filling the calorimetric ampoules, solutions were shared by 2 mL to the plastic tubes and placed into thermostat at 35 °C – these were the parallels to the calorimetric experiments. Glucose and lactic acid concentrations and pH determined at time intervals were considered appropriate by bacterial calorimetric growth curves. The routine samples were taken in all cases at the beginning and at the end of both exponential and deceleration growth phases and also during the mentioned phases to get the representative data sets. Sample solutions for the HPLC measurements were prepared by homogenizing the solid media in Falcon tubes with appropriate amount of MilliQ water in proportion of 1:2.

2.3. Composition of the medium used

CRM agar growth medium was prepared by adding Agar No.1 (LAB M, UK)(1%, v/v) to CRM broth medium of the following composition: casitone (DIFCO) 20 g/L, Tween 80 1.08 g/L, sodium chloride (MERCK) 25 g/L, magnesium sulfate 0.58 g/L, manganese sulfate 0.3 g/L, ferric sulfate 0.06 g/L, B-vitamin solution (SIGMA) 10 ml/L, MES (2-[N-Morpholino]ethanesulfonic acid, SIGMA) 8.5 g/L.

2.4. Analytical methods

The TAM III (24-channels, *TA Instruments, Delaware, USA*), a type of heat conduction multi-channel microcalorimeter, was used to monitor the growth of cells. The construction and functioning of the TAM III calorimeter was previously described (Wadsö, 2002). HPLC Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 × 7.8 mm for measuring glucose and lactate concentrations (solvent–sulfuric acid solution (2.2 mL per 1 L MilliQ water), flow rate 0.6 mL/min, measuring temperature 35 °C). pH was measured with pH meter S20 Seven Easy Mettler Toledo with InLab 413 Mettler Toledo electrode. The dimensions of colonies were measured using the microscope Zeiss Axiovert 200 M with Zeiss AxioCam MRc5 camera and Zeiss AxioVs40 V45.0.0 software. The same system was used to photograph the colonies.

2.5. Analysis of power-time curves

The curves that are the output of microcalorimeter are called calorimetric thermograms or power–time curves. Power–time curve is a derivation of a standard bacterial growth curve in batch cultures. Calorimetric power–time curves (see Fig. 1) could be divided into three growth phases (Fig. 1a) essentially the same way as the bacterial growth curves, but with some reservations. Firstly goes the lag-phase, which determines the adjustment period during which bacterial cells adapt to the new environment and start to divide exponentially. The length of the lag-phase was determined as shown on Fig. 1b. The calculation procedure was practically the same as in Swinnen et al. (2004). However, it should be emphasized that in calorimetric experiments length of the lag-phase is defined besides physiology of the cell also by the level of sensitivity of a microcalorimeter. Sensitivity of TAM III in registration of heat is $7^{*}10^{-4}$ J (0.5 μW), which means that only a certain number of growing (metabolizing) bacteria can produce heat fluxes surpassing the sensitivity threshold of the instrument. It means that the lag-phase duration measured on the basis of calorimetric curves should be calculated taking into account the time when the heat produced by the growing bacterial population exceeds the

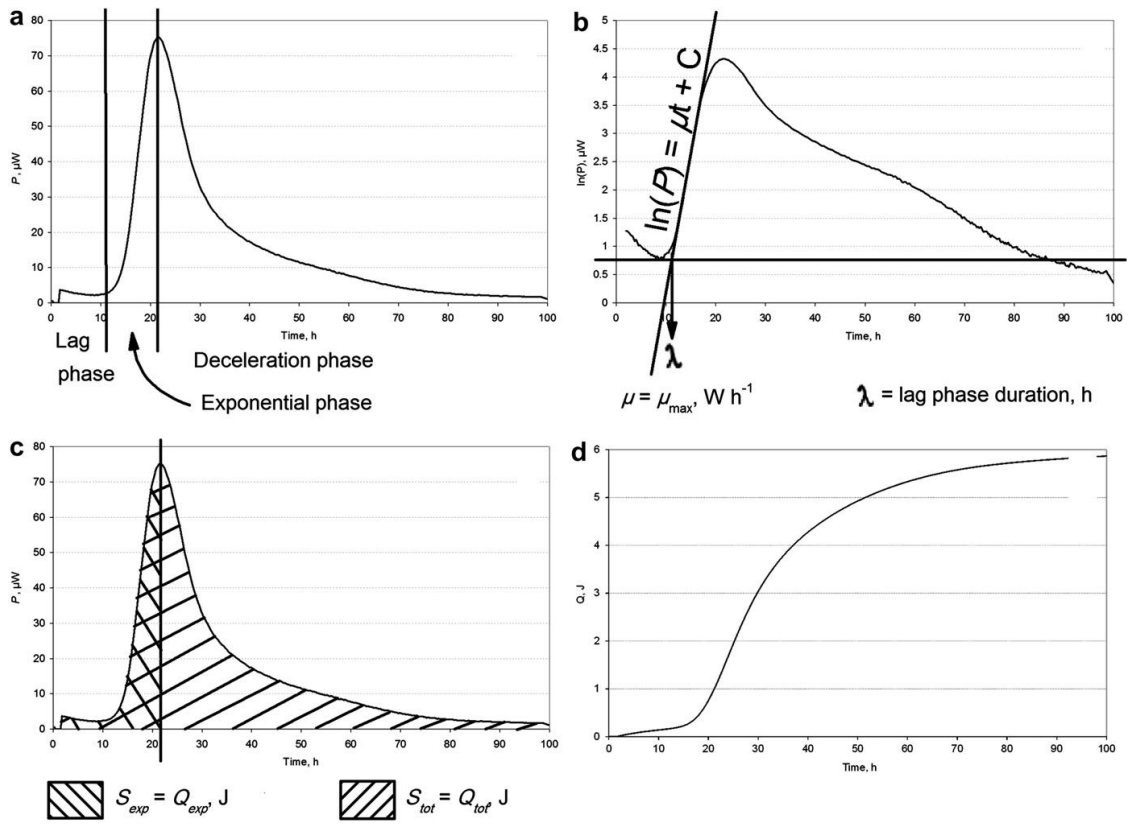


Fig. 1. The explanation of the processing of calorimetric power–time curves; (a) the division of calorimetric power–time curves into three phases – lag-phase, exponential growth phase, and deceleration phase; (b) determination of the maximum growth rate (μ_{max}, W/h) and the lag-phase duration (λ, h); (c) determination of heat amounts during the exponential phase (Q_{exp}, J) and during the total experiment (Q_{tot}, J); (d) an integrated power–time curve: accumulated heat change in time.

level of the sensitivity of the instrument. It should be noted that this is valid also if some other physical method is used, e.g. OD etc – see also Swinnen et al. (2004). The second phase of the power–time curve following lag-phase corresponds to the exponential growth phase during which bacteria grow with maximal growth rate μ_{max} possible in the experimental conditions under study – see Fig. 1. Taking into account that in the exponential growth phase the relationship between biomass concentration and specific growth rate may be described by the first order kinetics and assuming that the rate of biomass formation is proportional to the rate of heat production, maximum specific growth rate (μ_{max}) is measured as shown on Fig. 1b. The end of exponential phase of bacterial growth was defined in our data analysis by the peak of the power–time curves – see Fig. 1a. According to the results obtained by *plate count*, the end of the exponential growth could be put at some time after the peak of calorimetric curve, but then according to the power-time curve inhibitory processes and decrease of the growth rate have been obviously started already (see also Fig. 1d). Therefore, the exponential growth phase ending at the peak value of the power–time curves is justified. The parameters describing bacterial growth like Y_Q (J/cfu), heat Q_{exp} (J/mL), bacterial numbers N (cfu) etc. were calculated in the first place on the basis of power–time curves for the exponential growth phase.

The complex post-exponential growth phase that goes after the exponential growth phase is called deceleration phase in our studies, and it ends in principle with full stop of the growth of bacteria, and the experiment (see Fig. 1). The detailed systematic study of the deceleration phase is not a topic of the present paper.

The total heat produced during the whole process of growth (area between the power–time curve and baseline, Q_{tot}, J/mL) and the heat evolved during the exponential growth phase (area between the power–time curve, vertical line, that goes through the peak, and baseline, Q_{exp}, J/mL - see Fig. 1(a, c)) as well as the maximum growth rate (the slope of the tangent to the exponential phase, μ_{max}, W/h) and lag-phase duration (the crossing point of X-axis and the tangent, λ, h) (Fig. 1(b)) were determined using *TAM Assistant* program (v 0.9.1012.40, *SciTech Software AB, Thermometric AB*) and *Microsoft Excel*. The number of bacteria produced by the end of exponential phase was calculated on the basis of Q_{exp} (J/mL).

3. Results and discussion

3.1. Power–time curves of growth in liquid cultures

The growth of *L. lactis* was monitored both in agar gels and in broth in case of different inoculation rates (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu/mL) and glucose concentrations (2, 10, 20 g/L). The main topic of the present paper is investigation of the growth of the bacteria in solid 1% agar gels. However, some data obtained from the experiments with liquid cultures are used for better understanding and interpretation of solid-state growth curves.

The data obtained at glucose concentration of 2 g/L in case of broth and different inoculation rates (10^2–10^6 cfu/mL) are shown on Fig. 2. The same series of power–time curves were obtained for each mentioned initial concentration of glucose.

As seen from Fig. 2, length of lag-phase (for the definition see Section 2.5) of the growth curves is remarkably shortening on increasing of the initial inoculum. A separate study showed that in the case of *L. lactis* the level of sensitivity of the instrument is corresponding to growth of approximately 10^5 cells per mL. This would mean that the growth of the cells at inoculation rates higher than 10^5 cfu/mL is recorded from the beginning of the growth, after a "genuine" lag-phase, and at lower inoculation rates bacterial growth is recorded after the number of bacteria had reached 10^5 cfu/mL after the growth of different length. Indeed, changing the inoculum concentration we are turning the "observation window" of the instrument to the growth of populations of the same size but different history (past). In agreement with the said the experimentally determined length of the lag-phase should differ by 3.33 times generation times ($2^{3.33} = 10$) in case of different inoculation rates lower than 10^5 cfu/mL. Indeed, this was observed in our experiments – see Fig. 2 (the generation times in liquid cultures at high inoculation rates were about 1 h).

As seen from Fig. 2 maximal growth rates μ_{max} are slightly decreasing from 0.56 W/h at inoculation rate 10^6 cfu/mL to 0.44 W/h at inoculation rate 10^2 cfu/mL. The maximum values of the observed growth rates of the bacteria in the exponential phase in case of high inoculation rates is coinciding with the μ_{max} value measured for these bacteria in liquid cultures using other (conventional) methods. Decrease of the μ_{max} values at lower inoculations rates could be

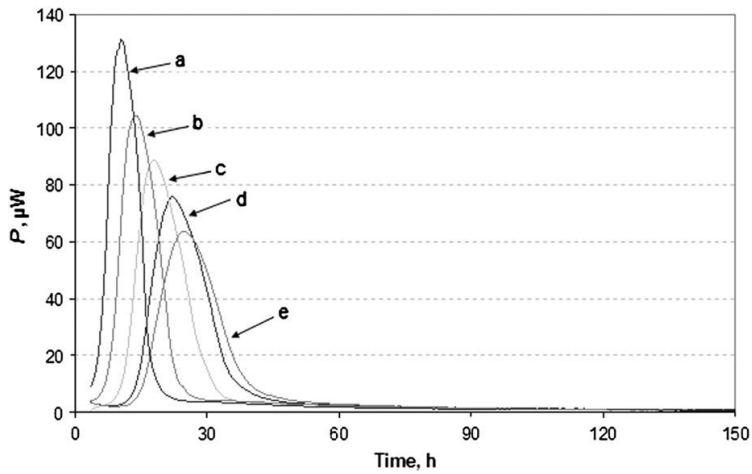


Fig. 2. Power–time curves describing bacterial growth in broth at different inoculation rates and at initial glucose concentration 2 g/L : a – inoculation rate - 10^6 cfu/mL; b – 10^5 cfu/mL; c – 10^4 cfu/mL; d – 10^3 cfu/mL; e – 10^2 cfu/mL.

explained by the fact that in these cases we observe the growth of the cultures with the same number of cells as in case of high inoculation rates, however, as the cells have been grown from the initial low numbers, they have synthesized growth inhibiting lactate to the media, which had led to the inhibition of the growth rate.

Besides μ_{max}, the exponential growth phase is characterized by the amount of heat produced by the growing cells that is proportional to the number of bacteria grown. Heat yield coefficient Y_Q (J/cfu) that links the amount of heat produced per one bacterium synthesized was calculated on the basis of the results obtained from the experiments of bacterial growth in liquid phase in case of different glucose concentrations (2, 5, 10, 20 g/L) and inoculation rates (10^2–10^6 cfu/mL). The *plate count* was carried out at the end of exponential growth phase and heat amounts produced were calculated from the power-time curves. The value of Y_Q (J/cfu) was determined to be $Y_Q = (2.58+/-0.44)*10^{-9}$ J/cfu. The Y_Q value is used for the description of exponential phase of the liquid culture also in the case of solid-state growth.

It was observed that the heat produced during the exponential growth phase in liquid cultures was practically the same at a value of 0.80+/−0.05 J/mL which corresponds to $3.10*10^8$ cells in case of different inoculation rates ranging from 10^2–10^6 cfu/mL and different glucose concentrations (2, 10 and 20 g/L). This fact means that during the exponential growth phase the same number of bacteria was produced independently from the size of inoculum which means that there was no substrate (glucose) limitations in the exponential phase and the termination of the exponential growth was caused by accumulating lactate either through direct inhibition or more probably through the change of the pH of the media. Essentially the same conclusion was drawn from the HPLC and pH measurements – data not shown.

3.2. Analysis of power–time curves of solid-state growth of bacteria

The calorimetric power–time curves of growth of the bacteria in solid matrices are presented on Fig. 3. As said above, the growth of *L. lactis* was monitored in agar gels in case of different inoculation rates (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cfu/mL) and glucose concentrations (2, 10, 20 g/L).

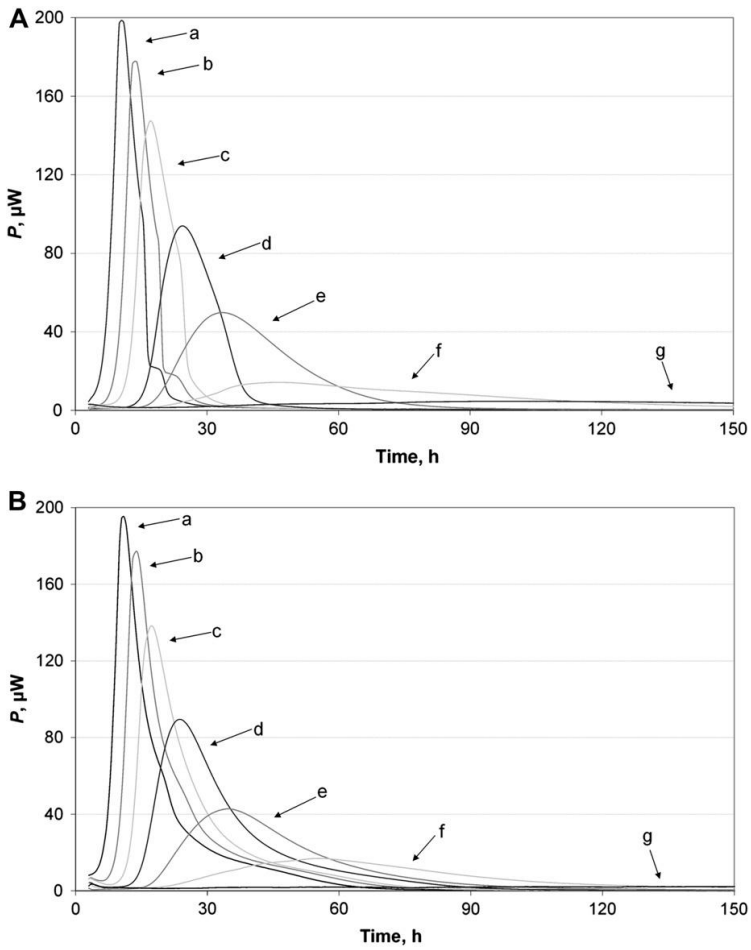


Fig. 3. Bacterial growth monitored by calorimetric power–time curves with different inoculation rates in case of two different glucose concentration: (A) 2 g/L and (B) 10 g/L in solid agar (1%) gels: a – 10^6 cfu/mL; b – 10^5 cfu/mL; c – 10^4 cfu/mL; d – 10^3 cfu/mL; e – 10^2 cfu/mL; f – 10^1 cfu/mL; g – 10^0 cfu/mL.

Maximum growth rates μ_{max} were determined from the power–time curves as described in *Materials and Methods*. On Fig. 4 the intervals where the maximum growth rate is constant are marked with bold black lines. Even at lower inoculation rates (10^1 and 10^0 cfu/mL) the linear correlation coefficients were equal to $R^2 = 0.999$ for a period of time up to 12 h indicating that bacteria were growing exponentially.

The values of the maximum growth rates measured from the power–time curves presented on Fig. 3 and also from the curves not presented in the text are given in Fig. 5. As seen from Fig. 5 the values of maximum growth rates μ_{max} depend notably on the inoculation rates but do not depend on the concentration of glucose in the media. It is seen also that in the case of inoculation rates 10^4–10^6 cfu/mL the μ_{max} values about 0.65 W/h are slightly higher, but in the same range with 0.54 W/h which is the μ_{max} value in broth. This means that in these cases bacteria grow in solid matrix with the same or even higher rate as in broth, and it could be concluded that no diffusion limitation of the growth by glucose was observed in case of growth of small colonies in 1% agar gels – the small colonies possibly containing one bacterium in the beginning are growing with the maximum growth rate μ_{max} characterizing the bacteria in excess of glucose.

The lower recorded growth rates μ_{max} of the colonies at lower inoculation rates are explained by the fact that in these cases we monitored the growth of the bigger colonies growing in the conditions of higher concentration of the lactate synthesized within the colonies and to the bulk media during the latent period of measurements of the heat flow. Comparison of the decrease of the growth rates in liquid culture and in solid-state at the same low inoculation rates (lower than 10^3 cfu/mL) showed that the decrease was more pronounced in the latter case. This could be explained by assuming that lactate is accumulating within the colonies and thus inhibiting the bacterial growth more efficiently than lactate equally distributed in the broth in the case of liquid cultures.

The heats produced during the exponential growth phase at different glucose concentrations and inoculation rates in the case of solid-state growth experiments are presented on Fig. 6.

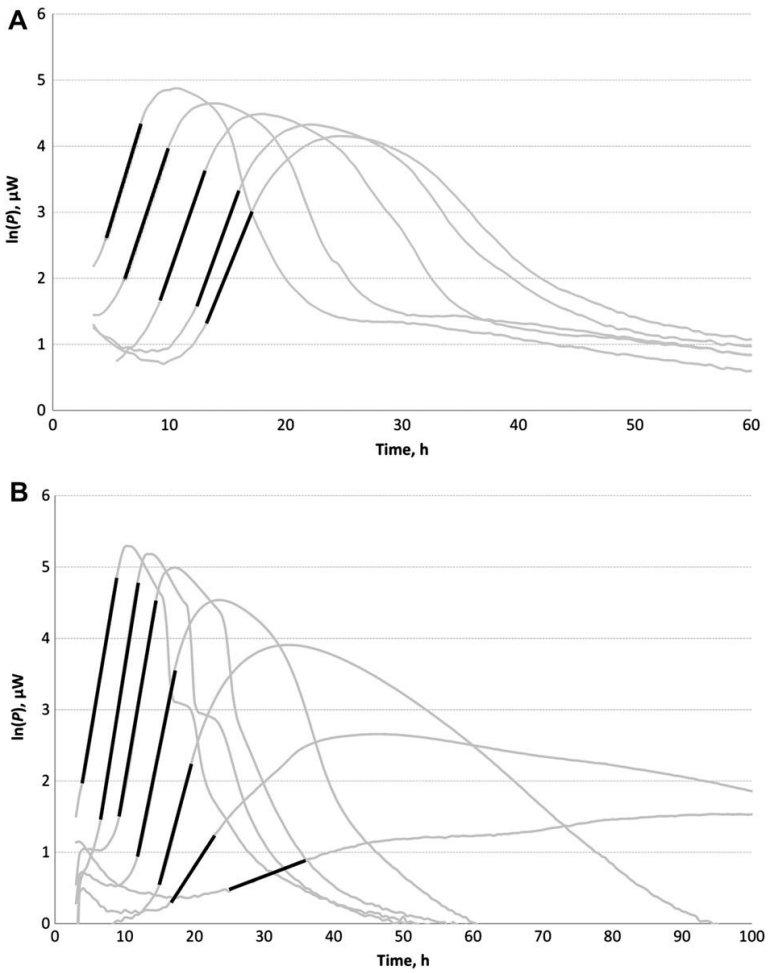


Fig. 4. The determination of maximum specific growth rate μ_{max} (W/h) from the calorimetric power–time curves in broth (A) and in solid agar gels (B) in case of different inoculation rates from 10^0 till 10^6 cfu/mL with the increment of 10-fold. The bold solid lines show the time interval of the curve where the specific growth rate is maximum and constant during the exponential growth phase.

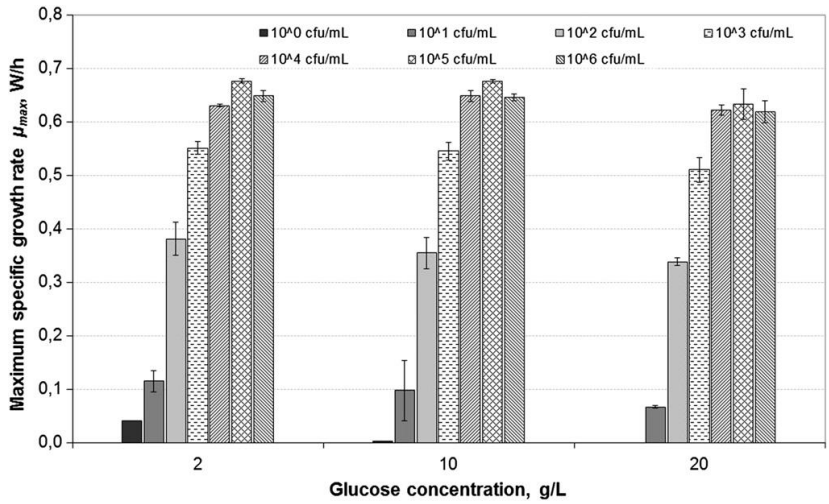


Fig. 5. The maximum specific growth rates at different initial glucose concentrations (2, 10, 20 g/L) and various inoculation rates from 10^0 cfu/mL to 10^6 cfu/mL.

According to Fig. 6, the heat produced during the exponential phase in the solid matrix has the highest value 0.65 J/mL ($2.52*10^8$ cfu/mL) at inoculation rates 10^2–10^4 cfu/mL and it decreases about 50% in case of inoculation rate decrease and increase. However, as the numbers of bacteria at the end of exponential growth in solid-state matrices are practically the same as in the liquid cultures (about 10^8 cfu/mL), thus the same conclusion – as for the liquid cultures that accumulating lactate was responsible for the termination of exponential growth – could be drawn. As said, pronounced decrease of the numbers of bacteria at the end of the exponential phase at lower and higher inoculation rates studied – 10^0–10^1 cfu/mL, and 10^5–10^6 cfu/mL respectively, was observed in solid-state growth. In case of lower inoculation rates mentioned this could be explained by the more pronounced inhibitory effect of lactate in solid-state cultures – by higher local concentrations of lactate in the colonies due to the diffusion limitations in comparison with the equal uniform distribution of lactate in liquid cultures. In case of high inoculation rates it could be assumed that the closely located growing colonies in solid-state matrices start to influence each other through diffusion of lactic acid earlier than in broth with the same number of bacteria.

Microcalorimeter is measuring the total heat evolution of a sample, and this data should be recalculated into growth of a culture, and eventually into growth of an average colony taking into account sensitivity of the instrument and inoculation rate. The scheme of calculation of the numbers of bacteria in case of different inoculation rates used by us is illustrated on a schematic drawing on Fig. 7. It is possible to calculate heats evolved during the growth,

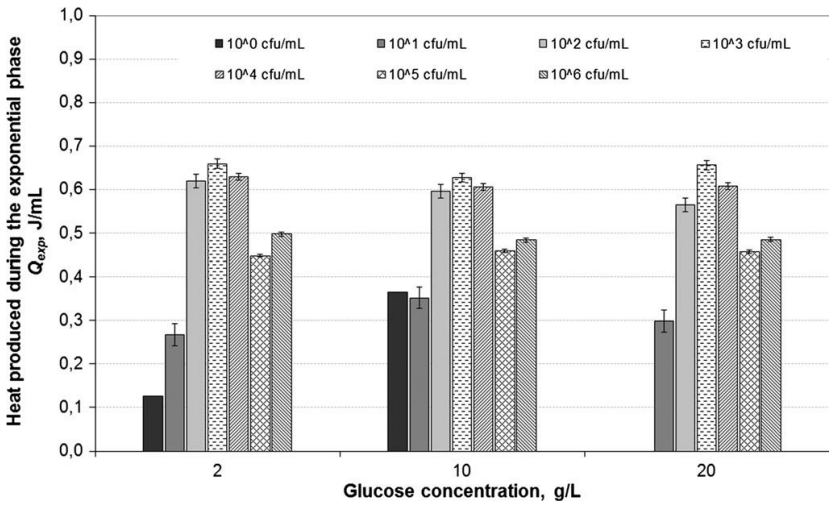


Fig. 6. The heat produced during the bacterial growth in exponential phase in agar at different initial glucose concentrations (2, 10, 20 g/L) and various inoculation rates from 10^0 cfu/mL to 10^6 cfu/mL.

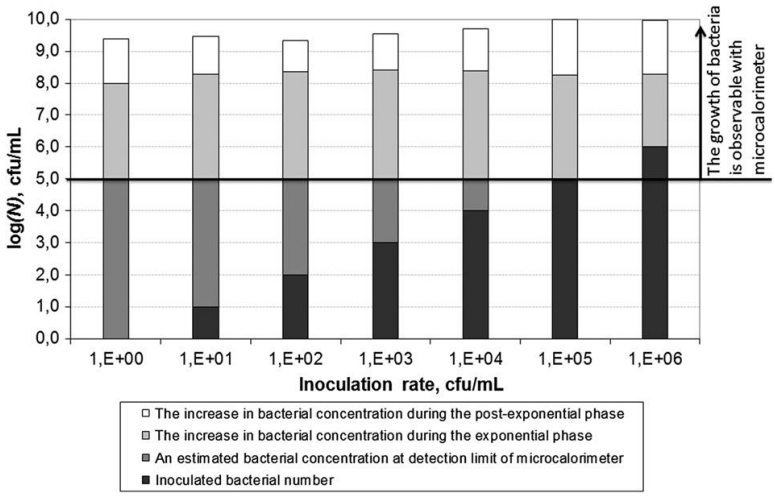


Fig. 7. The numbers of bacteria of different "origin" (inoculum, exponentially grown, grown during the post-exponential growth phase) in samples, cultures at different inoculation rates. Number of the bacteria grown in a sample is calculated by subtracting from the total number of bacteria, or from the number of bacteria at the end of exponential phase the number of bacteria inoculated into the sample in the beginning of the experiment. The number of bacteria in an average colony is calculated by dividing the number of bacteria grown in a sample by the inoculation rate. The detection limit of the instrument, and the number of bacteria needed to grow until power–time curves could be measured, are also presented.

numbers of bacteria in the sample, colonies etc on the basis of the Figure, and understand how "lower inoculation rate effects" and "colony-effects" should lead to the differences of the power–time curves in solid-state growth and growth in liquid cultures – as discussed already above. It should be mentioned that the total numbers of bacteria in the sample were calculated using microscopy (see Section 3.3). Microscopy was found more suitable for the determination of the total numbers of cells in average colonies in the end of the growth than power–time curves. However, these issues need more detailed further investigations.

A snapshot of the solid culture at the end of exponential growth phase calculated on the basis of the experimental results obtained is presented in Table 1. Taking into account the inoculation rates, it is possible to calculate distances of the colonies in idealized grids (third column).

Colony distribution in agar gel assumed by us is similar to that calculated for the spatial distribution of colonies in a model cheese by Jeanson et al. (2011). It was also shown by them that mean distances calculated from the image analyses of experimental data perfectly fitted the theoretical calculations. The distances between the colonies increase with inoculation rate decrease and colonies are randomly distributed through the matrix. At inoculation rate of 10^6 cfu/mL the distances between the colonies about 50–52 μm were determined according to Jeanson et al. (2011), whereas 100 μm was calculated in our study.

Numbers of bacteria in average colonies at the end of exponential growth phase calculated using Y_Q in case of different glucose concentrations and inoculation rates are presented in the fifth column of the Table 1.

Radii of average (spherical) colonies at the end of exponential growth were calculated using Kepler's conjecture. (Hales, 1994 and Hsiang, 1993). It should be noticed that the colonies are rather lenticular than spherical in the agar gels, but for bigger intuitive transparency a simplified calculation using spherical shapes of colonies were carried out in the paper.

Radii of average spheres from where glucose was scavenged by average colonies were calculated using the HPLC data on glucose

Table 1
Calculated parameters describing growth of the bacterial colonies at the end of exponential growth phase at different initial glucose concentrations (2, 10, 20 g/L) and inoculation rates (from 10^0 cfu/mL to 10^6 cfu/mL): yield coefficient Y_{XS} (g/g), amounts of produced heat during the exponential phase - Q_{exp} (J), the number of bacteria N_{exp} (cfu/col) formed in an average colony with the radius R_{COL} (μm), and R_{GLC} (μm) is the radius of an area from where glucose was scavenged for growth of an average colony.

Inoculation rate, cfu/mL	GLC, g/L	Distance between colonies, μm	Q_{exp}, J/mL	The number of bacteria[a] N_{exp}, cfu/col	R_{COL}[b], μm	R_{GLC}[c], μm	Y_{XS}, g/g
10^0	2	10000	0.13	4.91E+07	185	5149	1.36E-02
	10		0.36	1.41E+08	263	4281	1.36E-02
10^1	2	4642	0.27	1.03E+07	110	3063	1.36E-02
	10		0.35	1.36E+07	142	1963	1.36E-02
	20		0.30	1.16E+07	150	1475	1.36E-02
10^2	2	2154	0.62	2.40E+06	68	1876	1.37E-02
	10		0.60	2.31E+06	67	1086	1.36E-02
	20		0.57	2.19E+06	66	845	1.36E-02
10^3	2	1000	0.66	2.55E+05	32	876	1.44E-02
	10		0.63	2.55E+05	32	510	1.45E-02
	20		0.66	2.54E+05	32	381	1.85E-02
10^4	2	464	0.63	2.44E+04	15	409	1.34E-02
	10		0.61	2.34E+04	14	238	1.31E-02
	20		0.61	2.35E+04	14	174	1.69E-02
10^5	2	215	0.45	1.73E+03	6	190	9.53E-03
	10		0.46	1.78E+03	6	109	1.02E-02
	20		0.46	1.77E+03	6	77	1.45E-02
10^6	2	100	0.50	1.92E+02	3	88	1.05E-02
	10		0.48	1.87E+02	3	54	9.17E-03
	20		0.49	1.88E+02	3	37	1.40E-02

[a] The number of bacteria was calculated using the data on the heat evolved during the exponential growth, and Y_Q value determined.
[b] Radius of an average spherical model colony calculated from an average volume of the colony calculated using Kepler's conjecture of bacterial packing in colonies, sizes of which were measured using microscope.
[c] Radius of an average spherical area from where glucose was used by an average colony – radius was calculated from the volumes of glucose consumed by one colony determined using HPLC analysis.

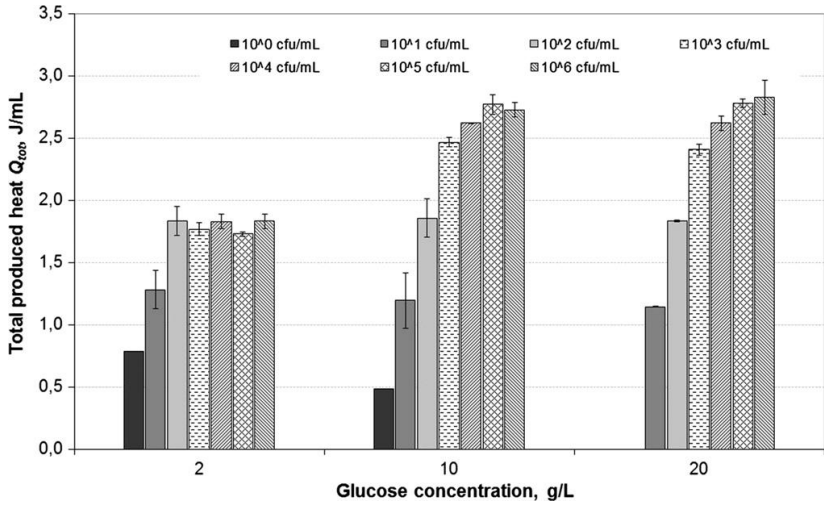


Fig. 8. Total heat produced during bacterial growth in 1% agar gels at different initial glucose concentrations (2, 10, 20 g/L) and various inoculation rates from 10^0 cfu/mL to 10^6 cfu/mL.

consumption (data not shown) – see the Table 1. As seen also in Table 1 the spheres of influence of average colonies do not overlap heavily which is in good agreement with the conclusion made on the basis of other data that during the exponential growth phase, colonies are growing essentially separately.

Using the data on heat amounts produced Q_{exp} and glucose consumed the values of yield coefficient Y_{XS} (g/g) were calculated for all inoculation rates 10^0–10^6 cfu/mL and initial glucose concentrations 2, 10 and 20 g/L for the growth during the exponential phase, for the conditions where there were no strong inhibitory effects influencing the growth. First, the bacterial number per ampoule was calculated on the basis of the heat amount produced during the exponential phase using heat yield coefficient Y_Q. To calculate the biomass in the sample the number of bacteria should be multiplied by the dry weight of one cell. The mass of a cell (an average cell) was obtained two different ways. Data on the volume and the density of the cell was used in the first method. According to literature (Kokkinos et al., 1998) the size of *L. lactis* subsp. *lactis* is around 0.35 μm^3 and multiplying it with the cell density (ρ = 1 g/cm^3) the dry weight of the cell would be $(1.05+/-0.49)*10^{-13}$ g. Mass of an average cell was also experimentally measured in chemostat cultures of *L. lactis*. The concentration of the bacteria was determined using *plate count*; dry weight of biomass was determined using gravimetry. Average mass of cells was calculated to be $(2.08+/-0.54)*10^{-13}$ g, which matches well the result of the first method. The values of growth yield coefficients Y_{XS} determined are presented in the last column of the Table 1. According to the data in Table 1, the Y_{XS} is constant irrespective of initial substrate concentration at inoculation rates lower than 10^3 cfu/mL. With further inoculation rate increase in the interval of 10^3–10^6 cfu/mL the decrease in Y_{XS} is observed which means that bacteria in smaller colonies are spilling more energy in comparison with bigger colonies – at higher inoculation rates in case of faster growing cells more substrate is consumed to synthesize the same amount of biomass. The opposite trend was observed in liquid continuous culture in Lahtvee et al. (2011). This could be explained assuming that *L. lactis* exercises different metabolic strategies in broth and solid-state media.

If the heats evolved (number of bacteria grown) during the exponential growth phase are practically the same at all glucose concentrations (see Fig. 6) then the total heats evolved during the complete growth processes are remarkably different – see Fig. 8.

The total amount of heat produced during the complete growth was in the range of 1.5–2.8 J/mL in practically all cases indicating that most of the biomass, about 3–5 times more than by the end of the exponential growth phase, was synthesized during the post-exponential growth phase. However, there are notable differences

Table 2
Consumed glucose (GLC, g/L) and produced lactic acid (LA, g/L) concentrations and pH values at the end of bacterial growth in solid matrices at different initial glucose concentrations (2, 10, 20 g/L) and inoculation rates (from 10^0 cfu/mL to 10^6 cfu/mL).

Initial glucose concentration, g/L	2 g/L			10 g/L			20 g/L		
Growth parameters at the end of experiment Inoculation rate, cfu/mL	GLC, g/L	LA, g/L	pH	GLC, g/L	LA, g/L	pH	GLC, g/L	LA, g/L	pH
10^0	0.88	1.00	5.00	2.07	1.03	4.97	4.20	0.74	–
10^1	1.46	1.56	4.62	2.88	1.43	4.70	4.30	1.06	4.86
10^2	1.85	2.15	4.59	3.42	2.30	4.45	4.65	2.04	4.51
10^3	1.88	1.98	4.66	3.70	3.19	4.18	4.70	3.03	4.13
10^4	1.91	1.92	4.59	3.75	3.34	4.03	4.89	3.15	3.94
10^5	1.91	1.85	4.60	3.65	3.45	4.02	4.29	3.27	3.97
10^6	1.93	1.88	5.52	4.29	3.40	4.02	4.70	3.36	3.94

– data is absent.

between the glucose concentration 2 g/L, and other glucose concentrations studied. In the case of initial glucose concentration of 2 g/L the total heat evolved was 1.8 J/mL in all inoculation rates except the lowest -10^0 and 10^1 cfu/mL. This fact supports the conclusion according to which the number of bacteria in the end of the growth was limited by the amount of glucose present in the media at 2 g/L (Table 2). This conclusion was supported also by the HPLC measurements, which showed the exhaustion of glucose in the media in the case of liquid cultures (Kabanova et al., 2009). As seen from the Table 2, the energy metabolism of the *L. lactis* cells was homofermentative in the whole range of inoculation rates in the case of glucose concentration 2 g/L. Taking into account that more than 2/3 of the cells were synthesized during the post-exponential growth phase, the conclusion could be drawn that the energy metabolism was not changed during the whole growth of bacteria on 2 g/L, and Y_Q value calculated for the exponential growth phase could be used for the characterization of the growth during the whole experiment in this case.

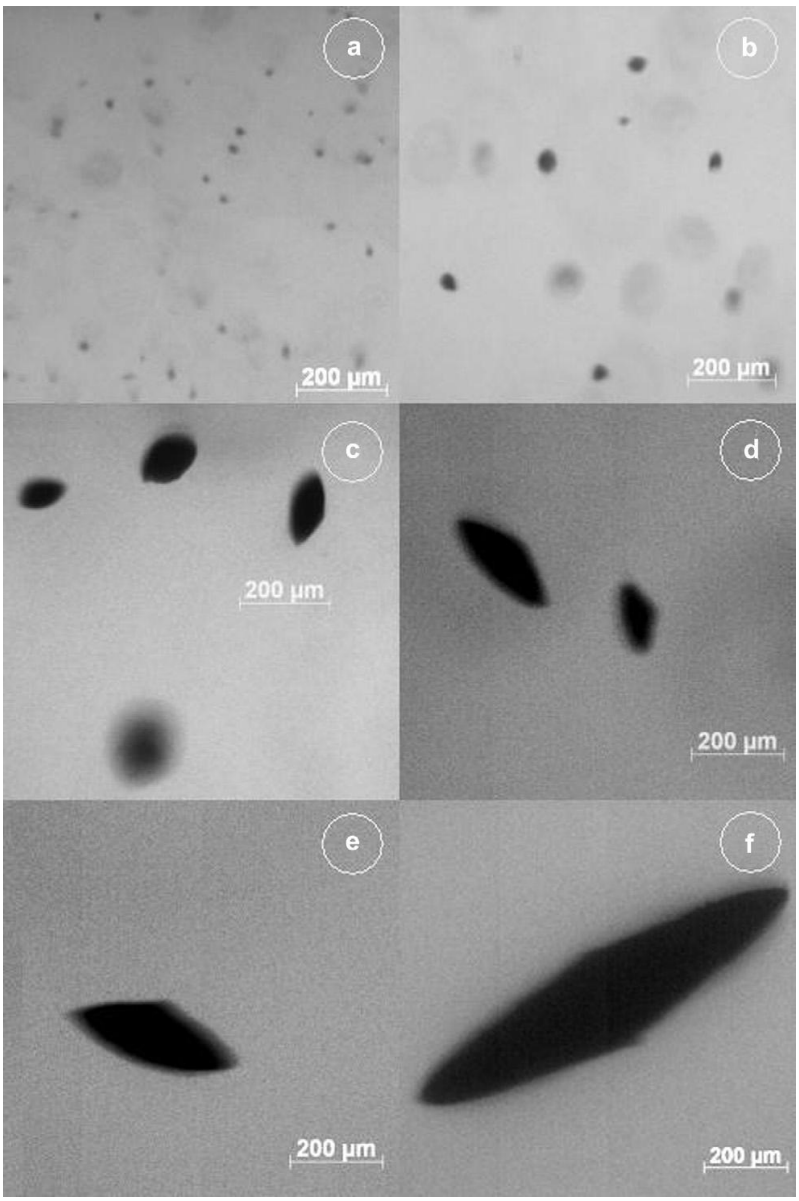


Fig. 9. Photos of bacterial colonies in agar (1% wt/vol) in case of different inoculation rates (cfu/mL) under 10× magnification: (a) 10^6 cfu/mL (b) 10^5 cfu/mL (c) 10^4 cfu/mL (d) 10^3 cfu/mL (e) 10^2 cfu/mL f) 10^1 cfu/mL.

Table 3
Changes in dimensions (μm) of the colonies in sample depending on initial glucose concentration (g/L) and on inoculation rate (cfu/mL). Standard deviations of the values are also presented.

Inoculum concentration, cfu/mL	Initial glucose concentration, g/L	Colony dimension, μm	Standard deviation, μm
10^0	2	2206.2	
	10	1439.9	
	20	1093.5	
10^1	2	775.7	147.2
	10	1060.5	295.5
	20	1046.2	195.9
10^2	2	332.4	16.3
	10	416.7	29.8
	20	384.2	31.8
10^3	2	201.0	28.7
	10	202.5	36.8
	20	184.8	37.5
10^4	2	92.7	24.9
	10	86.4	16.4
	20	105.5	26.5
10^5	2	45.3	9.5
	10	45.2	5.3
	20	49.5	5.7
10^6	2	19.7	3.5
	10	23.2	4.2
	20	21.1	2.6

Amounts of heat evolved during the growth at high glucose concentrations −10 g/L and 20 g/L are remarkably higher than in case of 2 g/L. However, in these cases the initial concentrations of glucose could support also growth of notably higher numbers of cells $-2*10^9$–$4*10^9$ bacteria per mL. It was shown by us that high lactic acid concentration was the factor eventually stopping the growth in these cases – see also (Kabanova et al., 2009). The mechanisms leading to the change of the metabolism of the bacteria from homofermentative to more efficient heterofermentative mechanisms ensuring prolongation of the production of heat, prolongation of post-exponential growth at high glucose concentrations (10 g/L, 20 g/L), however, require further investigation. Uncoupling of energy production is the most probable factor involved in determining the peculiarities observed as proposed earlier also by Belaich et al. (1972); Senez (1962) and Russel and Cook (1995).

3.3. The sizes and shapes of the colonies determined by microscopy

The lenticular shape of the colonies could be visually well observed starting from 10^4 cfu/mL and with further decrease of colony number in samples (Fig. 9). The shape of the colonies is hardly observable at higher than 10^4 cfu/mL inoculation rates using 10-fold magnification, but taking into account the structure of agar gel it could be assumed that the colonies are also lenticular in these conditions. According to Pernodet et al. (1997) the sizes of the agar gel pores are around 350–400 nm in case of the agar concentration of 1% (wt/vol). When bacteria are starting to multiply in solid media, they are forming colonies in the pores of agar gel overcoming the resistance of the media by breaking the bonds between the chains of polysaccharides and thus "enlarging" pores. It could be assumed that colonies are growing lenticular-shaped forced by agar structure as it is easier to break bonds between the chains and form lenticular colonies than stretching the interchain bonding, and form spherical colonies.

The size of an average colony is varying from 17 μm at the highest inoculation rate 10^6 cfu/mL to 2200 μm at the inoculation rate 10^0 cfu/mL (see Table 3). As it is also seen from Table 3, standard deviation of colony sizes is high at low inoculation rates (10^1 cfu/mL) and vice versa. Limited number of colonies (less than 10–20) could be orientated in solid samples differently, and this leads to the differences in sizes and the distances between the colonies, that in its turn also influences the size of the colonies. At higher inoculation rates the colonies are evenly distributed through the matrix in sample and thus their dimensions are less variable. The same circumstances are determining the variability of power–time curves. In case of higher inoculation rates (number of colonies higher than 10^2) the differences are "averaged out" and in these conditions the reproducibility of the microcalorimetric method is (very) high - $\sigma = 0.37$ μW.

The sizes of the colonies determined by microscopy were used for the calculation of the numbers of cells in the colonies in the end

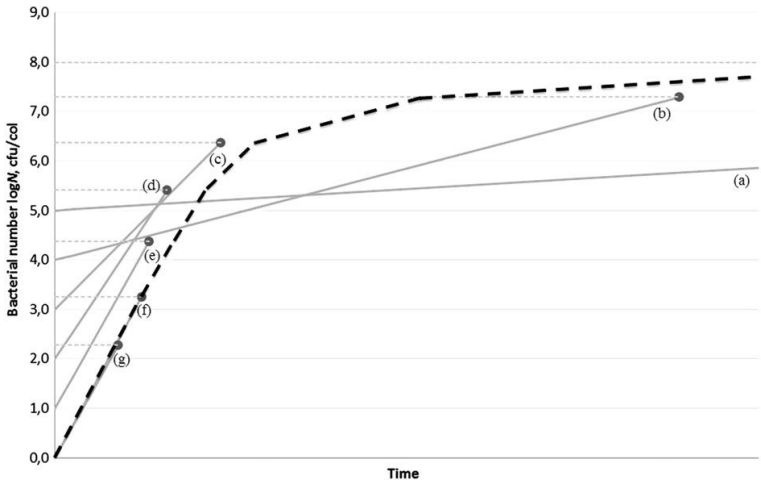


Fig. 10. The growth of an averaged colony of *L. lactis* in agar gel constructed on the basis of the data obtained from the analysis of the calorimetric power–time curves. Solid lines show the μ_{max} values and the ranges of colony sizes where these values were measured. Broken line is a constructed growth curve of an average colony of *L. lactis* in solid agar gel.

of the growth. For more detailed comparison of the microscopic and calorimetric data, the values of Y_Q for the deceleration phase of growth in different conditions should be determined more precisely.

3.4. The model of growth of an average individual colony of L. lactis in agarose gel

A model of the changes of specific growth rate of individual (average) colonies of different sizes using data on maximum specific growth rates μ_{max} (Fig. 5) and on the numbers of bacteria, both, in the beginning and in the end of exponential growth phase in different growth conditions was developed (Fig. 10). As seen from Fig. 10 an average colony is growing exponentially until the number of bacteria reaches approximately 10^5, and radius of an average colony is 46 μm. Growth rate of bigger colonies containing 10^5–10^8 cells is decreasing due to the accumulation of lactic acid leading to the lowering of pH. The growth of bacteria inside the colonies is restricted and the size of the colony is increasing thanks to the division of the bacteria on the surface of the colony (McKay et al., 1997). Power–time curves of the growth of bacteria in big colonies at the inoculation rates 10^0 and 10^1 cfu/mL are observed in the microcalorimeter in the conditions where the number of bacteria in colony is reaching 10^4–10^5 bacteria and lactate is starting to inhibit the growth. This fact is explaining lower growth rates and heat productions at 10^0 and 10^1 cfu/mL in comparison with higher inoculation rates (Figs. 3,5 and 8).

Similar results were obtained by Malakar et al. (2002a) who showed that mass transfer limitations can be significant in the development of bacterial colonies in solid systems if the colonies contain about 10^5 or more cells. Growth of smaller colonies containing less than 10^5 cells in solid matrices at higher inoculation rates than 10^2 cfu/mL is similar to growth in broth cultures – small colonies have sizes at which mass transfer limitations are not significant.

4. Conclusion

The present study has shown that the maximal specific growth rate in small growing colonies in agar gel at high inoculation rates was practically the same as in the liquid cultures. Small colonies grow with the maximal growth rate until 10^5 cells per colony, the growth rate of bigger colonies was gradually decreasing, however, exponential growth with lower than maximal growth rates was observed in big colonies until 10^8 cells per colony. It was shown on the basis of HPLC and pH measurements that accumulation of lactate and decrease of pH was the reason for the termination of the exponential growth of the colonies. A prolonged post-exponential growth was observed at higher initial glucose concentrations (10 g/L and 20 g/L) with the considerable change towards more efficient intracellular metabolism in the end of growth. Yield coefficients $Y_Q = (2.58+/-0.44)^*10^{-9}$ J/cfu, and $Y_{XS} = (1.34+/-0.22)^*10^{-2}$ g/g were determined. Use of these constants together with the dry weight of an individual cell of *L. lactis* (1.05^*10^{-13} g) allowed develop a quantitatively coherent growth description of an average colony from one cell to 10^8 cells on the basis of power–time curves measured.

All the observations mentioned show that microcalorimetry used in combination with other relevant measurements is a very powerful instrument in studying quantitative detailed peculiarities of solid-state fermentations.

Acknowledgments

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

Alklint, C., Wadsö, L., Sjöholm, I., 2005. Accelerated storage and isothermal microcalorimetry as methods of predicting carrot juice shelf-life. Journal of the Science of Food and Agriculture 85, 281–285.
Andersen, A.Z., Carvalho, A.L., Neves, A.R., Santos, H., Kummer, U., Olsen, L.F., 2009. The metabolic pH response in *Lactococcus lactis*: an integrative experimental and modelling approach. Computational Biology andChemistry 33, 71–83.
Antwi, M., Geeraerd, A.H., Vereecken, K.M., Jenne, 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.
Beezer, A.E., 2001. An outline of new calculation methods for the determination of both thermodynamic and kinetic parameters from isothermal heat conduction microcalorimeter. Thermochimica Acta 380, 205–208.
Belaich, J.P., Belaich, A., Simonpietri, P., 1972. Uncoupling in bacterial growth: effect of pantothenate starvation on growth of *Zymomonas mobilis*. Journal of General Microbiology 70, 179–185.
Birou, B., Marison, I.W., Von Stockar, U., 1987. Calorimetric investigation of aerobic fermentations. Biotechology and Bioengineering 30, 650–660.
Boe, I., Lovrien, R., 1990. Cell counting and carbon utilization velocities via microbial calorimetry. Biotechnology and Bioengineering 35, 1–7.
Braissant, O., Wirz, D., Goepfert, B., Daniels, A.U., 2010a. 'The heat is on': rapid microcalorimetric detection of mycobacteria in culture. Tuberculosis 90, 57–59.
Braissant, O., Wirz, D., Goepfert, B., Daniels, A.U., 2010b. Use of isothermal microcalorimetry to monitor microbial activities. FEMS Microbiology Letters 303, 1–8.
Guan, Y., Evans, P.M., Kemp, R.B., 2000. Specific heat flow rate: an on-line monitor and potential control variable of specific metabolic rate in animal cell culture that combines microcalorimetry with dielectric spectroscopy. Biotechology and Bioengineering 58, 464–477.
Gustafsson, L., 1991. Microbiological calorimetry. Thermochimica Acta 193, 145–171.
Hales, T.C., 1994. The status of the Kepler conjecture. Mathematical Intelligencer 16, 47–58.
Hsiang, W.-I., 1993. On the sphere packing problem and the proof of Kepler's conjecture. Proceedings of Kaist Mathematics Workshop, 41–52.
Hutkins, R.W., Nannen, N.L., 1993. pH Homeostasis in lactic acid bacteria. Journal of Dairy Science 76, 2354–2365.
Jeanson, S., Chadoeuf, J., Madec, M.N., Aly, S., Floury, J., Brocklehurst, T.F., Lortal, S., 2011. Spatial distribution of bacterial colonies in a model cheese. Applied and Environmental Microbiology 77, 1493–1500.
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.
Kokkinos, A., Fasseas, C., Eliopoulos, E., Kalantzopoulos, G., 1998. Cell size of various lactic acid bacteria as determined by scanning electron microscope and image analysis. Lait 78, 491–500.
Lahtvee, P.-J., Adamberg, K., Arike, L., Nahku, R., Aller, K., Vilu, R., 2011. Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. Microbial Cell Factories 10, 1–12.
Lamprecht, I., 2003. Calorimetry and thermodynamics of living systems. Thermochimica Acta 405, 1–13.
Ma, J., Qi, W.T., Yang, L.N., Yu, W.T., Xie, Y.B., Wang, W., Ma, X.J., Xu, F., Sun, L.X., 2007. Microcalorimetric study on the growth and metabolism of microencapsulated microbial cell culture. Journal of Microbiological Methods 68, 172–177.
Malakar, P.K., Brocklehurst, T.F., Mackie, A.R., Wilson, P.D.G., Zwietering, M.H., van't Riet, K., 2000. Microgradients in bacterial colonies: use of fluorescence ratio imaging, a non invasive technique. International Journal of Food Microbiology 56, 71–80.
Malakar, P.K., Martens, D.E., Van Breukelen, W., Boom, R.M., Zwietering, M.H., van't Riet, K., 2002a. Modeling the interactions of *Lactobacillus curvatus* colonies in solid medium: consequences for food quality and safety. Applied and Environmental Microbiology 68, 3432–3441.
Malakar, P.K., Zwietering, M.H., Boom, R.M., Brocklehurst, T.F., Wilson, P.D.G., Mackie, A.R., van't Riet, K., 2002b. Diffusion of lactic acid in a buffered gel system supporting growth of *Lactobacillus curvatus*. Journal of the Science of Food and Agriculture 82, 1729–1734.
Malakar, P.K., Barker, G.C., Zwietering, M.H., van't Riet, K., 2003. Relevance of microbial interactions to predictive microbiology. International Journal of Food Microbiology 84, 263–272.
McKay, A.L., Peters, A.C., Wimpenny, J.W.T., 1997. Determining specific growth rates in different regions of *Salmonella typhimurium* colonies. Letters in Applied Microbiology 24, 74–76.

Menert, A., Liiders, M., Kurisoo, T., Vilu, R., 2001. Microcalorimetric monitoring of anaerobic digestion processes. Journal of Thermal Analysis Calorimetry 64, 281–291.
Mercade, M., Lindley, N.D., Loubiere, P., 2000. Metabolism of *Lactococcus lactis* subsp. *cremoris* MG 1363 in acid stress conditions. International Journal of Food Microbiology 55, 161–165.
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.
Mitchell, A.J., Wimpenny, J.W.T., 1997. The effects of agar concentration on the growth and morphology of submerged colonies of motile and non-motile bacteria. Journal of Applied Microbiology 83, 76–84.
Papagianni, M., Avramidis, N., Filiousis, G., 2007. Glycolysis and the regulation of glucose transport in *Lactococcus lactis spp. lactis* in batch and fed-batch culture. Microbial Cell Factories 6, 16.
Pelaez, C., Requena, T., 2005. Exploiting the potential of bacteria in the cheese ecosystem. International Dairy Journal 15, 831–844.
Pernodet, N., Maaloum, M., Tinland, B., 1997. Pore size of agarose gels by atomic force microscopy. Electrophoresis 18, 55–58.
Russel, J.B., Cook, G.M., 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiological Reviews 59, 48–62.
Senez, J.C., 1962. Some considerations on the energetics of bacterial growth. Bacteriological Reviews 26, 95–107.
Settanni, L., Giancarlo, M., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiology 27, 691–697.
von Stockar, U., van der Wieler, L.A.M., 1997. Thermodynamics in biochemical engineering. Journal of Biotechnology 59, 25–37.
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.
Theys, T.E., Geeraerd, A.H., Verhulst, A., Poot, K., Van Bree, I., Devlieghere, F., Moldenaers, P., Wilson, D., Brocklehurst, T., Van Impe, J.F., 2008. Effect of pH, water activity and gel micro-structure, including oxygen profiles and rheological characterization, on the growth kinetics of *Salmonella typhimurium*. International Journal of Food Microbiology 128, 67–77.
Vandenhove, H., 1998. Microcalorimetric characterization of bacterial inocula. Advanced Instrumentation, Data Interpretation and Control of Biotechnological Processes 25, 121–158.
Wadsö, I., 2002. Isothermal microcalorimetry in applied biology. Thermochimica Acta 394, 305–311.
Wadsö, L., Galindo, F.G., 2009. Isothermal calorimetry for biological applications in food science and technology. Food Control 20, 956–961.
Wilson, R.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.
Wimpenny, J.W.T., Leistner, L., Thomas, V.L., Mitchell, A.J., Katsaras, K., Peetz, P., 1995. Submereged bacterial colonies within food and model systems: their growth, distribution and interactions. International Journal of Food Microbiology 28, 299–315.
Winkelmann, M., Hüttl, R., Wolf, G., 2004. Application of batch-calorimetry for the investigation of microbial activity. Thermochimica Acta 415, 75–82.
Yi, L., Than, T.T., Zhong, C., Songsheng, Q., Ping, S., 2000. Microcalorimetric studies on the metabolism of *Chlorella vulgaris*. Chemosphere 40, 845–849.

PUBLICATION III

I. Stulova, **N. Kabanova**, T. Kriščiunaite, A. Taivosalo, T.-M. Laht, R. Vilu. Fermentation of reconstituted milk by *Streptococcus thermophilus*: effect of irradiation on skim milk powder. *Int. Dairy J.* 2013, http://dx.doi.org/10.1016/j.idairyj.2013.02.004[4]

[4] Copyright 2013, reprinted with permission from Elsevier

International Dairy Journal xxx (2013) 1–11

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 Published by Elsevier Ltd.
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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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.

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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.

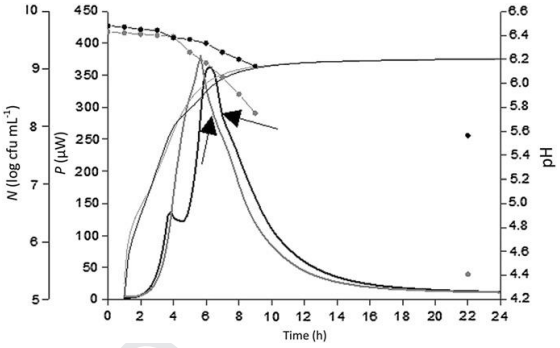


Fig. 2. Calorimetric power–time (P) curves (bold lines) of the growth of *Streptococcus thermophilus* ST12, number of bacteria (N) calculated from the power–time curves (thin lines), and pH changes (circles) in reconstituted skim milk powder (RSM; black) and in irradiated RSM (grey) at an inoculation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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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

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

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

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

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

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

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

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

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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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

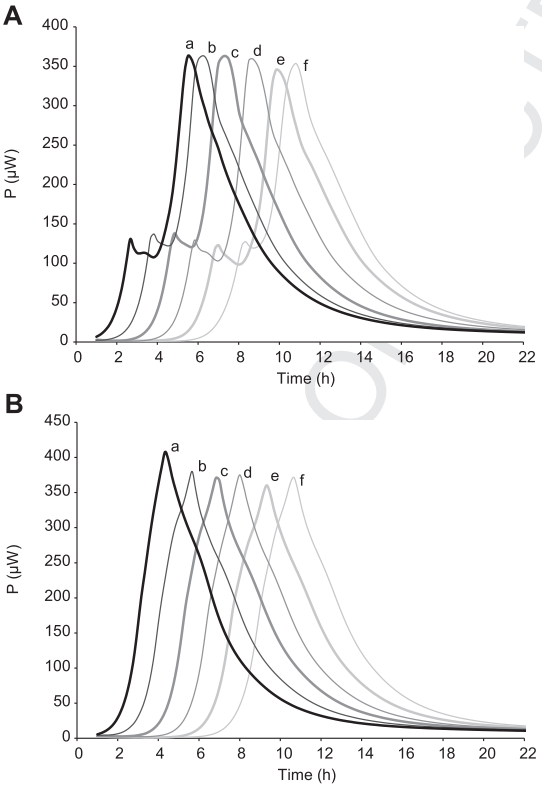


Fig. 3. Calorimetric power–time (P) curves of the growth of *Streptococcus thermophilus* ST12 in (A) reconstituted skim milk powder (RSM) and (B) irradiated RSM at different inoculation rates (cfu mL^{-1}): a, 10^6; b, 10^5; c, 10^4; d, 10^3; e, 10^2; f, 10^1.

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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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

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

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

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

3.3. Carbohydrate utilisation and lactic acid production

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

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

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

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

3.4. Change of free amino acids during fermentation

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

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

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

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

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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

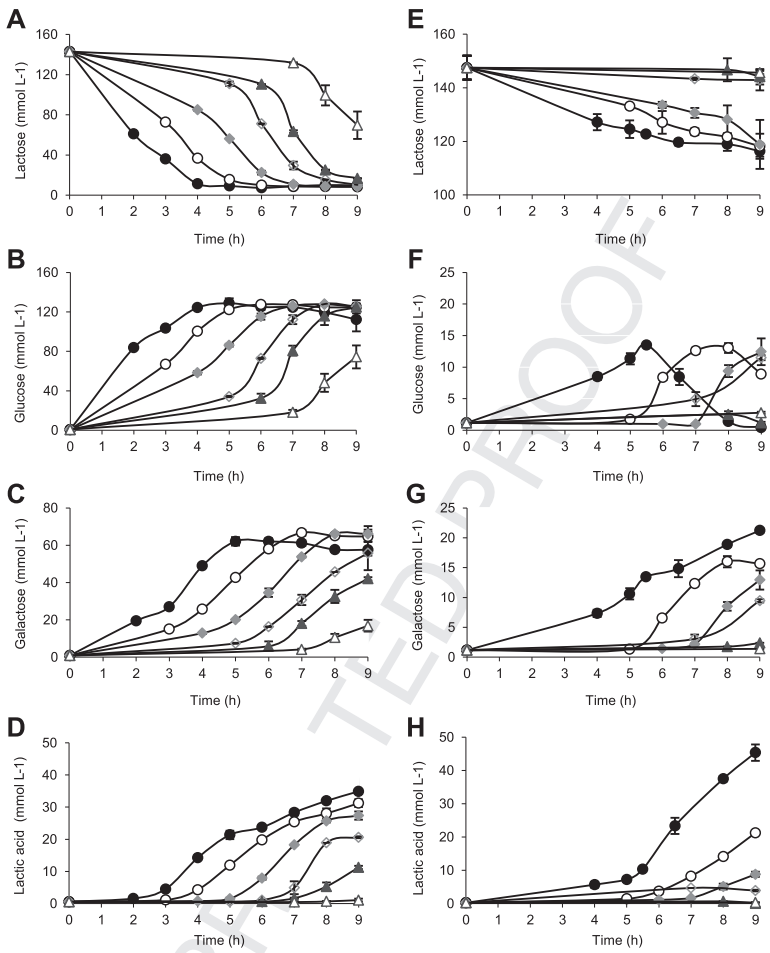


Fig. 4. Comparison of lactose (A, E), glucose (B, F), galactose (C, G) and lactic acid (D, H) profiles during growth of *Streptococcus thermophilus* ST12 in (A, B, C, D) reconstituted skim milk powder (RSM) and (E, F, G, H) irradiated RSM at different inoculation rates (cfu mL^{-1}): ●, 10^6; ○, 10^5;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

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

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

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

3.5. Dynamic rheological measurements

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

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

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

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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

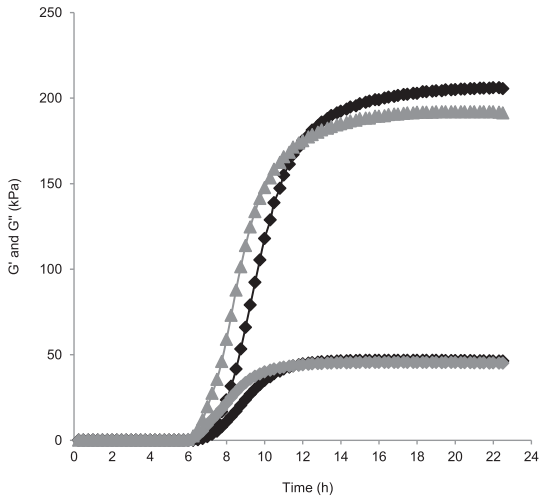


Fig. 5. Development of storage (G′; diamonds) and loss (G″; triangles) moduli during fermentation of reconstituted skim milk powder (RSM; black symbols) and irradiated RSM (grey symbols) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}.

3.6. Monitoring of fermentation by front-face fluorescence spectroscopy

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

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

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

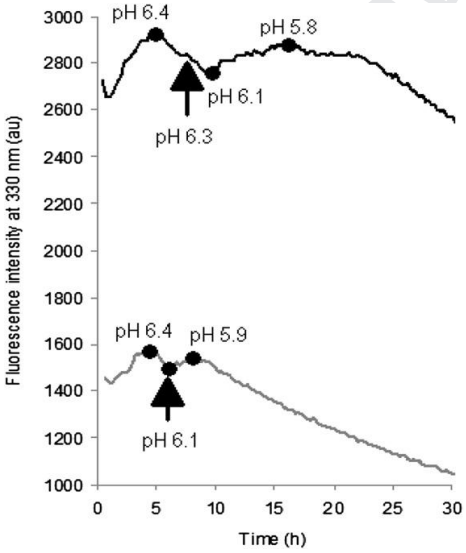


Fig. 6. Development of fluorescence intensity at 285/330 nm during fermentation of reconstituted skim milk powder (RSM; black line) and irradiated RSM (grey line) with *Streptococcus thermophilus* ST12 at an inoculation rate of 10^5 cfu mL^{-1}. Arrows mark the mean gelation times determined rheologically.

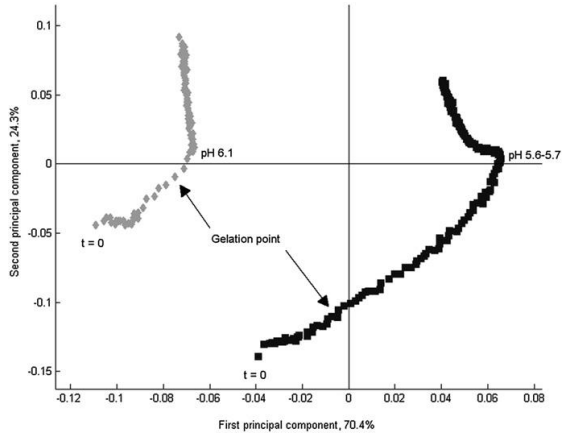


Fig. 7. Principal component analysis map defined by two first principal components for spectral fluorescence signatures data recorded during fermentation of reconstituted skim milk (RSM; black squares) and irradiated RSM (grey diamonds) with *Streptococcus thermophilus* ST12 at an inoculation rate of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

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

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.

Please cite this article in press as: Stulova, I., et al., Fermentation of reconstituted milk by *Streptococcus thermophilus*: Effect of irradiation on skim milk powder, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.02.004

PUBLICATION IV

A. Kazarjan, **N. Kabanova**, R. Vilu. Microcalorimetric study of extruded dog food containing probiotic microorganisms. *Adv. in Microbiol*. 2012, 2, 436-440[5]

[5] Copyright 2012, Scientific Research

Advances in Microbiology, 2012, 2, 436-440
doi:10.4236/aim.2012.24055 Published Online December 2012 (http://www.SciRP.org/journal/aim)

Microcalorimetric Study of Extruded Dog Food Containing Probiotic Micro-Organisms

Aram Kazarjan[1], Natalja Kabanova[1,2], Raivo Vilu[1,2]
[1]Tallinn University of Technology, Tallinn, Estonia
[2]Competence Center of Food and Fermentation Technologies (CCFFT), Tallinn, Estonia
Email: kazarjan@gmail.com

Received August 23, 2012; revised September 23, 2012; accepted October 10, 2012

ABSTRACT

Extruded dry dog food products claimed to have a probiotic ingredient of *Enterococcus faecium* (NCIMB10415) in the commercial available formulations under the brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) were studied in the present work using a multichannel thermal activity monitor TAM III. Maximum specific growth rate, heat produced during different growth phases, and lag-phase duration were determined. The length of the lag-phase that can be used to determine the time necessary for the probiotic ingredient to restore its activity after consumption of probiotic containing extruded products was also measured. The calorimetric data confirmed the ability of the *Enterococcus faecium* to grow at the acidic pH conditions, modeling conditions of gastro-intestinal tract of dogs, and preserve its metabolic activity (viability) at the same level as at the neutral pH. The results obtained indicated that microcalorimetry was a precise and convenient tool for monitoring probiotic activity in complicated solid-state matrices.

Keywords: *Enterococcus faecium*; Probiotic Dog Food; Microcalorimetry

1. Introduction

Various attempts have been made to produce extruded feed or food product(s) with stable and viable probiotic bacteria count over the entire shelf-life period of the carrier product [1-3]. However, determination of the number of bacteria, and especially evaluation of their growth and metabolic activity in extruded food and feed products by means of *outplating* etc. cannot be considered sufficiently precise and convenient.

It has been shown that generation of heat by microbial cultures can be used for on-line monitoring of growth and metabolism of cells [4-10]. Very sensitive measurements of heat flows using microcalorimetry is one of the most attractive techniques for studying and monitoring of the metabolic activity of bacteria in opaque liquid media, and solid matrices [11-13]. It could be successfully applied in the studies of spoilage processes and shelf-life determination of products containing microbes [8,14-16], in fact in all cases where the possibilities of optical and other physical methods are limited due to the non-transparency of media [17,18].

Growth of bacteria in extruded dry dog food products under the brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) claimed to contain a probiotic ingredient of *Enterococcus faecium* (NCIMB10415) were studied in the present work using a multichannel thermal activity monitor TAM III.

2. Materials and Methods

2.1. Samples

The experiments were performed with dry dog food in the form of extruded kibbles under a brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) containing according to the producer viable probiotic bacteria *Enterococcus faecium* (NCIMB10415) during the entire period of product shelf-life (15 months) at room temperature. The concentration of bacteria as declared on the packaging is 10^6 cfu per gram of dog food in average. Three commercially available dog food products differentiated by dog age (adult and senior) and by taste (salmon and chicken formulations) were used in the experiments: ProBiotic LIVE Adult Chicken & Rice (AC), ProBiotic LIVE Adult Salmon & Rice (AS) and ProBiotic LIVE Senior Chicken & Rice (SC). All dog food samples used in the experiments were initially received in hermetically closed separate 1.5 kg bags, with expiry date/lot. nr. 23.11.2012 and used freshly in the middle of the claimed product shelf-life. All the bags were aseptically opened immediately before the experiments. Additionally, dry bacterial concentrate of the same strain of the bacteria as in the dog food (*Enterococcus faecium* NCIMB10415) provided by Probiotics International Ltd. (UK, Batch No BN 29094, expiry date 11.2013) was studied as a reference sample to dog food formulations.

The bacterial concentrate contained 10^{10} cfu per gram of the powder according to the producer.

2.2. Sample Preparation and Experimental Conditions

Dry dog food kibbles of different types were ground and sifted to fine particles (less than 0.05 mm). Afterwards samples were weighed in sterile plastic Falcon tubes. The mass of each sample was approximately ~0.5 g. Samples were aseptically transferred into 3 mL autoclaved microcalorimetric ampoules. Depending on experimental conditions sterile MilliQ water, adjusted to pH = 2 (dog stomach equivalent pH [19]), or to pH = 7 (neutral value), was added to the ground dry dog food in mass ratio 1:1. pH was measured with pH meter S20 Seven Easy Mettler Toledo with calibrated InLab 413 Mettler Toledo electrode. All the solutions were previously sterilized by autoclaving at 121˚C for 15 min. As a reference, samples of dry non-diluted bacterial concentrate were also analyzed.

In order to determine the initial bacterial concentration in dog food kibbles *outplating* on PCA (Plate Count Agar, Scharlau, Spain) was carried out for all three dog food formulations and dry bacterial concentrate.

2.3. Microcalorimetry

A TAM III (24-channels, TA Instruments, US), a heat conduction multi-channel microcalorimeter was used to monitor the growth of cells in dog food and bacteria concentrate samples. The construction and functioning of the TAM III calorimeter were described earlier by Wadsö [8]. Microcalorimetric power-time curves were analyzed as described by Kabanova [10]. The calorimetric trials were performed at fixed temperature of incubation at 37˚C (equivalent of dogs body temperature [20]) using 3 mL calorimetric ampoules. All the calorimetric experiments were carried out in three parallels and power-time curves obtained were normalized per gram of dog food. Averaged power-time curves of three parallel runs were used for the further analysis.

3. Results and Discussion

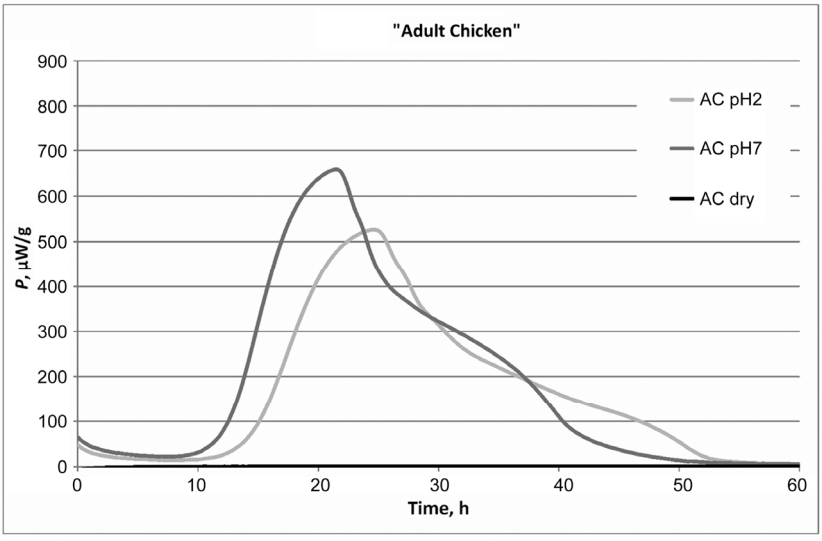
The averaged power-time curves of growth of *Enterococcus faecium* (NCIMB10415) in different formulations of dog food in case of dry samples and in moisturized samples at two different pH values are presented in **Figure 1**.

The standard deviation of the averaged power-time curves was 0.55 μW, which was quite low, confirming high reproducibility of the calorimetric method.

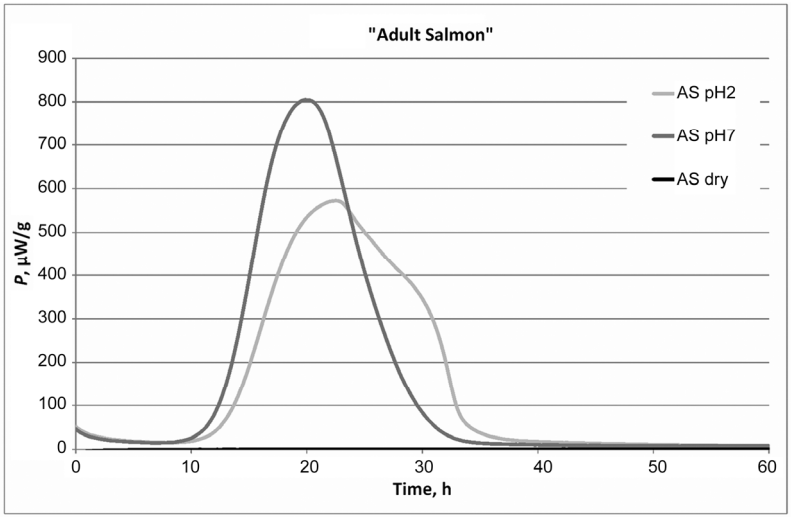
Power-time curves were analyzed according to the Kabanova [9] and all numerical values of processed power-time curves are presented in **Table 1**.

The length of the lag phase was 3.9 ± 0.72 hours (in average) at pH 7 (neutral pH). The lag phase was prolonged by 1 hour, up to 4.9 ± 0.2 hours (in average) at pH 2 (low pH), which is the pH level in the stomach of dogs [13]. Prolongation of the lag-phase at low pH can be explained by acidic stress effect on the probiotic bacteria.

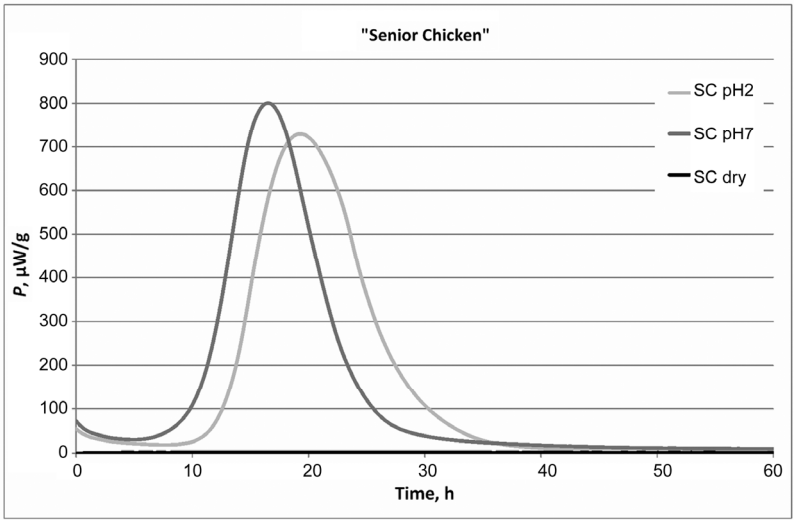
The average specific maximum growth rate (μ_{max}, W/h) of the *Enterococcus faecium* bacteria was practically the



(a)



(b)



(c)

Figure 1. Power-time curves of growth of probiotic bacteria in three different dog food: dry and moisturized samples (AC, AS and SC) at two different pH (pH = 2 and pH = 7) values.

same at low and neutral pH levels (0.5209 ± 0.0634 W/h and 0.5391 ± 0.0539 W/h correspondingly).

The average heat produced during the exponential phase (S_{exp}, J/g) at neutral pH was 15.33 ± 0.90 J/g and 13,67 ± 0.74 J/g at acidic pH condition. Using the Y_Q value 2.58 ± 0.44 × 10^{-9} J/cfu determined by us for *L. lactis* IL1403 [10] the average number of bacteria grown during the exponential growth (N_{exp}, cfu/g—5.93 × 10^9 cfu/g at neutral pH and 5.29 × 10^9 cfu/g at low pH) was calculated—see **Table 1**.

The estimates of biomass obtained should be considered reliable, as the values of Y_Q have been practically the same in the different papers: 5.53 × 10^{-8} J/cfu for soil samples [21], 4.22 × 10^{-9} J/cfu and 3.4 × 10^{-8} J/cfu for *Staphylococcus aureus* and *E. coli* respectively [22].

The total heat produced during the entire period of thermodynamic activity (full length of power-time curves registered, all bacteria growth phases included S_{tot}, J/g) was also practically the same at both pH-s studied. The average total heat produced at neutral pH was 35.92 ±

Table 1. Parameters describing bacterial growth of three dog food formulations (AC, AS, SC) at two different pH (pH = 2 and pH = 7) levels obtained from the processed power-time curves: lag phase duration (λ, h), maximal specific growth rate (μ_{max}, W/h), heat produced during exponential growth phase (S_{exp}, J/g) and during the total growth (S_{tot}, J/g) and the respective numbers of bacteria produced during the both growth phases (N_{exp}, cfu/g and N_{tot}, cfu/g respectively).

		λ, h	σ, h	μ_{max}, W/h	σ, W/h	S_{exp}, J/g	σ, J/g	N_{exp}, cfu/g	S_{tot}, J/g	σ, J/g	N_{tot}, cfu/g
pH 2	AC	4.94	0.77	0.4547	0.05	14.28	0.86	5.53E+09	37.09	8.58	1.44E+10
	AS	5.08	0.49	0.5271	0.01	13.89	4.96	5.38E+09	32.18	7.77	1.25E+10
	SC	4.68	0.39	0.5809	0.00	12.85	0.92	4.97E+09	32.37	1.74	1.25E+10
pH 7	AC	3.54	0.33	0.5039	0.03	17.24	1.13	6.67E+09	41.62	3.78	1.61E+10
	AS	4.73	0.09	0.6012	0.04	15.52	1.11	6.01E+09	34.45	2.10	1.33E+10
	SC	3.42	0.50	0.5123	0.17	13.21	0.47	5.11E+09	31.68	3.40	1.23E+10

0.78 J/g and 33.88 ± 1.23 J/g at low pH, which correspond to 1.39×10^9 cfu/g and 1.31×10^9 cfu/g respectively. The numbers of bacteria grown during the exponential growth phase (N_{exp}, cfu/g) and bacteria grown during the complete growth (N_{tot}, cfu/g) achieved the same levels of 10^9 and 10^{10} cfu/g correspondingly at the two pH values.

All three formulations of ProBiotic LIVE dog food (AC: Adult Chicken & Rice, AS: Adult Salmon & Rice and AS: Senior Chicken & Rice) enriched with probiotic bacteria of *Enterococcus faecium* (NCIMB10415) had the same initial bacterial count in average 10^6 cfu/g confirmed by *outplating* of the samples on the PCA presented in **Table 2**. Average concentration of bacteria in all the three dog food formulations was $2.86 \times 10^6 \pm 5.31 \times 10^5$ cfu/g according to the results presented in **Table 2**. This confirms the claimed by a producer average *E. faecium* load of 10^6 cfu per gram in the product.

It is known that the low acidic pH in the stomach of dogs is considered as a barrier for the probiotic bacteria. Ability to preserve the viability during the passage through the stomach acidic barrier is a prerequisite for the further successful colonization of the host intestine by the probiotic bacteria. The *Enterococcus faecium* bacteria present in the dog food of ProBiotic LIVE formulations were able to show strong metabolic activity at low acidic pH value, which confirmed the fact that *Enterococcus faecium* can tolerate the passage through the acidic barrier of the dog's stomach and populate the intestine afterwards. The ability of the probiotic bacteria *Enterococcus faecium* (NCIMB10415), to preserve the activity throughout the passage through the stomach acidic barrier of dogs was also confirmed in the trials of the European Food Safety Authority (EFSA, [23]). Analysis of the power-time curves revealed that the duration of the time needed for the probiotic bacteria to regain their activity in the host organism after consumption, the length of the lag-phase, was about 5 hours (see **Table 1**). This time is sufficient for the digest flow to pass the acidic barrier of stomachs of dogs.

Table 2. Plate counts of the bacteria on PCA and pH values of different dog food formulations and bacteria concentrate.

Sample	N, cfu/g	σ, cfu/g	Bulk pH
AC	2.35E+06	2.19E+04	5.77
AS	3.41E+06	6.17E+04	5.87
SC	2.82E+06	5.14E+04	5.94
Bact. concentrate	2.24E+10	3.28E+08	5.23

There was no bacterial metabolic activity observed in the case of non-diluted dry dog food samples analyzed, see **Figure 1**. Also the freeze-dried *Enterococcus faecium* (NCIMB10415) bacteria powder concentrate (both dry and diluted samples) showed no thermal activity (data not shown).

4. Conclusions

The results obtained showed clearly the ability of the probiotic *Enterococcus faecim* (NCIMB10145) bacteria strain present in the extruded dog food kibbles to preserve its viability and growth characteristics at acidic and neutral pH. This indicated that the extruded kibbles containing *E. faecium* bacteria are able to tolerate the dog's stomach acidic barrier with further successful colonization of the dog's intestine.

The results obtained showed that calorimetric technique is a prospective and sensitive method for the continuous *in-situ* monitoring of bacterial activity in such complicated matrices as extruded feed or food products, and for shelf-life determination of probiotic ingredients in the products.

5. Acknowledgements

This investigation was financially supported through the grant ETF9192 of Estonian Science Foundation, through the grant SF0140090s08 of Estonian Ministry of Education and Research, and by the European Regional Development Fund project EU29994.

The authors wish to thank the Probiotics International Ltd. (UK) for providing the sample of the bacteria concentrate and Bacterfield S.A. (Luxembourg) for providing the dog food samples.

REFERENCES

[1] S. D. Forssten, C. W. Sindelar and A. C. Ouwehand, "Probiotics from an Industrial Perspective," *Anaerobe*, Vol. 17, No. 6, 2011, pp. 410-413. doi:10.1016/j.anaerobe.2011.04.014

[2] A. C. Ouwehand, "Recent Advances in Probiotic Research: A Conference Update," *Future Microbiology*, Vol. 6, No. 9, 2011, pp. 981-984. doi:10.2217/fmb.11.76

[3] W. Kneifel and S. Salminen, "Probiotics and Health Claims," Wiley-Blackwell, London, 2011. doi:10.1002/9781444329384

[4] L. Yi, L. Xi, Q. Songsheng and S. J. Ping, "Microcalorimetric Investigation of the Toxic Action of Cd2+ on *Rhizopus nigricans* Growth," *Journal of Biochemical and Biophysical Methods*, Vol. 45, No. 2, 2000, pp. 231-239. doi:10.1016/S0165-022X(00)00115-9

[5] L. Gustafsson, "Microbiological Calorimetry," *Thermochimica Acta*, Vol. 193, 1991, pp. 145-171. doi:10.1016/0040-6031(91)80181-H

[6] H. Vandenhove, "Microclaorimetric Characterization of Bacterial Inocula," *Advanced Instrumentation*, *Data Interpretation*, *and Control of Biotechnological Processes*, Vol. 25, 1998, pp.121-158.

[7] I. Lamprecht, "Calorimetry and Thermodynamics of Living Systems," *Thermochimica Acta*, Vol. 405, No. 1, 2003, pp. 1-13. doi:10.1016/S0040-6031(03)00123-0

[8] L. Wadsö and F. G. Galindo, "Isothermal Calorimetry for Biological Applications in Food Science and Technology," *Food Control*, Vol. 20, 2009, pp. 956-961. doi:10.1016/j.foodcont.2008.11.008

[9] N. Kabanova, A. Kazarjan, I. Stulova and R. Vilu, "Microcalorimetric Study of Growth of *Lactococcus lactis* IL1403 at Different Glucose Concentrations in Broth," *Thermochimica Acta*, Vol. 496, No. 1-2, 2009, pp. 87-92. doi:10.1016/j.tca.2009.07.003

[10] N. Kabanova, I. Stulova and R. Vilu, "Microcalorimetric Study of the Growth of Bacterial Colonies of *Lactococcus lactis* IL1403 in Agar Gels," *Food Microbiology*, Vol. 29, No. 1, 2012, pp. 67-79. doi:10.1016/j.fm.2011.08.018

[11] D. A. Mitchell, O. F. von Meien, N. Krieger and F. D. H. Dalsenter, "A Review of Recent Developments in Modeling of Microbial Growth Kinetics and Intraparticle Phenomena in Solid-State Fermentation," *Biochemical Engineering Journal*, Vol. 17, No. 1, 2004, pp. 15-26. doi:10.1016/S1369-703X(03)00120-7

[12] I. Stulova, N. Kabanova, T. Kriščiunaite, T.-M. Laht and R. Vilu, "The Effect of Milk Heat Treatment on the Growth Characteristics of Lactic Acid Bacteria," *Agronomy Research*, Vol. 9, 2011, pp. 473-478.

[13] A. Mihhalevski, I. Sarand, E. Viiard, A. Salumets and T. Paalme, "Growth Characterization of Individual Rye Sourdough Bacteria by Isothermal Microcalorimetry," *Journal of Applied Microbiology*, Vol. 110, No. 2, 2011, pp. 529-540. doi:10.1111/j.1365-2672.2010.04904.x

[14] M. Riva, D. Fessas and A. Schiraldi, "Isothermal Calorimetry Approach to Evaluate Shelf Life of Foods," *Thermochimica Acta*, Vol. 370, 2001, pp. 73-81. doi:10.1016/S0040-6031(00)00782-6

[15] C. Alklint, L. Wadso and I. Sjoholm, "Accelerated Storage and Isothermal Microcalorimetry as Methods of Predicting Carrot Juice Shelf-Life," *Journal of the Science of Food and Agriculture*, Vol. 85, No. 2, 2005, pp. 281-285. doi:10.1002/jsfa.1942

[16] U. von Stockar and L. A. M. van der Wieler, "Thermodynamics in Biochemical Engineering," *Journal of Biotechnology*, Vol. 59, No. 1-2, 1997, pp. 25-37. doi:10.1016/S0168-1656(97)00167-3

[17] M. Antwi, K. Barnaerts, J. F. Van Impe and A. H. Geeraerd, "Modelling the Combined Effects of Structured Food Model System and Lactic Acid on *Listeria innocua* and *Lactococcus lactis* Growth in Mono- and Coculture," *International Journal of Food Microbiology*, Vol. 120, 2007, No. 1-2, pp. 71-84. doi:10.1016/j.ijfoodmicro.2007.04.015

[18] P. D. G. Wilson, T. F. Brocklehurs, S. Arino, D. Thuault, M. Jakobsen, M. Lange, J. Farkas, J. W. T. Wimpenny and J. F. Van Impe, "Modelling Microbial Growth in Structured Foods: Towards a Unified Approach," *International Journal of Food Microbiology*, Vol. 73, No. 2-3, 2002, pp. 275-289. doi:10.1016/S0168-1605(01)00660-2

[19] M. Akimoto, N. Nagahata, A. Furuya, K. Fukushima, S. Higuchi and T. Suwa, "Gastric pH Profiles of Beagle Dogs and Their Use as an Alternative to Human Testing," *European Journal of Pharmaceutics and Biopharmaceutics*, Vol. 49, 2000, pp. 99-102. doi:10.1016/S0939-6411(99)00070-3

[20] D. G. Carlson and J. M. Griffin, "Dog Owner's Home Veterinary Handbook," Howell, New York, 1992.

[21] T. Kimura and K. Takahashi, "Calorimetric Studies of Soil Microbes: Quantitative Relation between Heat Evolution during Microbial Degradation of Glucose and Changes in Microbial Activity in Soil," *Journal of General Microbiology*, Vol. 131, No. 11, 1985, pp. 3083-3089. doi:10.1099/00221287-131-11-3083

[22] S. Bayne-Jones and H. S. Rhees, "Relationship of Heat Production to Phases of Growth of Bacteria," *Journal of Bacteriology*, Vol. 17, No. 2, 1929, pp. 123-140.

[23] The European Food Safety Authority, "Opinion of the Scientific Panel on Additives and Products or Substances Used in Animal Feed on the Safety of Product Oralin for Dogs," *The European Food Safety Authority Journal*, Vol. 51, 2004, pp. 1-6.

CURRICULUM VITAE

Personal data

Name	Natalja Kabanova
Date and place of birth	11.01.1985, Tallinn
Citizenship	Estonian

Contact information

Address	Tallinn University of Technology (TUT), Department of Chemistry, Akadeemia tee 15, 12618 Tallinn, Estonia
Phone	+372 56476827
E-mail	nataljakabanova@yahoo.com

Education

2009 – Ph.D studies in Tallinn University of Technology – Chemistry and Gene Technology
2003-2008 – B.Sc and M.Sc in Tallinn University of Technology – Applied Chemistry and Biotechnology

Defended thesis

Master's Degree (M.Sc) in Applied Chemistry and Biotechnology, 2008. Study of the growth of lactic acid bacteria *Lactococcus lactis* IL1403 in solid gelatine matrices (*cum laude*)

Special courses

2011 (5 days)	PhD course in Advanced Food Microbiology. Copenhagen, Denmark
2010 (2 days)	Seventh Winter Symposium on Chemometrics. Introductory school, St. Petersburg, Russia
2008 (3 days)	Basic Microcalorimetry, RH perfusion calorimetry, Isothermal Titration Microcalorimetry. Stockholm, Sweden

Professional Employment

2008 - ... Competence Centre of Food and Fermentation Technology (CCFFT) – researcher.

• Development and use of a microcalorimetric method for the study of bacterial growth in different environments

	• Study of the growth of *Lactococcus lactis* and *Streptococcus thermophilus* in liquids and during solid state fermentation processes in transparent and opaque media: broth, agar, gelatine, casein gels and milk
2008 - 2013	TUT, Department of Chemistry, Chair of Inorganic Chemistry – teacher (*autumn term*)

List of original publications

I. **N. Kabanova**, A. Kazarjan, I. Stulova, R. Vilu. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth. *Thermochim. Acta* 2009, 498, 87-92

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

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

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

V. A. Kazarjan, **N. Kabanova**, R. Vilu. Microcalorimetric study of extruded dog food containing probiotic micro-organisms. *Adv. in Microbiol*. 2012, 2(4), 436-440.

VI. I. Stulova, **N. Kabanova**, T. Kriščiunaite, A. Taivosalo, T.-M. Laht, R. Vilu. Fermentation of reconstituted milk by *Streptococcus thermophilus*: effect of irradiation on skim milk powder. *Int. Dairy J.* 2013 (*accepted*)

VII. **N. Kabanova**, I. Stulova, R. Vilu. Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at low glucose concentration in liquids and solid agar gels. *Thermochim. Acta* 2013, 559, 69-75.

ELULOOKIRJELDUS

Isikuandmed

Ees- ja perekonnanimi	Natalja Kabanova
Sünniaeg ja -koht	11.01.1985, Tallinn
Kodakondsus	Eesti

Kontaktandmed

Aadress	Tallinna Tehnikaülikool (TTÜ), Keemiainstituut, Akadeemia tee 15, 12618 Tallinn, Eesti
Telefon	+372 56476827
E-posti aadress	nataljakabanova@yahoo.com

Hariduskäik

2009 – Doktoriõpe TTÜ, Keemia ja Geenitehnoloogia
2003-2008 – Bakalaureuse- ja magistrikraad, TTÜ – Rakenduskeemia ja biotehnoloogia

Kraad

Magistrikraad (MSc) Rakenduskeemia ja biotehnoloogia, 2008. Piimhappebakterite *Lactococcus lactis* IL1403 kasvu uurimine tahkefaasilises želatiini maatriksis (*cum laude*)

Täiendusõpe

2011 (5 päeva)	PhD kursus „Advanced Food Microbiology“. Kopenhaagen, Taani
2010 (2 päeva)	Seventh Winter Symposium on Chemometrics, kemomeetria sissejuhatav koolitus, St. Peterburg, Venemaa
2008 (3 päeva)	TA Instruments kalorimeetriliste seadmete koolitus “Basic Microcalorimetry, RH perfusion calorimetry, Isothermal Titration Microcalorimetry”, Stockholm, Rootsi

Töökogemus

2008 - …	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus (TFTAK) – teadur
2008 - 2013	TTÜ, Keemiainstituut, Anorgaaniline õppetool, õppejõud (*sügissemestriti*)

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.