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Investigation of the physiology of genetically modified strains of Lactococcus lactis, and their potential for accelerated ripening of cheese

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Preface

The present thesis was carried out by Mia Ryssel at the Department of Food Science, Food Microbiology, Faculty of Life Sciences, University of Copenhagen as well as the Department of System Biology, Centre for Systems Microbiology at the Technical University of Denmark. The work fulfils the requirements for a Ph.D. degree at the Research School FOOD, Centre for Advanced Food Studies (LMC), Frederiksberg, Denmark. The work has partly been financed by the the Danish Dairy Research Foundation.

I would like to thank my supervisor Associate Professor Henrik Siegumfeldt for excellent guidance and many fruitful discussions throughout this project. I would also like to thank Professor Mogens Kilstrup for letting me work with his group and for the many hours he spend on discussing results obtained in the laboratory.

My thanks also to my colleagues at the Food Microbiology Section for a pleasant and friendly working atmosphere, a special thanks to Jacqueline, Mette, Line T. and Line O. whom I have also spent a great deal of time with outside of work. Thanks to all my friends and my soccer team for taking my mind of the thesis and providing me with new energy.

Warm thanks to my family; Jan, Lonnie, Heidi and Sarah, for always supporting and believing in me.

Summary

The overall objective of the present Ph.D. thesis was to investigate the physiology of genetically modified strains of *Lactococcus lactis* and investigate whether these could be used to accelerate the ripening time of cheese. This was done by examining the growth of mutants on a solid surface, examining the stress phenotype of different mutants and finally examining how proteome and transcriptome analysis could be performed on cells embedded in a matrix.

Autolysis of the starter culture is essential in the ripening of cheese and it is desirable to have a high amount of autolysis at the ripening stages. As the cells will be present in the cheese matrix as discrete colonies, rather than dispersed individual cells, we developed a method to quantify growth and death of single cells on a surface. It was first attempted to use the measurements of intracellular pH to determine the status of the cells, but this did was unsuccessful because of the very large variations in the measurements within replicates. Instead we used a combination of propidium iodide, which penetrates only dead cells with a permeabilised membrane, and bright field, for visualizing all cells. When we examined the growth by microscopy, very large variations were found at different fields of view within the same specimen. This underlines the difficulty in quantitatively interpreting individual microscopic images of cell growth. By taking the average of many positions, in this case 16, we were able to clearly differentiate between the growth behaviour of different strains. Growth and death of *Lactococcus lactis* strains, with different mutations in the purine biosynthesis pathway, were examined by this method. The mutants had different levels of *guaB* (IMP dehydrogenase) expression, and it was shown that reduced levels of *guaB* resulted in a decrease of growth rate. The death of the cells was also influenced by the *guaB* expression level but not in the same linear way as for the cell growth and a strain with an intermediate expression of *guaB* demonstrated the slowest death rate.

Previously it has been reported that *Lactococcus lactis* can acquire multiple stress resistance preferentially by mutations in genes involved in purine nucleotide metabolism. A hypothesis was therefore that purine nucleotides have an influence on the stress phenotype of *Lactococcus lactis*. To examine this, genetically engineered mutants with deletion in genes involved in the purine metabolism were constructed; the genes involved were *hpt, hprT, guaA*, and *guaB*. Also a mutant with an inactive *pup* gene was examined. Stress phenotypes for *L. lactis* MG1363 were found to

be different in the chemically defined SA medium compared to the complex M17 medium. At pH 3.0 *L. lactis* MG1363 was fully stress resistant in SA medium, while sensitive in M17 medium. Consistency in stress phenotypes could be obtained in SA medium and it was shown that when salvage of purines (by addition to the medium) and subsequent conversion to GTP was possible in various genetic backgrounds, *L. lactis* MG1363 and derivatives of *L. lactis* MG1363 became sensitive to acid stress. This indicates that an excess of guanine nucleotides induces stress sensitivity. Furthermore the addition of phosphate to the acid stress medium increased the stress sensitivity of *L. lactis* MG1363. However, the role of the phosphate in the stress phenotype is not completely clear, but could be explained by an increased level of nucleotides as a result of an improved conversion of nucleosides by phosphorylases in the salvage pathway. Based upon these findings, it is suggested that *L. lactis* MG1363 is naturally multi-stress resistant in habitats devoid of purine sources (as in milk), but the presence of any exogenous purine that results in increased guanine nucleotide pools renders the bacterium sensitive to environmental stresses.

It is reasonably easy to purify bacteria grown in liquid medium in order to examine their proteome and transcriptome, but when the cells are embedded in matrixes, such as casein gels (cheese), the analysis of proteome and transcriptome is more difficult. We developed a method where bacteria that had been captured in the matrix, could be released by dissolving them in 3M guanidinium chloride. The bacteria are killed by treating with 3M guanidinium chloride (shown as a decrease in CFU), but the cells were still physically intact (shown by phase contrast and LIVE/DEAD staining). It was therefore possible to use the extracted bacteria from the cheese for subsequent proteome and transcriptome analysis. This provides new opportunities to investigate cell behaviour throughout cheese production, which has previously been very difficult. The methods could also be useful for other matrixes for example other food matrixes and biofilms.

Sammendrag

Det overordnede formål med dette Ph.D. projekt har været at undersøge fysiologien for genetisk modificerede stammer af *Lactococcus lactis,* og hvordan disse stammer kan anvendes til at øge modningshastigheden af ost. Dette blev gjort ved at undersøge væksten af forskellige mutanter på en fast overflade, undersøge stress fænotyper af forskellige mutanter og endeligt ved at undersøge, hvordan proteom- og transcriptomeanalyser kan foretages på celler indkapslet i en matrice.

Autolyse af starterkulturen er af afgørende betydning for modning af ost, og høj grad af autolyse er ønskværdigt. I ost er bakterierne tilstede som små kolonier og ikke spredt som enkelte celler. Vi udviklede en metode til at kvantificere vækst og død af enkelte celler på en fast overflade. Der blev forsøgt at bruge målinger af intracellulær pH til at bestemme cellernes status, dette var dog ikke succesfuldt, da der var store variationer indenfor målinger i den samme prøve. I stedet blev propidium iodid brugt til at farve døde celler, da dette kun trænger ind i celler med permeabel cellemembran, og lys mikroskopi blev brugt til at visualisere alle celler. Indenfor samme bakteriestamme gav vækstundersøgelserne ved mikroskopi store variationer i hvert felt der blev observeret. Dette understreger problemet ved kvantitativt at undersøge cellevækst ved enkelte billeder taget ved mikroskopi. Ved at følge vækst i flere felter, i dette tilfælde 16, var det muligt, klart at se forskel på vækst af forskellige stammer. Denne metode blev brugt til at følge vækst og død af *Lactococcus lactis* stammer med forskellige mutationer i purinbiosynthesen. Mutanterne havde forskellig ekspression af *guaB* (IMP dehydrogenase), hvor lavere udtryk af *guaB* resulterede i nedsat væksthastighed. Hastigheden, hvor med cellerne døde, var også afhængig af ekspressionen af *guaB*, men ikke i samme lineære grad som væksten. Mutanten med den største overlevelse havde et middelsvagt udtryk af *guaB*.

Det er tidligere vist, at *Lactococcus lactis* kan blive multiresistent overfor stress ved genmutationer, der er involveret i purinbiosyntesen. Hypotesen var derfor, at purinnukleotider har en indflydelse på stressfænotypen af *Lactococcus lactis*. For at undersøge dette, blev der konstrueret genetisk modificerede mutanter med deletioner i purinmetabolismen. Generne, der blev undersøgt, var *hpt, hprT, guaA* og *guaB*. En mutant med inaktivt *pup* gen blev også undersøgt. Det blev vist, at stressfænotypen af *L. lactis* MG1363 var anderledes i kemisk

defineret SA medie sammenlignet med det komplekse M17 medie. Ved pH 3.0 var *L. lactis* MG1363 stress resistent i SA medie, mens den var sensitiv i M17 medie. Det var muligt at gentage bestemmelser af stressfænotyper i SA medie, og det blev vist, at når optag af puriner (tilsætning til mediet) og videreomdannelse til GTP var muligt, blev *L. lactis* MG1363 og dets mutanter sensitive overfor syrestress. Endvidere blev stresssensitiviteten af *L. lactis* MG1363 øget, når fosfat blev tilsat til syrestressmediet. Hvilken rolle fosfat har i forhold til stressfænotypen, er imidlertid ikke helt afklaret. En mulig forklaring kan være, at fosfat øger omdannelsen af nukleosider til nukleotider og derved hæver mængden af nukleotider i cellen. Vores resultater indikerer, at *L. lactis* MG1363 naturligt er multi-stressresistent i miljøer uden purinkilder (så som mælk), mens tilstedeværelsen af purinkilder omvendt resulterer i en øget mængde guaninnukleotider i cellen og dermed øget sensitivitet overfor stress.

Det er forholdsvis let at oprense bakterier fra et flydende medie og anvende disse til proteom- og transkriptomanalyser, men når bakterierne er indkapslet i en matrice, som f.eks. en kaseingel (ost), bliver analysen af proteomet og transkriptomet meget besværlig. Vi udviklede en metode, hvor celler indkapslet i en matrice kan blive frigivet ved opløsning i 3M guanidinium klorid. Bakterierne dør ved behandling med 3M guanidinium klorid, hvilket er vist som fald i CFU, men de beholder en fysisk intakt membran, hvilket kunne ses mikroskopisk ved fasekontrast og LIVE/DEAD farvning. Det var derfor muligt at bruge de ekstraherede celler fra ost til proteom- og transkriptomanalyser. Dette giver mange nye muligheder for at undersøge bakteriel adfærd i alle stadier i osteproduktionen, hvilket tidligere har været meget besværligt. Metoden kan desuden bruges til andre matricer f.eks. andre fødevarer matricer eller biofilm.

List of Abbreviations

AR	adenosine	**NSLAB**	non-starter lactic acid bacteria
CEP	cell-envelope proteinase	**Opp**	oligopeptide transport system
CFU	colony forming unit	**PEP/PTS**	phosphoenol pyruvate-phosphotransferase
CoA	coenzyme A	**ΔpH**	pH gradient
CSLM	confocal scanning laser microscopy	**pH$_{ex}$**	external pH
FAD	flavin adenine dinucleotide	**pH$_i$**	intracellular pH
G	guanine	**PI**	propidium iodide
GR	guanosine	**ppGpp**	3'-pyrophosphorylguanosine-5'-pyrophosphate
Hx	hypoxantine	**pppGpp**	3'-pyrophosphorylguanosine-5'-triphosphate
IL1403	*L. lactis* subsp. *lactis* IL1403	**PRA**	phosphoribosyl amine
IR	inosine	**PRPP**	5-phosphoribosyl-α-1-pyrophosphate
L. lactis	*Lactococcus lactis*	**Prt$^+$**	ability to degrade proteins
LAB	lactic acid bacteria	*S. thermophilus*	
Lac$^+$	ability to utilize lactose		*Streptococcus thermophilus*
MG1363	*L. lactis* subsp. *cremoris* MG1363	**subsp.**	subspecies
NAD$^+$	nicotinamide adenine dinucleotide	**UHT**	ultra high temperature
NADP$^+$	nicotinamide adenine dinucleotide phosphate		

Contents

Appendix 1:

M. Ryssel, Z. Duan, and H. Siegumfeldt. (2010) Quantitative Examination of Cell Growth and Death of *Lactococcus lactis* by Microscopy. *International Dairy Journal.* Submitted.

Appendix 2:

M. Ryssel, J. Haaber, M. Liu, M.S. Dawish, K. Hammer, J. Martinussen & M. Kilstrup. (2010) Purine Induced Stress Sensitivity in *Lactococcus lactis*. *Molecular Microbiology*. Submitted.

Contents

Appendix 3:

M. Ejby, M. Ryssel, L. Nesic & M. Kilstrup. Fractionation of physically intact bacteria from casein gels for proteome and RNA analysis. *In preparation*.

1 Introduction

Lactic acid bacteria (LAB) have been used for fermenting foods for thousands of years. Fermentation is a way to conserve otherwise perishable foods. A large number of foods that are eaten every day, e.g. cheese, yoghurt, sausages and sauerkraut, obtain their characteristics from fermentation with LAB. Nowadays, a starter culture is usually added and the fermentation is carried out under highly controlled conditions. Especially in the dairy sector large amounts of different starter cultures are used, and these are crucial for the quality of the final product.

In 2008 a total of 320 tons of cheese were produced in Denmark and from this 240 tons were exported for a value of 7 billion DKK (www.danishdairyboard.dk), showing the importance of the cheese industry in Denmark. Cheese production is a complex process, which is dependent on the microbial activities, during both cheese manufacturing and ripening. Therefore there is a need to strictly control the whole cheese process. Research within starter cultures and the development of new starter cultures are therefore important areas of investigation. Better understanding of the behaviour of starter cultures implemented in production could lead to more uniform and thereby higher quality products and also reduce loss from failed fermentation.

During cheese production the role of the starter culture changes. Initially the acidification of the milk depends on the starter culture, at a later stage the autolysis of the starter culture releases intracellular enzymes, which are essential for the aroma and texture of the cheese (ripening). A thorough knowledge on cell physiology during the different steps of cheese production helps the interpretation of how the cells are influenced by the processes and how the processes influence the cells.

The nature of the cheese fermentation process causes phage problems to continuously appear in the dairy industry, resulting in slow or dead "vats" and substantial economic losses (Walker *et al.*, 2000). The key problems in the process are that (i) the milk cannot be heat treated properly to kill phages without spoiling the cheese properties of the milk, and therefore the fermentation is not sterile, (ii) the fermentation is conducted under a stringent schedule and the efficiency of the process is easily disrupted, (iii) the number and diversity of starter cultures available are limited,

since the products rely on very specialized strains and (iv) continuous use of the same starter cultures provides an ever present host for phage attack (Klaenhammer *et al.*, 1994). Although the problems of phages in cheese production are an important issue it will not be dealt with in this thesis.

This thesis aims to review the LAB and how these affect the manufacturing and ripening of cheese. Additionally, the ability of LAB to cope with acid stress and the influence of nucleotide metabolism on the stress phenotype are being described.

2 Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) represent a group of bacteria that are functionally related by their ability to produce lactic acid during fermentation. Other characteristics for LAB are: Gram positive, katalase negative (pseudokatalase does exist), obligate fermentative, non spore-formers, acid-tolerant, usually non motile and they have extensive growth requirements. There have been some controversy about which genera to include in LAB, but the following are included from a practical and food-technological point of view: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Axelsson, 2004; Walstra *et al.*, 2006b).

A variety of fermented foods are reliant on LAB to provide the correct flavour, texture and preservative qualities. The most important genus/species for industrial application are: *Lactococcus* (milk), *Lactobacillus* (milk, meat, vegetables, cereal), *Leuconostoc* (vegetables, milk), *Pediococcus* (vegetables, meat), *Oenococcus oeni* (wine) and *Streptococcus thermophilus* (milk) (Klaenhammer *et al.*, 2002).

Due to their practical significance in fermentation, bioprocessing, food, and more recently, medicine, LAB have been subject to considerable research and commercial development. One effort has been to determine the genome sequence of a representative collection of LAB species and strains (Klaenhammer *et al.*, 2002). Until now 73 LAB have been sequenced completely (www.ncbi.nlm.nih.gov/genomes/genlist.cgi) and more are in the pipeline. The size of the chromosome for LAB ranges from 1.8 to 3.4 Mbp (Davidson *et al.*, 1996).

For LAB used in the production of cheese the metabolism of lactose and the degradation of proteins (caseins) are important properties. These properties will be described below.

Transport and Metabolism of Sugars
There are two different mechanisms in LAB for actively transporting sugar across the cell membrane: The permease and the phosphoenol pyruvate-phosphotransferase system (PEP/PTS). Thermophilic LAB species and leuconoctocs use the permease, whereas the PEP/PTS system is

the mechanism used in Lactococci. The permease system transports the lactose across the membrane at the expense of ATP. Inside the cell, lactose is hydrolysed to glucose and galactose by β-galactosidase. In the PEP/PTS system, the transport of lactose into the cell is mediated via a complex system where lactose is phosphorylated and transported across the cell membrane. Inside the cell, the lactose phosphate is hydrolyzed to glucose and galactose-6-phosphate by phospho-β-galactosidase (McKay *et al.*, 1970; Hickey *et al.*, 1986; Thompson, 1987; Fox *et al.*, 1990; Teuber, 1995; Mayra-Makinen *et al.*, 2004).
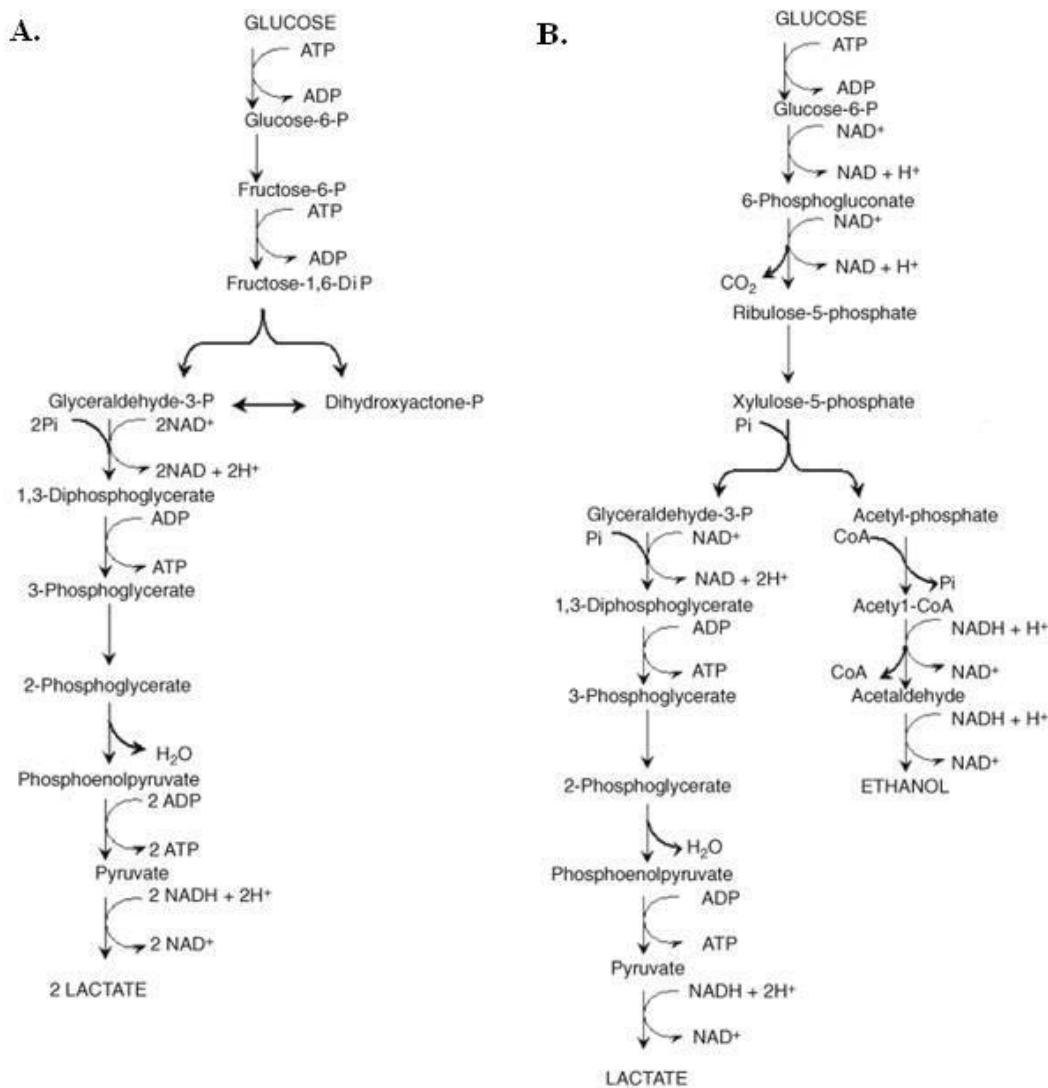

Figure 1. Major fermentation pathways of glucose. A) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway). B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway) (Axelsson, 2004).

LAB can be either homo- or heterofermentative. Glycolysis (Embden-Meyerhof-Parnas pathway) is the homolactic fermentation where pyruvate is produced. Pyruvate can further be converted to lactic acid. In homofermentation, 1 mole glucose results in 2 moles of lactic acid and 2 mole of ATP. The heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway) results in lactic acid and a significant amount of other end products such as ethanol, acetate and CO_2 (Figure 1). In heterofermentation, 1 mole glucose results in 1 mole of lactic acid, ethanol, CO_2 and ATP (Axelsson, 2004; Walstra *et al.*, 2006b). Glycolysis is used by all LAB except the leuconostoc, oenococci, weissella and some lactobacilli (group III) (Axelsson, 2004).

In cheese production the pathway for lactose metabolism is dependent on the type of starter used for the cheese production. *Lactococcus lactis* produces L-lactate from lactose. *Streptococcus thermophilus* hydrolyzes lactose to glucose and galactose, and glucose is further metabolized to L-lactate, while galactose cannot be metabolized and is secreted in antiport with lactose, which constitutes a different transport system altogether. Some strains of lactobacilli are galactose-positive and are able to convert galactose (secreted by e.g. *Streptococcus*) to DL-lactate (Hickey *et al.*, 1986; Fox *et al.*, 1997; Axelsson, 2004).

The fermentation of galactose depends on the uptake system. If the PEP/PTS system is used, galactose-6-phosphate is converted to pyruvate by the tagatose-6-phosphate pathway. When the permease is used for the uptake of lactose, the Leloir pathway is used for converting galactose to pyruvate (Figure 2) (Axelsson, 2004).
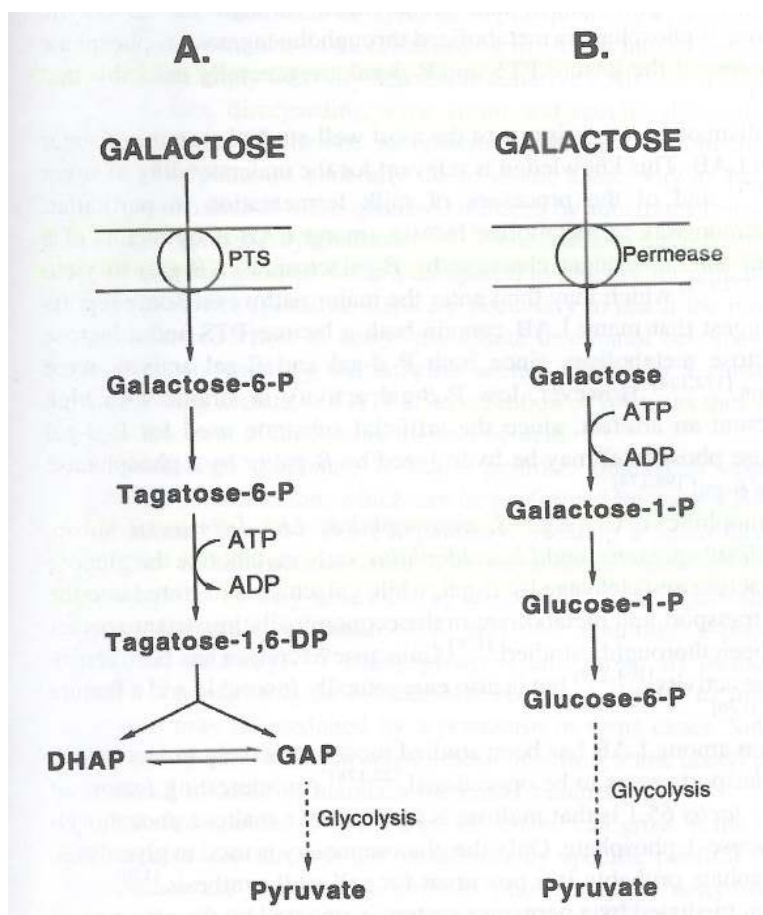
Figure 2. Galactose metabolism in lactic acid bacteria. A) tagatose-6-phosphate pathway. B) Leloir pathway (Axelsson, 2004).

The proteolytic system of LAB

Milk is rich in nitrogen, but most of it is present as proteins in the form of caseins. The breakdown of caseins by LAB plays an important role in generating peptides and amino acids for bacterial growth and in the formation of metabolites that contribute to flavour formation of fermented products. The proteolytic system of LAB comprises three major components: (i) cell-envelope proteinase (CEP) that initiates the degradation of extracellular casein into oligopeptides, (ii) peptide transporters that transport the peptides into the cell, and (iii) various intracellular peptidases that degrade the peptides into shorter peptides and amino acids (Figure 3) (Teuber, 1995; Parente *et al.*, 2004; Savijoki *et al.*, 2006; Liu *et al.*, 2010)

The CEP of *Lactococcus lactis* (PrtP) are the best described proteinase of LAB. The *prtP* gene can be plasmid- or genome encoded (Parente *et al.*, 2004; Savijoki *et al.*, 2006). In Appendix 1 we used a plasmid with *prtP* to make the examined strains able to degrade proteins. The caseins in milk can be divided into α, β- and κ-caseins and the CEPs of LAB have a different specificity for the caseins (Kunji *et al.*, 1996; Parente *et al.*, 2004). The CEPs have a strong preference for hydrophobic proteins, which are the most abundant in milk (Swaisgood, 1982; Savijoki *et al.*, 2006). When the caseins have been degraded to ologipeptides, they can be transported into the cell by the oligopeptide transport system (Opp). The Opp system of *Lactococcus lactis* is able to transport peptides of up to at least 18-mer's (Detmers *et al.*, 1998). Other transport systems in LAB are able to transport amino acids or di- and tripeptides across the membrane, but in milk the Opp system is the most important, as only very small amounts of di- and tripeptides are present in milk (Parente *et al.*, 2004). After the casein-derived peptides are taken up by the LAB, they are degraded to amino acids by the action of several peptidases with different specificity (Kunji *et al.*, 1996). The amino acids can be used for biosynthesis, as an energy source or to regulate the intracellular pH, and in cheese production catabolism of the free amino acids is important for the development of flavour during ripening (Curtin *et al.*, 2004; Ardo, 2006).
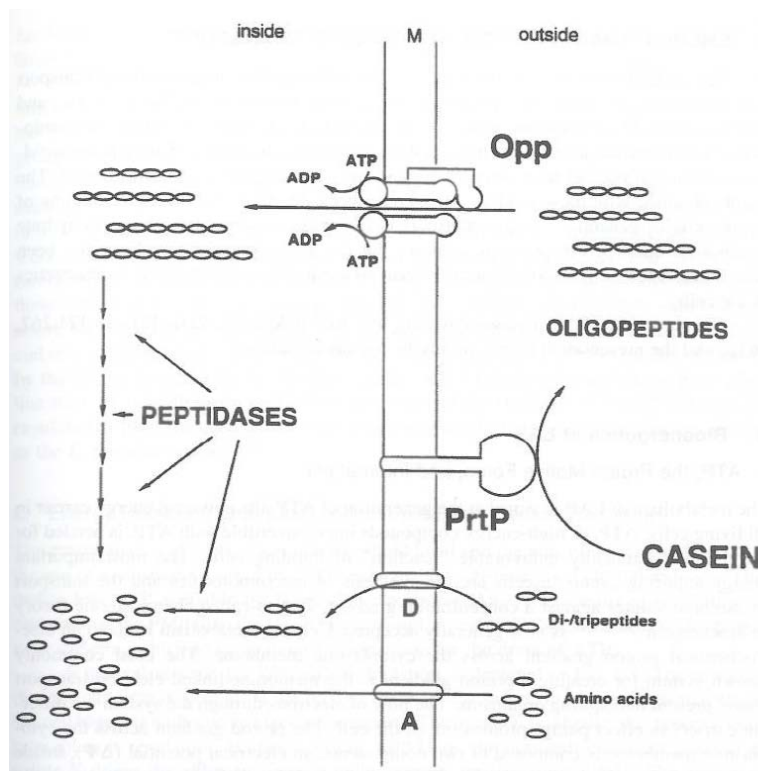
Figure 3. Model of the proteolytic pathway in *Lactococcus lactis*. Included is transport of di- and tripeptides and free amino acids, even though they contribute very little to the proteolytic system when the cells are grown in milk. PrtP, cell-envelope proteinase; Opp, oligopeptide transport system; D, di-/tripeptide transport systems; A, amino acid transport systems; M, cytoplasmic membrane (Axelsson, 2004).

2.1 Lactococcus lactis

Lactococci are homofermentative, they are cocci and occur singly, in pairs or in chains. The cells are often elongated in the direction of the chain. They grow at 10°C and 30°C, but not at 45°C. Usually they can grow in 4% (w/v) NaCl (although *Lactococcus lactis* subsp. *cremoris* only tolerates 2% (w/v) NaCl) (Teuber, 1995). Lactococci are closely associated with the fermentation of dairy products, but only one species, *L. lactis,* is actually used in the dairy industry. Two subspecies of *L. lactis* are important in the dairy industry; *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, (Schleifer *et al.*, 1985; Axelsson, 2004). The two subspecies have been intensely studied, mainly because of their industrial interest, and they have become excellent models for research in metabolism, physiology, genetics, and molecular biology of LAB (Bolotin *et al.*, 2001). Some phenotypic characteristics are different for the two subspecies and can be used to differentiate between them; *L. lactis* subsp. *lactis* produces ammonia from arginine and can grow at 40°C, whereas *L. lactis* subsp. *cremoris* does neither of those things (Davidson *et al.*, 1996). Also sequencing of 16S rRNA and DNA hybridization can differentiate between the subspecies (Salama *et al.*, 1991; Godon *et al.*, 1992). Liu *et al.* (2010) found that the pattern of proteolytic enzymes differs between the two subspecies, this again can be used as a method to identify the subspecies.

L. lactis are anaerobic bacteria, but are aerotolorant (Walstra *et al.*, 2006b). They possess certain oxygen metabolizing enzymes like superoxide dismutase or NADH oxidases, which enable them to grow in the presence of oxygen. Therefore they have an aerotolerant phenotype, which has the advantage that their medium, e.g. milk, does not have to be completely free of oxygen. This is convenient when used in large scale industrial application (Teuber, 1995).

L. lactis often contains plasmids, and most strains contain between 4 and 7 different plasmids per cell. Certain plasmids carry important properties for the industrial process (McKay, 1983; Teuber, 1995). The ability to utilize lactose (Lac[+]) and degrade proteins (Prt[+]) is essential for

growth in milk. These abilities, along with others, e.g. the ability to utilise citrate, are associated with plasmids and IS elements (McKay, 1983; Romero *et al.*, 1993; Liu *et al.*, 2010). This suggests that, in the adaptation of *L. lactis* to its predominance in the dairy environment, plasmids and IS elements have played a crucial role (Davidson *et al.*, 1996).

LAB are nutritionally fastidious and are generally auxotrophic for the amino acids isoleucine, valine, leucine, histidine, methionine, arginine and proline (Chopin, 1993; Teuber, 1995). For exponential growth *Lactococcus lactis* needs a complement of 19 amino acids (Jensen *et al.*, 1993). In milk the content of free amino acids is too low to support growth of Lactococci to more than about 10^7 CFU/ml. For sufficient acidification of the milk for cheese production, the cell number needs to reach more than 10^9 CFU/ml (Teuber, 1995). This can only be accomplished by use of the proteolytic system of the dairy Lactococci, described in the previous section.

The first LAB to be sequenced was *Lactococcus lactis* subsp. *lactis* IL1403 by Bolotin *et al.* (2001). The genome size was 2.4 Mb. Some unexpected sequences were found; biosynthetic pathways for all 20 amino acids, a complete set of late competence genes, five complete prophages and partial components for aerobic metabolism. Some of these systems are not functional or complete, but they may indicate an evolutionary trend towards minimizing the chromosome and deactivating unnecessary systems during adaptation to nutritionally complex but specific environments, such as milk (Chopin, 1993; Bolotin *et al.*, 2001). Comparison of the genomes of *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363 showed that MG1363 had a larger genome (160 kb) than IL1403 and contained several additional genes for carbohydrate metabolism and transport, thereby enabling MG1363 able to grow on various carbohydrates from plants (Wegmann *et al.*, 2007). This could indicate that the original habitat of Lactococci is plants, and actually many strains of Lactococci are still found in plant environments.

When genomes from plant-associated strains, *L. lactis* subsp. *lactis* KF147 and KF282 (Siezen *et al.*, 2008) were compared with genomes of *L. lactis* subsp. *lactis* IL1403 (Bolotin *et al.*, 2001) and *L. lactis* subsp. *cremoris* SK11 (Makarova *et al.*, 2006) from the dairy environment, it was found that the plant isolates carried genes e.g. for growth on plant carbohydrates (as was also the case for MG1363), but these were not seen in the dairy isolates (Siezen *et al.*, 2008). Analysis of

the "unique" genes in the plant isolates, on the basis of the G+C content, indicates that these were ancient genes that were lost from the dairy isolates, supporting the hypothesis that dairy strains have adapted to a rich environment and not the other way around (Siezen *et al.*, 2008).

Efficient use of Lactococci by the dairy industry requires understanding of the many aspects of bacterial physiology, such as the use of sugars and proteins in milk for growth, conversion of sugars to lactate and synthesis of substances involved in flavours (Bolotin *et al.*, 2001). Not surprisingly, many studies on Lactococci have been performed, and still more research will have to be performed before we understand the nature of these mechanisms.

2.2 Intracellular pH (pH$_i$)

Intracellular pH (pH$_i$) is an important aspect of cell physiology, and the cells exercise relatively tight regulation of the pH$_i$. Cells of growing *Lactococcus* have an initial pH gradient (ΔpH) of 0.8 to 1.0 (alkaline inside), which causes accumulation of acids inside the cell and thereby a reduction in pH$_i$ (Booth, 1985). The lowering of pH$_i$ by dissociation of intracellular protons affects ΔpH, which contributes to the proton motive force. The proton motive force is used as an energy source in numerous transports across the membrane (Slonczewski *et al.*, 1996; van de Guchte *et al.*, 2002). A decrease in pH$_i$ also changes the enzymatic activity and may denature proteins and damage nucleic, thereby having a great effect on the cell (Champomier-Verges *et al.*, 2002).

LABs are able to maintain a ΔpH over a wide range of low external pH (pH$_{ex}$) values (Hutkins *et al.*, 1993). A pH$_i$ of 7.5 to 6.0 (pH$_{ex}$ > 5.0) is the optimal pH for growth of LAB and a pH$_i$ of 5.0 is critical for growth (Nannen *et al.*, 1991). Cook and Russell (1994) showed that *Lactococcus lactis* ML3 were able to grow in MRS media until pH$_{ex}$ reached 5.3, pH decreased as a consequence of lactate accumulation. The pH$_i$ decreased as the pH$_{ex}$ decreased, so an almost constant ΔpH was maintained. If the cells maintained a high pH$_i$ it would cause a large accumulation of anions from the fermentation. It therefore appears that for fermentative bacteria it is better to decrease pH$_i$ when pH$_{ex}$ is decreased, resulting in less accumulation of anions. Hutkins and Nannen (1993) and Poolman *et al.* (1987) observed that some LAB, including Lactococci, maintain an almost neutral internal pH until the pH$_{ex}$ drops to a certain threshold

value and then the pH_i starts to decrease. This is in contrast with results reported from other groups as described above.

Lowering the pH_{ex} by HCl or lactate has a different effect on the pH_i. A higher pH_i could be maintained when the pH_{ex} was decreased by HCl compared to lactate (Cook *et al.*, 1994). This suggests that when comparing experimental results, it is important to note if the pH is manipulated by adding organic or inorganic acid, and that organic acid should be used if the experiment should resemble a milk fermentation.

Measuring pH_i

In the early stages, the distribution of radio labelled weak acids or weak bases was used to measure pH_i (Kashket, 1985) but this method has some temporal limitations. Also the pH_i can only be measured under steady-state conditions or if the changes in pH occur slowly (Molenaar *et al.*, 1991). More convenient methods have now been developed.

Carboxyfluorescein is a ratiometric pH probe that can be used for pH_i measurements in the cells. When excited at 435 nm it shows no pH sensitivity, but excitation at 490 nm results in emissions that are pH dependent. A ratio, which is independent of the concentration of the probe, can be obtained from the intensities of the two wavelengths. This ratio can be converted to a pH_i-value (Siegumfeldt *et al.*, 2000). Siegumfeldt *et al.* (2000) showed that when the pH_{ex} was lowered from 7.0 to 5.0, the pH_i for *Lactococcus lactis* decreased over a time span of 20 min. *L. lactis* was able to maintain a ΔpH of 0.8 at both 7.0 and 5.0. For four strains of *Streptococcus thermophilus* the authors showed that they were able to maintain a ΔpH of 0.5 at pH_{ex} of 7.0 to 5.0. The decrease in pH_i for *S. thermophilus* was faster than for *L. lactis*.

The method of measuring pH_i by carboxyfluorescein is a method that could be used in the dairy industry to evaluate the physiological status of the starter culture both before addition to the cheese milk and also within the cheese. If the pH_{ex} is acidic and the pH_i is the same as pH_{ex}, it indicates that the cells can be dead, whereas cells with a higher pH_i are metabolic active, and therefore more likely to be viable. An intermediate pH_i will indicate that the cells are stressed, but trying to counter the stress. Early in this project, we tried to use the measurements of pH_i to determine the condition of single cells in a starter culture. After trying to optimize the

experimental procedure it was recognized that the variation in the measurements was very large and even replicates within the same sample were significantly different (Figure 4). As we have shown in Appendix 1, large variations within a sample can still lead to useful results, but when the average pH_i of three replicas showed statistical differences, it was decided to abandon the use of pH_i to predict the status of the starter culture in this project, although this method has proven very useful in other experiments. Another problem with this method was autofluorescence, especially when measurements were performed in milk or cheese, the milk proteins seemed to fluoresce to such a degree that it was difficult to observe the single cells and measure their pH_i with a sufficient precision.

If these methods are to be used for measuring pH_i within a solid product e.g. cheese it is very important to obtain results that are representative of the whole sample. In Appendix 1 we showed that large variations in the growth of cells were observed in different spots on the solid surface. The variation could be levelled out by performing many (in this case 16) observations. The observations were chosen automatically, thereby reducing any bias from the researcher who might prefer to choose a specific area for examination.
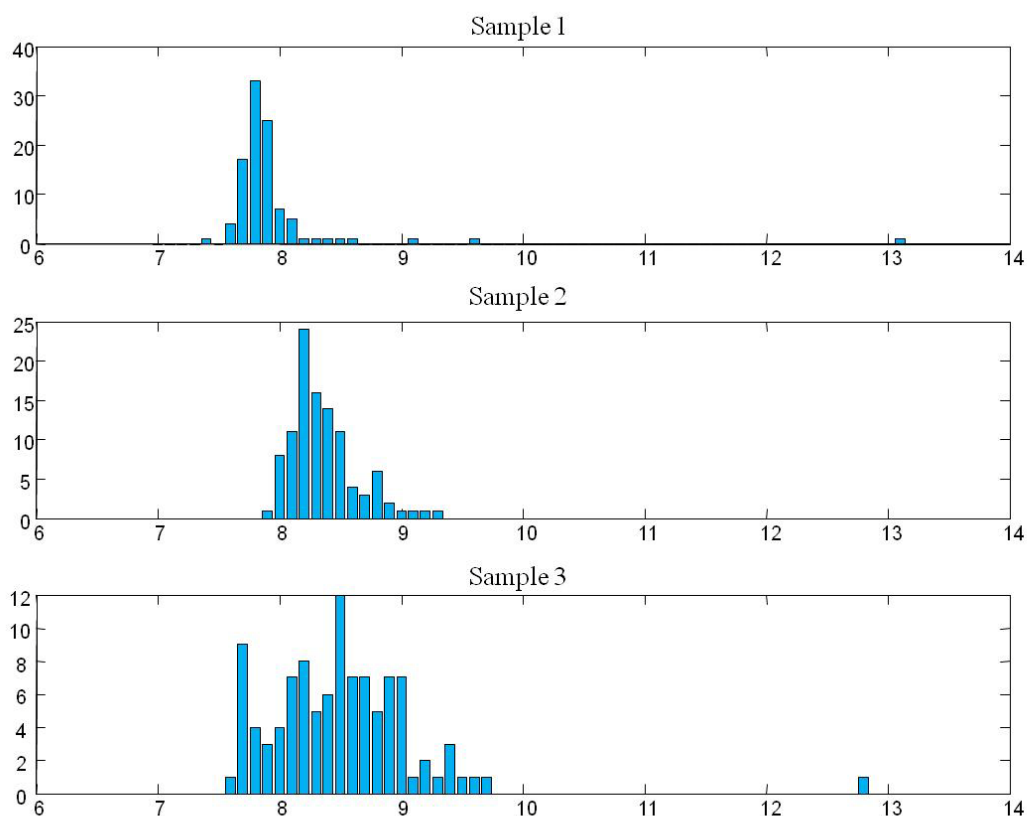
Figure 4. Histogram of the distribution of pH$_i$ in three samples taken from the same replicate, measured by the use of carboxyfluorescein (see text for explanation). Each set represent measurements of 100 cells. The x-axis represents the pH$_i$ measured in the cells and the y-axis is the number of cells with the same pH$_i$. The difference in distribution is very large between the samples and sample 1 is significant (5% level) different from the two other samples.

3 Cheese Production

Many hundreds of different cheeses exist. They differ due to the starter culture, the composition of the milk used, the manufacturing steps (degree of heating, cutting, stirring, water addition, salting, moulding and pressing), the ripening time and temperature. All these factors affect the texture, flavour, colour etc., which gives each cheese its characteristics.

The production of cheese includes different steps that are principally the same for most cheese varieties; I) acidification, II) coagulation, III) syneresis, IV) moulding, V) salting and VI) ripening (Fox *et al.*, 1990; Fox *et al.*, 1996b). Cheese production is basically a dehydration process. Fat and casein in milk are concentrated 6-12 times depending on variety. The hydration is dependent on the cheese manufacturing steps and on the milk composition (Fox *et al.*, 1990; Fox *et al.*, 1996b).

The production of cheese comprises manufacturing and ripening. The manufacturing of Cheddar cheese, for example, contains the following steps: acidification of cheese milk by the starter, coagulation by the rennet and cutting. The curd is then cooked, drained, cheddared, milled, salted and pressed (Figure 5). The cheese is then left to ripen at around 8°C. During ripening, hydrolysis of proteins and polypeptides takes place as well as the hydrolysis of fats to free fatty acids and glycerol. The ripening affects the flavour and texture of the cheese. Modification of amino acids and free fatty acids, which contribute to the flavour, take place late in the ripening process. Cheddar is a common cheese worldwide and is special from many other cheeses in the way that it is salted before moulding. Danbo, which is the most common cheese in Denmark, is moulded and then salted in brine.

In the following sections the manufacture and ripening of cheese are described, with emphasis on the Cheddar cheese.

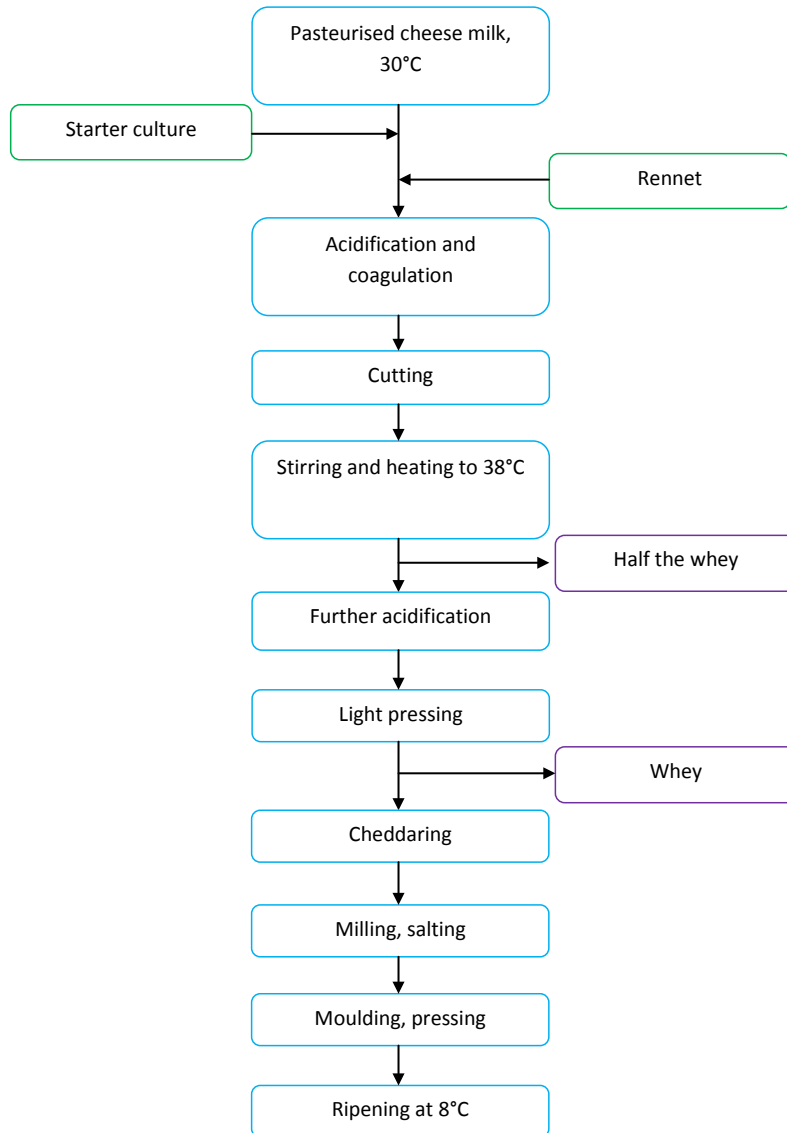**Figure 5. Flow diagram of Cheddar cheese manufacturing. The starter culture is added to the milk and after acidification starts, rennet is added. During the process first half the whey and finally the rest of the whey is removed.**

3.1 Cheese Manufacturing

The manufacturing of cheese includes different steps. In this part the acidification, coagulation, syneresis, moulding and salting are described.

Acidification

One of the primary processes in cheese manufacturing is the acidification of the milk. The acidification has a huge impact on cheese production and the final quality of the cheese (Fox *et al.*, 1996b). The starter culture is responsible for the acidification of the milk and the rate of acidification. E.g. *L. lactis* subsp. *lactis* acidifies faster than *L. lactis* subsp. *cremoris* (Dawson *et al.*, 1957). The pH of the cheese curd reaches pH 5.2 to 4.5 depending on the variety of cheese (Fox *et al.*, 1990). Cheddar cheese is salted in an amount that retards further acidification and therefore the pH of the Cheddar curd at salting (pH~5.3) is close to the final pH (~5.1) in the cheese. For cheeses salted in brine the acidification continues after the salting and these cheeses can be salted at a higher pH (Fox *et al.*, 1996b; Shakeel *et al.*, 2004).

Acidification is important because the lower pH raises the activity of the rennet and inhibits the growth of unwanted organisms. The pH also affects the texture of the cheese. Acid production has an effect on almost all steps in the manufacture of cheese and therefore affects both the composition and quality of the final cheese (Fox *et al.*, 1990).

Coagulation

After the starter culture has started to grow, thereby lowering the pH of the milk, the rennet is added. Rennet is able to degrade the caseins of the milk, which in the end leads to coagulation. The rennet coagulation of milk comprises two stages that overlap: The primary phase consists of the enzymatic breakdown of κ-casein to produce *para*-κ-caseins and glycomacropeptides, the second phase consists of aggregation of *para*-κ-casein Ca^{2+} (Visser, 1976; Fox, 1988; Dalgleish, 1992; Dalgleish, 1993; Leaver *et al.*, 1995). When 60-90% of the κ-casein has been hydrolyzed, the micelles are destabilized and start to coagulate (Figure 6) (Dalgleish, 1979; Carlson *et al.*, 1986; Van Hooydonk *et al.*, 1986). The coagulation of micelles is critically dependent on the Ca^{2+} concentration and temperature. The coagulation will not take place if the temperature is too low (<18°C for bovine milk) (Fox *et al.*, 1996b). Calcium ions might contribute to crosslinking of the micelles or neutralization of the micelles charges and thereby influences the coagulation of the micelles.
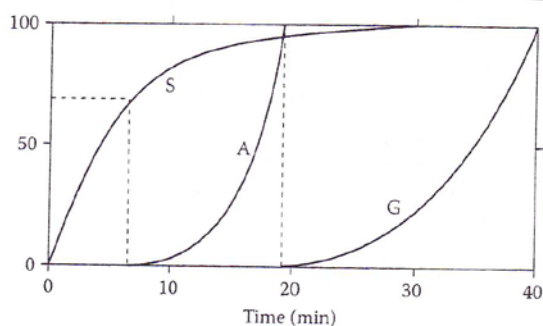
Figure 6. Renneting of milk. Percentage of κ-casein split (S), degree of aggregation of paracasein micelles (A, e.g. deduced from viscosity increase), and shear modulus of the formed gel (G), as a function of the time after adding rennet. A and G, arbitrary scale. At physiological pH and about 30°C (Walstra *et al.*, 2006a).
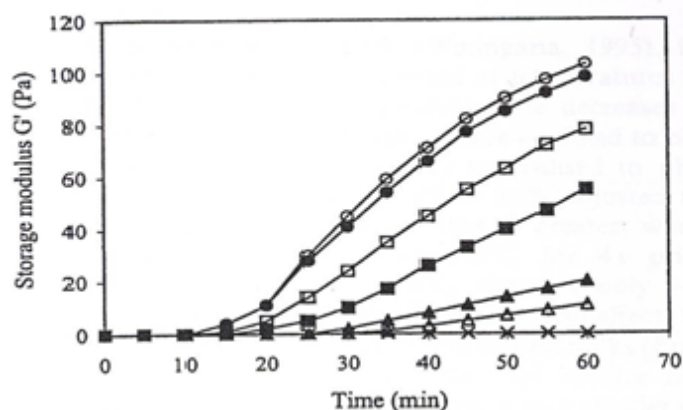
Figure 7. The effects of temperature for a holding time of 4s on the storage modulus (G'; gel firmness) as a function of time after addition of rennet in skim milk. Heating temperatures: unheated milk (○), 80 (●), 90 (□), 100 (■), 110 (▲), 120 (Δ), 130 (+) or 140 (X). All samples were renneted at pH 6.5 at 32°C. Modified from Waungana *et al.* (1996).

The optimum pH in the first stage of rennet action in milk has found to be around pH 6.0 independent of the temperature (Van Hooydonk *et al.*, 1986). Often CaCl$_2$ is added to the cheese milk to increase the [Ca^{2+}], [colloidal calcium phosphate] and to decrease the pH, which together contributes to reduce rennet coagulation time and increased gel strength (Fox, 1988).

Heat treatment affects the coagulation of milk by rennet. Some of the heat treatments used in the dairy industry is thermization (57-68°C, >15 sec), pasteurization (72°C, 15 sec) and UHT (115 to 120°C, 3 sec).

Figure 7 shows how the temperature affects the firmness of the gel and coagulation time. It shows that an increase in temperature prolonges the gelation time and treatment by temperatures of 130-140°C prevent any gel formation (Van Hooydonk *et al.*, 1987; Waungana *et al.*, 1996). The heat treatment causes denaturation of whey proteins, which is why the aggregation of micelles and the gel formation process are influenced (Leaver *et al.*, 1995). Denaturation of whey protein will lead to retention of these in the cheese curd. This results in a higher cheese yield, but unfortunately there is also a change in texture and flavour (Leaver *et*

al., 1995). If the milk is acidified or $CaCl_2$ is added the effect of the heat treatment can be reversed (Van Hooydonk *et al.*, 1987).

Syneresis

When the coagulation of the milk has led to a desired firmness, the gel is cut in order to promote syneresis. At syneresis whey are expelled from the cheese curd. The syneresis does not occur spontaneously but handling of the rennet gel will cause the syneresis to occur (Walstra *et al.*, 2006a).

The rate and extent of the syneresis depends on temperature, pH, degree of stirring, protein concentration and Ca^{2+} concentration (Fox *et al.*, 1990). The syneresis will be enhanced by increasing temperature, decreasing pH, addition of $CaCl_2$, fine cutting of the gel, vigorous stirring and high casein concentration, whereas heat treatment of the milk and increasing fat content will retard syneresis (Fox *et al.*, 1996b; Dejmek *et al.*, 2004). The pH at the time of the syneresis has a great influence on the mineral content of the cheese. The loss of calcium and phosphate from the casein micelles determines the disruption of the micelles and this has a considerable influence on the structure and texture of the cheese (Lawrence *et al.*, 1983). Low pH in the curd gives a crumbly texture (Cheshire) whereas a higher pH gives a more elastic cheese (Emmental) (Fox *et al.*, 1990). Another effect of low pH is that more rennet is retained in the curd and being more active than at higher pH, meaning increased proteolysis occurs in cheeses with low pH (Holmes *et al.*, 1977; Dalgleish, 1979; Creamer *et al.*, 1985). The knowledge of the influence of these factors can help the cheesemaker to control the syneresis of the curd and thereby control the final water content of the cheese, which affects both the texture and ripening of the cheese (Fox *et al.*, 1996b).

Moulding

The composition of the curd greatly affects the deformation of the cheese grains. At pH 5.2-5.3 the highest deformability is obtained, at higher or lower pH the deformability of the cheese grains decreases. With higher water content and higher temperature the deformability also increases. If the curd has a low pH, and a low water content and the moulding is done at low

temperature, holes can remain in the cheese even if it is heavily pressed (Walstra *et al.*, 2006a). In Cheddar cheese where the curd is more acidic than for most other cheeses and the curd has a low water content, it is necessary to apply a large press when the curd is shaped into the moulds, in order to avoid holes in the cheese (Walstra *et al.*, 2006a).

The size of the mould may have an influence on the final composition of the cheese e.g. for unsalted cheese curds a larger cheese will end up with lower water content than a smaller one (Figure 8) (Geurts, 1978; Walstra *et al.*, 2006a). At salting, by brining, the salt gradient will be levelled out faster for smaller cheeses than for larger cheeses. Again this has an influence on the ripening and final quality of the cheese.
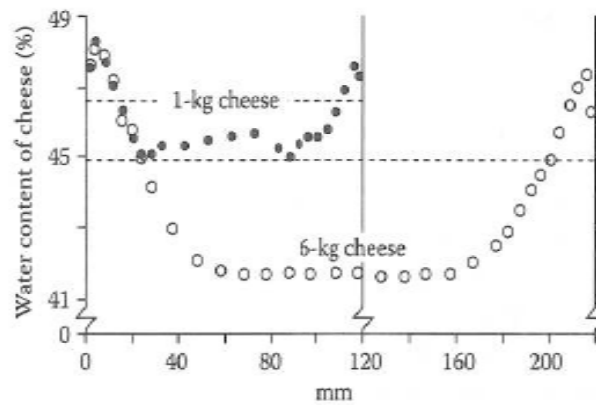
Figure 8. Distribution of moisture throughout unsalted spherical loaves of cheese of 1 and 6 kg. They have been moulded from the same curd, lightly pressed and left for three days. The broken lines represent the average moisture content in the different size cheese (Geurts, 1978).

Salting

Salting is an important step in cheese production. Salt preserves the cheese, affects the ripening process, the final flavour and the texture. Most cheeses contain about 4-5% salt-in-water (Walstra *et al.*, 2006b). Dry salting, rubbing and brining are the most common methods used for salting cheeses (Guinee *et al.*, 2004).

Schroeder *et al.* (1988) have shown that cheeses with lower NaCl levels have a higher moisture content. By salting the cheese it absorbs salt, but simultaneously some water is lost from the

cheese and results in a weight loss of approximately 3% for brined cheeses (Walstra *et al.*, 2006a).

The water content and the microbiology in cheeses changes with different levels of NaCl. Cheeses with the lowest levels of NaCl generally contain higher numbers of LAB. A NaCl level of less than 0.85% supports a significantly higher LAB population than cheeses containing higher concentrations (Schroeder *et al.*, 1988).

In an experiment made by Schroeder *et al.* (1988) cheeses were produced with 1.44, 1.12, 0.73, 0.37 and 0.07% NaCl. The 1.44% NaCl level being the typical salt level for Cheddar cheeses on the market. Trained judges in a taste panel were able to differentiate the NaCl levels in the cheeses and rated them from highest to lowest NaCl content. No other discriminating flavours were found between the cheese with 1.44% and 1.12% NaCl. Below 1.12% NaCl flavour scores decreased, higher acidity, lower Cheddar intensity, more intense bitterness and an unpleasant aftertaste were noted by the taste panel. Another result of the lower NaCl was a reduction in firmness, while adhesiveness and cohesiveness increased. This is consistent with the results obtained by Ryssel *et al* (unpublished), which show that a reduction in salt of 25% did not have any effect on the texture or sensory properties of Cheddar cheese. These results indicate that a reduction of salt in cheese is possible without changing the flavour significantly. This can be an advantage for the many consumers who have a too high intake of salt, which is detrimental to their health.

3.2 Cheese Ripening

The manufacturing steps have a very large influence on the quality of the final cheese. However, it is during ripening that the flavour and texture develop, which characterise the different varieties of cheese (Fox *et al.*, 1996b; McSweeney, 2004). The biochemical changes that take place during ripening are very complex. The composition of the curd has an influence on the ripening process, as well as the rennet and indigenous milk enzymes (plasmin, lipoprotein lipase). Starter cultures and their enzymes, non-starter lactic acid bacteria (NSLAB) and their enzymes also participate in the ripening process (Fox, 1989; Fox *et al.*, 1996b; Fox *et*

al., 1996c; Fox *et al.*, 2004). The focus in this section will be primarily on the influence of the starter culture on cheese ripening.

The ripening time for cheeses ranges from 2 weeks (Mozzarella) to more than 2 years (e.g. Parmesan or extra-mature Cheddar) (Fox *et al.*, 1996a; Fox *et al.*, 1996b). In the ripening of cheese different reaction take place: (1) metabolism of residual lactose and catabolism of lactate, (2) lipolysis and catabolism of free fatty acids and (3) proteolysis and further break down of amino acids (Fox *et al.*, 1996a; Fox *et al.*, 1996b; McSweeney, 2004).

In the production of cheese the primary starter culture have different roles. Initially, they are responsible for the rapid acidification of the milk through efficient conversion of lactose into lactic acid. In this later stage of the process, the proteolytic, peptidolytic, and amino acid-converting enzymes of the starter bacteria play a crucial role in the generation of flavour components. Most of these enzymes are located in the cytoplasm, while their substrates are mostly present outside the cells in the cheese matrix. Therefore, the effect of lysis of the starter culture, whereby the enzymes are released, is generally considered an essential part of the ripening process (Crow *et al.*, 1995a; deRuyter *et al.*, 1997; Cibik *et al.*, 2000; Ouzari *et al.*, 2002). At the start of the ripening, the starter cultures have reached a level of 10^9 CFU/g, but after 2-3 months of ripening the viability is below 1% of this level (Martley *et al.*, 1972; Wilkinson *et al.*, 1994b). Although starter culture autolysis is usually beneficial, undesirable consequences, such as insufficient acid production and removal of residual lactose, can occur if autolysis occurs too soon. In practice, a balance in autolysis is necessary for optimal Cheddar cheese ripening and flavour development (Crow *et al.*, 1995a). The autolysis of LAB in cheese will be discussed further later.

Fate of Lactose in Cheese

Most of the lactose (98%) from the cheese milk is removed with the whey, while the remaining lactose is metabolised to lactate early in the ripening process (Huffman *et al.*, 1984; Fox *et al.*, 1996b; Fox *et al.*, 1996c). Fermentation of lactose by homo- and heterofermentation is described earlier. The starter primarily converts the lactose into L-lactate, but if a high population of NSLAB are present a considerable amount of D-lactate will be formed. Calcium

D-lactate is less soluble than calcium-L-lactate and can crystallize especially on the surface of the cheese (Fox *et al.*, 2000).

Lactate may be oxidized to acetate, but this will depend on the NSLAB population and the availability of oxygen (Fox *et al.*, 1996b). Very extensive metabolism of lactose is seen in surface mould-ripened varieties, e.g. Camembert and Brie (Fox *et al.*, 1996b; McSweeney *et al.*, 2004). The metabolism of lactate by *Clostridium* spp. to butyrate, H_2 and CO_2 leads to "late blowing" and off-flavours that are common defects in many cheeses (Fox *et al.*, 1997).

Lipolysis

LAB have low lipolytic activities, but when present in high numbers for long periods, as in the cheese ripening, the milk fat will be hydrolyzed to a significant degree (Fryer *et al.*, 1967; Fox *et al.*, 1997). Lipolysis only occurs to a low degree in most cheese varieties, and is considered undesirable in most varieties because it may impart a rancid taste to the cheese. Exceptions are Blue cheeses and a few other varieties where lipolysis is considered a part of the characteristic taste of the cheese (Fox *et al.*, 1996b; Fox *et al.*, 1996c; Fox *et al.*, 1997).

Proteolysis

Proteolysis is an important process in all cheese varieties and is often the rate-limiting process in the ripening of cheese. The indigenous milk enzymes, rennet, starter culture and NSLAB contribute to the proteolysis in the cheese (Fox *et al.*, 1996c). Rennet and to some degree plasmin are mainly responsible for the initial proteolysis of caseins leading to large and intermediate sized peptides, which can be further degraded by the rennet and other enzymes from the starter culture and NSLAB. Further breakdown to small peptides and free amino acids are due to enzymes from the starter culture and NSLAB (Reiter *et al.*, 1969; Okeeffe *et al.*, 1976; Visser *et al.*, 1977; Okeeffe *et al.*, 1978; Fox, 1989; Lane *et al.*, 1996; Lynch *et al.*, 1997). Small peptides and amino acids are very important components in cheese as they contribute to the flavour (Engels *et al.*, 1994). The flavours of different amino acids are very distinctive and a combination of these flavours is believed to give each cheese its characteristic taste (Visser, 1993; Fox *et al.*, 1996b). The proteolysis does indeed vary considerably between different cheese varieties due mainly to differences in the manufacturing process. In cheeses with a high cooking temperature (e.g. Mozzarella, Emmental) the plasmin activity is increased

by increased conversion of plasminogen to plasmin (Visser, 1993). High cooking temperatures may also lead to inactivation of the rennet leaving plasmin as the main contributor to the primary proteolysis (Matheson, 1981; Singh *et al.*, 1990). In other cheeses, e.g. Cheddar, with low cooking temperatures the rennet contributes to the primary proteolysis of these types of cheeses (Fox *et al.*, 1996b). About 10-20% of the rennet added to the cheese milk will be retained in the cheese curd of e.g. Cheddar and Gouda (Visser, 1993; Bansal *et al.*, 2009) and thus contribute to the proteolysis in the cheese curd.

During ripening proteolysis modifies the texture of the cheese. In Cheddar with its low pH the caseins are hydrolyzed faster than in cheeses with a higher pH, because the colloidal calcium phosphate is solubilised and therefore micelles are dissociated and more susceptible to proteolysis. Additionally, more rennet is retained in the curd and this is more active at low pH (O'Keffe et al 1975, Holmes et al 1977, Creamer et al 1985).

The rate of secondary proteolysis in cheese depends on the autolysis of the starter culture. If strains with a high rate of autolysis are used, higher levels of intracellular enzymes are present and thereby higher secondary proteolysis are observed in the cheese (O'Donovan *et al.*, 1996). This is further described in section 3.3 Autolysis of LAB in Cheese.

Non-Starter Lactic Acid Bacteria

The NSLAB found in cheese can either be contaminant bacteria entering the milk from the environment or originates from the milk. These NSLAB include mainly homofermentative and heterofermentative mesophilic lactobacilli and pediococci and are present at low numbers in the milk. During the acidification of the milk the starter culture dominates, but during ripening the NSLAB will grow and reach a maximal number. NSLAB population in Cheddar cheese ranges from 10^1 to 10^4 CFU/g during the first 10 days of ripening and after a few weeks the level ends at approximately 10^8 CFU/g (Crow *et al.*, 1993; Wilkinson *et al.*, 1994b; Folkertsma *et al.*, 1996; Fox *et al.*, 1996c; Crow *et al.*, 2001). It has been shown that NSLAB contributes to the cheese flavour development. Especially in cheeses with extended ripening time, their intracellular content are released in the cheese curd after inactivation of enzymes from the starter culture (Crow *et al.*, 1995a).

Proteinases and peptidases from NSLAB play an important role in proteolysis in some cheese varieties (Broome *et al.*, 1990; Broome *et al.*, 1991; Fox *et al.*, 1996b). E.g. for Cheddar the NSLAB seems to supplement positively to the peptidolytic activity of the starter and especially for the production of amino acids (Lynch *et al.*, 1997). Addition of both heat treated lactobacilli (Ardo *et al.*, 1988; Ardo *et al.*, 1989) and live lactobacilli (Lee *et al.*, 1990b; Lynch *et al.*, 1997) appear to accelerate the ripening and are closely related to the higher concentration of free amino acids in cheese (Broome *et al.*, 1990; Lee *et al.*, 1990a).

Acceleration of Ripening

For the dairy industry the ripening of cheese is a time consuming process and thereby an expensive process. It would obviously be beneficial for the industry to accelerate ripening. The problem is how to maintain the characteristic flavour and texture of the cheese varieties while decreasing the ripening time. Of the biochemical processes involved in ripening, proteolysis seems to be the rate limiting process (Fox *et al.*, 1996b). Elevated temperature, exogenous enzymes or modified starter culture are some of the ways the ripening can be accelerated (Wilkinson, 1993; Fox *et al.*, 1996b). Folkertsma *et al.* (1996) and Aston *et al.* (1983; 1985) have shown that the ripening of Cheddar cheese can be accelerated by raising the temperature from 8°C to 12-16°C or to 20°C for the first four weeks and then left at 13°C for the rest of the ripening time. Addition of proteinases and peptidases have been shown to increase ripening (Law *et al.*, 1982; Law *et al.*, 1983; Hayashi *et al.*, 1990). Also starter cultures that autolyse easier than normal have shown to increase the ripening activity (Chapot-Chartier *et al.*, 1994; Wilkinson *et al.*, 1994a).

Sheehan *et al.* (2009) examined a relative small block of Cheddar (12 kg) and found that there were considerable variations throughout the block in some factors that influence cheese quality. E.g. the number of NSLAB was greater in the interior of the block compared to the outer layer, and this can lead to a variable concentration of released intracellular enzymes when the cells lyse. This could have an influence on the pH and salt-in-moisture, which also varied throughout the block. These regional differences in the interior and outer layer of a cheese block are important in understanding the production of a uniform high quality cheese.

It is in general difficult to examine microbial activity in cheese because the cells are embedded in the cheese matrix, but in Appendix 3 we have demonstrated a way to purify the cells from the cheese. This gives us the ability to analyse the proteome and transcriptome of bacterial cells during cheese manufacturing and ripening. Changes in gene expression can be followed during cheese production and this could provide novel knowledge on which genes that are important during the different processes in cheese.

3.3 Autolysis of *Lactococcus* in Cheese

In the early stages of ripening the starter culture enters the stationary phase, become nonviable and the cell wall and membrane are disintegrated in varying degrees (autolysis) (Crow *et al.*, 1995a). Therefore, cells in the cheese may be found in many different stages where some are viable, whereas others are nonviable and may be fully disintegrated.

The autolysis of the starter culture has a prominent effect on biochemical reactions involved in flavour development (Law *et al.*, 1974; Chapot-Chartier *et al.*, 1994; Crow *et al.*, 1995a; Crow *et al.*, 1995b; Gatti *et al.*, 1999; Cibik *et al.*, 2000; Ouzari *et al.*, 2002). Intracellular enzymes access their substrates in the cheese curd via autolysis of the cells (Chapot-Chartier *et al.*, 1994; Niven *et al.*, 1998; Bunthof *et al.*, 2001). In particular, it has been shown that the release of intracellular peptidases accelerates amino acid production and results in lower bitterness by hydrolysis of large hydrophobic peptides. In addition, the amino acid catabolism leading to aroma formation can also be stimulated by autolysis (Gatti *et al.*, 1999; Bourdat-Deschamps *et al.*, 2004).

Autolysis of the starter culture in cheese depends on several different factors. The strain has an influence, but also the cheese-processing conditions such as pH, temperature and salt concentrations have a big influence (Chapot-Chartier *et al.*, 1994; Wilkinson *et al.*, 1994b; Crow *et al.*, 1995a; Crow *et al.*, 1995b; O'Donovan *et al.*, 1996; Buist *et al.*, 1998; Meijer *et al.*, 1998; Hickey *et al.*, 2004; Kozakova *et al.*, 2010). Flavour development may be enhanced during ripening if strains that lyse rapidly are selected, and/or if processes that favour lysis are used (Crow *et al.*, 1993; Fox *et al.*, 1996c). A difference in autolysis can be seen between the two subspecies of *Lactococcus lactis; L. lactis* subsp*. lactis* survives better in cheese than *L.*

lactis subsp. *cremoris*, this suggests that the level of autolysis of the latter is highest (Dawson *et al.*, 1957; Martley *et al.*, 1972; Crow *et al.*, 1993; Chapot-Chartier *et al.*, 1994).

Kozakova *et al.* (2010) examined an autolytic strain (*L. lactis* subsp. *lactis*) and a non-autolytic strain (*L. lactis* subsp. *cremoris*) and how different factors influenced their autolysis. The authors found in their setup that both of them had the highest degree of autolysis when they were exposed to 200 mM sodium citrate, 6.5 g/l NaCl, pH 6.0, temperatures between 13 and 30°C and when cells in early exponential phase were used. The study also demonstrated that a medium with 50 mM sodium citrate, 15 g/l NaCl, pH 5.0 and cells cultivated for 6 hours showed the highest differences in autolysis of the autolytic and non-autolytic strain after 12 days of incubation at 13°C. This medium can be used to screen strains for their autolytic abilities (Kozakova *et al.*, 2010). Another way to determine differences in autolysis is reported in appendix 1. It was found that mutants with a lower level of *guaB* expression (IMP dehydrogenase) lead to a lower degree of autolysis.

As already mentioned, the caseins of the milk are initially broken down by rennet and the lactococcal cell envelope proteinase, hereafter the autolysis of the starter culture releases enzymes beneficial for the cheese ripening (Crow *et al.*, 1995a; Fox *et al.*, 1996c). In cheese, lysis may be slow because of the stabilizing effect of the matrix and this can leave many cells in a permeable stage of cell disruption. Peptides from the cheese curd may freely diffuse inside permeable cells where they are hydrolyzed by intracellular enzymes, thus contributing to the protein degradation and flavour formation in the cheese (Bunthof *et al.*, 2001).

Lysis of the starter is important for the ripening of cheese, but it is a balance. Intact cells are required for the utilization of lactose and maybe for the formation of certain aroma compounds. On the other hand, significant autolysis of the starter is necessary for the proteolysis. The cheese manufacturing conditions, the composition of the cheese, the inherent characteristics of the starter with respect to their proteolytic and flavour potentials, and the susceptibility of the starters to autolysis, will in the end influence the result of the cheese (Crow *et al.*, 1995a; Crow *et al.*, 1995b).

Monitoring Autolysis in Cheese

Cell viability can be assessed rapidly and directly by fluorescence microscopy (Mcfeters *et al.*, 1995). This is an advantage when compared to measuring the cell viability by plate counting, but a limitation is that particular strains cannot be identified (Auty *et al.*, 2001). Fluorescent indicators for the viability of cells can be based on membrane integrity, enzymes activity, membrane potential, respiration or pH gradient (Stubberfield *et al.*, 1990; Molenaar *et al.*, 1991; Rodriguez *et al.*, 1992; Gant *et al.*, 1993; Mcfeters *et al.*, 1995).

The *Bac*Light LIVE/DEAD is a commercial kit from the Invitrogen corporation. In the kit two nucleic acid stains are used; Propidium iodide (PI) and SYTO 9. PI does not penetrate intact cell membranes in contrast to SYTO 9 that diffuses through the plasma membrane. SYTO 9 therefore stains all cells regardless of the membrane integrity, whereas PI only stains cells with damaged membranes. However, PI competes for nucleic acid binding sites with SYTO 9 and SYTO 9 may be replaced by PI. This means that live cells will fluoresce green (SYTO 9) and dead cells will fluoresce red (PI). By using fluorescence microscopy with a suitable optical filter set, the live and dead cells can be viewed separately or simultaneously. The LIVE/DEAD staining has several advantages compared to other staining methods. It is reliable, rapid and both viable and total counts are obtained in one step (Boulos *et al.*, 1999).

Figure 9. CSLM analysis of cheese curd for the lytic strain *L. lactis* AM1 (top) and nonlytic *L. lactis* 320 (bottom). The images are taken from a section of curd after overnight pressing. AM1 shows that a high proportion of cells stain red (indicating the majority of cells are nonviable). In contrast, the image taken from a section of curd manufactured with *L. lactis* 320 illustrates high numbers of viable (i.e., the membrane is intact) cells that stain green as shown by CSLM observation (O'Sullivan *et al.*, 2000).

Autolysis of the starter culture can be monitored by staining cells and, at different time points, counting the number of intact and permeable cells (Bunthof *et al.*, 2001). O'Sullivan *et al.* (2000) showed that different strains have different rates of autolysis and this could be seen by in situ confocal scanning laser microscopy (CSLM) where the cells were stained by LIVE/DEAD *Bac*ligth (Figure 9). Also Bunthof *et al.* (2001) have shown that it is possible to stain cells with LIVE/DEAD staining in a cheese (Figure 10). Many researchers have used

LIVE/DEAD staining as a method for analysing the viability of a bacterial population (Virta *et al.*, 1998; Boulos *et al.*, 1999; Berney *et al.*, 2007).

Figure 10. CSLM image of 2-week-old Gouda cheese stained with SYTO 9 and PI. Bar, 10 mm (Bunthof et al., 2001).

In Appendix 1 we have used PI for staining dead cells, while total cells are estimated in bright field. When the media used are transparent, staining with SYTO 9 is not required, as cells can be visualized by bright field. We were able to show a difference in the growth and death rate of different mutants. It was shown that, within each strain, there were large variations in each field of view of growth. The large variations that would normally prevent a differentiation of the mutant, could be overcome by taking the average of several spots. Figure 11 shows an example of growth of *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *lactis* GuaB41 (Appendix 1). IL1403 have a shorter generation time than GuaB41 in a synthetic minimal media (SA) and this is consistent with the pictures in Figure 11.

Figure 11. Pictures of IL1403 and GuaB41 are shown after 0, 24 and 134 hours. Pictures are taking with

IL1403

GuaB41

	Bright Field	**Fluorescence**	**Bright Field**	**Fluorescence**
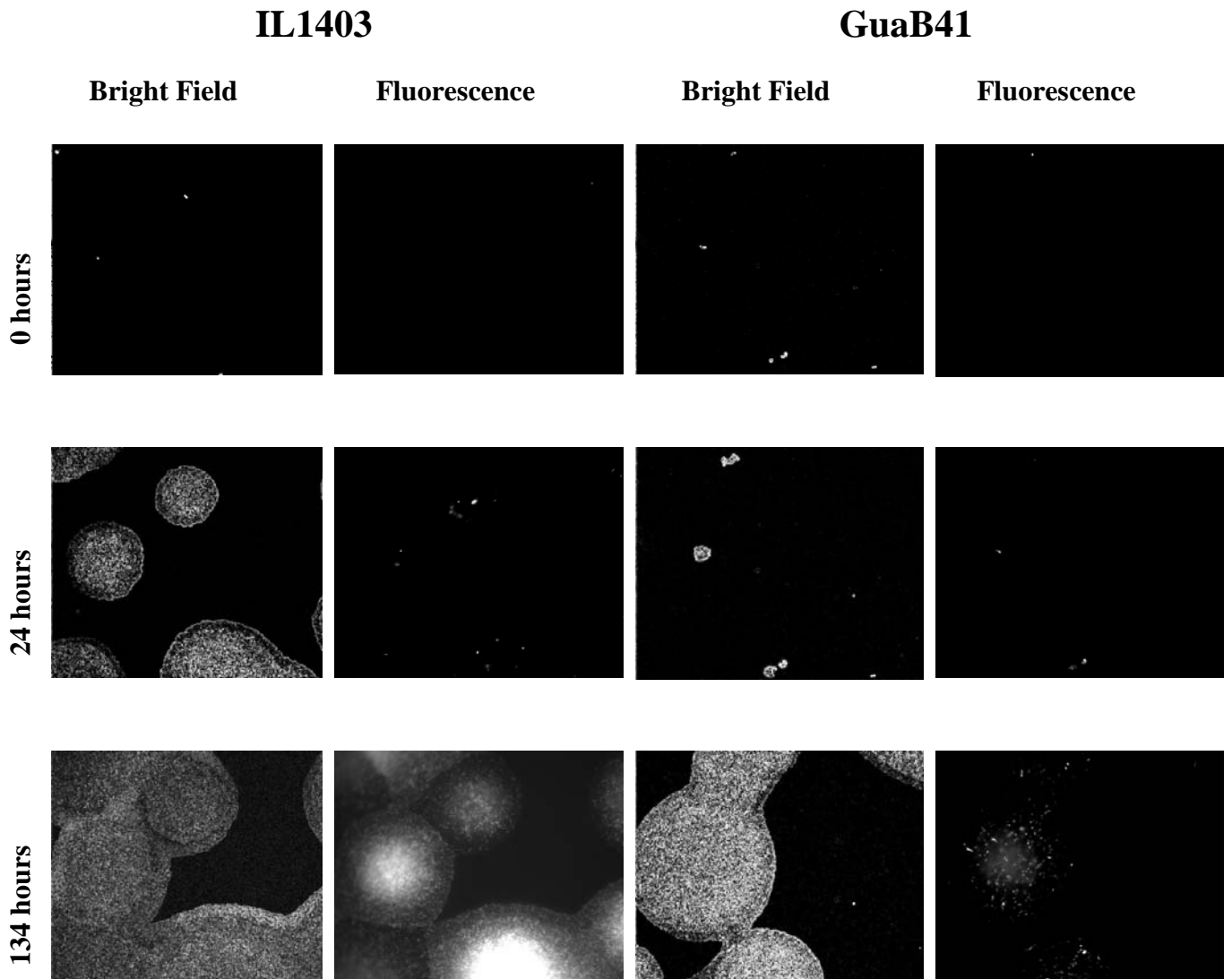
Bright Field and Fluorescence microscopy. The samples have been dyed with Propidium Iodide (PI) which diffuses through the cell membrane of permeable cells. Cells are defined as dead when they have taken up PI. Growth of cells as well as the death of cells can be followed and differences can be seen between the two strains displayed. Modified from Appendix 1.

4 Acid Stress in *Lactococcus*

In cheese production, the first challenge that the starter culture encounters is acid stress. The cells thus have to grow at low pH, in order for the production of cheese to succeed. Lactococci are acid tolerant and their optimal pH conditions for growth are 6.3-6.9 (Harvey, 1965; Bibal *et al.*, 1988; Andersen *et al.*, 2009). During the fermentation of milk the pH drops because of the production of lactic acid. At external pH values higher than 5, the Lactococci are able to maintain a ΔpH of around 1, with alkaline interior. However, by the end of the fermentation the pH in the milk is around 4.5. At this pH, the cells can maintain a ΔpH of around 0.6 for at least 72 hours. It is the low pH_i that causes the arrest in growth, while the nutrients are still in excess (Nannen *et al.*, 1991). It has been shown that for *L. lactis* subsp. *cremoris* 712, pH_i values between 7.2 and 5.9 enables growth, but with decreasing growth rate at low pH_i (O'Sullivan *et al.*, 1997).

Most Lactoccoci appear to have several inducible responses to low pH. Carbon starvation and mildly acidic media induce resistance to acid stress (Hartke *et al.*, 1994; Rallu *et al.*, 1996; Hartke *et al.*, 1996; Kim *et al.*, 1999). Exposure to sublethal concentrations of ethanol, H_2O_2 and NaCl did not increase tolerance to potentially lethal acid concentration in *L. lactis* subsp. *cremoris* 712. On the other hand, heat and acid did increase acid tolerance (O'Sullivan *et al.*, 1997), thus indicating that heat and acid stress responses are somehow overlapping (Frees *et al.*, 2003). A physical maintenance of pH homeostasis might be involved when acid-adapted cells, during an acid challenge, maintain a slightly higher pH_i (around 0.2) than non-adapted cells (O'Sullivan *et al.*, 1997).

A large number of proteins are induced in LAB during acid adaptive response. Some of the proteins induced at acid stress adaptation are heat shock proteins (mostly chaperons) and subunits of the H^+-ATPase (O'Sullivan *et al.*, 1999; Champomier-Verges *et al.*, 2002). In *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363, acid adaptation induced 33 and 23 proteins, respectively (Hartke *et al.*, 1996; Frees *et al.*, 2003). Surprisingly, *de novo* synthesis of proteins did not seem to be necessary in *L. lactis* subsp. *lactis* IL1403 for survival of acid stress (Hartke *et al.*, 1996), whereas *L. lactis* subsp. *cremoris* MG1363, was dependent on

de novo synthesis of proteins for acid adaptation (Rallu *et al.*, 1996) and this also resulted in resistance from other stresses (heat, ethanol, H_2O_2 and NaCl) (O'Sullivan *et al.*, 1997; O'Sullivan *et al.*, 1999).

Budin-Verneuil *et al.* (2005) showed that the acid tolerance response of *L. lactis* subsp. *cremoris* MG1363 was different when the cells were grown in M17 compared to SA medium. This might explain the results from Rallu *et al.* (1996) where it was found that *de novo* synthesis of proteins was necessary for the acid tolerance response in *L. lactis* subsp. *cremoris* MG1363 in SA, whereas Hartke *et al.* (1996) found that the acid tolerance response in *L. lactis* subsp. *lactis* IL1403 was not depending on *de novo* synthesis of proteins in M17. This was confirmed by Budin-Verneuil *et al.* (2005) using the same strain MG1363 in the two media showing that the results were not related to difference in subspecies, but a difference in media used. MG1363 was dependent on *de novo* protein synthesis for acid tolerance response in SA medium but not in M17 medium. This should be taken into consideration when results from different experiments are being compared. In appendix 2 we also confirmed that acid tolerance is different in M17 and SA medium. At pH 3.0 MG1363 survived 100% in GSA medium but only 0.1% survived in GM17 medium after 25 min. This shows a big difference between the two media and how the rich medium GM17 renders the strain sensitive to acid stress.

Exponential growing cells are much more sensitive to acid stress than cells in the stationary phase (Rallu *et al.*, 2000). During exponential growth there were only small changes in pH_i, suggesting that other factors than pH_i were responsible for the acid resistance in the exponential phase (Alemayehu *et al.*, 2000). The level of autolysis of *Lactococcus lactis* greatly decreases when the cells have entered stationary phase before they are exposed to e.g. acid stress. Also within the exponential phase there is a difference in the level of autolysis. By examining strains in the exponential phase Kozakova *et al.* (2010) showed that early exponential phase cells survived poorly due to autolysis compared to cells in a later growth stage when exposed to acid stress. This emphasizes the importance of controlling the growth phase of the cells when stress response is examined.

Rallu *et al.* (2000) tested 14 mutants that were resistant to acid stress which were constructed by insertional mutagenesis and selecting survivors at pH 5 and 37.5°C. 10 of these mutants grew

with the same rate as the wild type (*L. lactis* subsp. *cremoris* MG1363) with a doubling time of around 32 min in M17 media while the last 4 mutants grew slower (doubling time ~43 min). Auxotrophy in some of the mutants might be correlated to their resistance to acid stress, although it does not necessarily mean that they grow slow. Six of the mutants survived 120 to 1000-fold better than MG1363 after 2 days at pH 4.3. Four of these mutants were affected in purine metabolism. These mutants were also 20 to 110-fold better at surviving carbon starvation at pH 6.8. Since carbon starvation often involves global stress resistance, this could suggest multistress resistance. This was confirmed, as they were resistant to heat shock and three of them to oxidative stress (Rallu *et al.*, 2000).

5 Nucleotide Metabolism in *Lactococcus*

Nucleotides are vital for living cells because they are needed for the synthesis of DNA, RNA and several coenzymes. Therefore the synthesis and salvage of nucleobases, nucleosides and nucleotides are some of the most important biochemical pathways in the cell (Martinussen *et al.*, 2003).

Nucleotides play a role in nearly all biochemical processes. They are precursors of DNA and RNA, and serve as cofactors in sugar and lipid metabolism, in methylation reactions and as currency of energy in biological systems. They are also constituents of the redox coenzymes NAD^+, $NADP^+$, FAD and CoA (Stryer, 1999). Nucleotides are found as either ribo- or deoxyribonucleotides, and these are either mono- di- or triphosphates. The triphosphates are substrates for DNA and RNA polymerases. The mono- and diphosphates are intermediates in the biosynthetic reactions and they may also arise from hydrolytic or phosphorylytic breakdown of nucleic acids or other metabolic activities in the cell. Nucleosides and nucleobases may be converted to nucleotides by the salvage pathways (Kilstrup *et al.*, 2005). Figure 14 shows the purine biosynthesis and salvage pathways found in Lactococci.

Some LAB are able to produce nucleotides *de novo*, whereas others require access to either purines or pyrimidines for growth. After transport into the cell, salvage pathways in auxotrophic bacteria are used for conversion of the nucleobases or nucleosides to nucleotides. Nucleotides cannot be transported into the cell but need to be dephosphorylated to nucleosides by extracellular nucleotidases before entering the cell (Kilstrup *et al.*, 2005). In prototrophic bacteria, salvage systems for the uptake and utilization of nucleobases and nucleosides, are also present, and under conditions with excess of purine or pyrimidine sources the *de novo* pathway can be shut down (Kilstrup *et al.*, 2005).

5-phosphoribosyl-α-1-pyrophosphate (PRPP) is a common precursor in both purine and pyrimidine metabolism. PRPP is used for the biosynthesis of nucleotides, nicotinamide coenzymes (NAD^+, $NADP^+$), the amino acids histidine and tryptophane and for the salvage of nucleobases (Figure 12). Other roles for PRPP are described in the purine metabolism section.
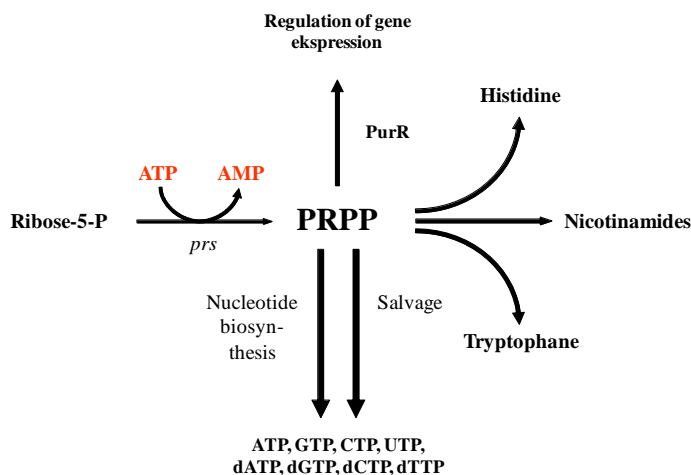
Figure 12. 5-phosphoribosyl-α-1-pyrophosphate (PRPP) synthesis and utilization. PRPP is synthesized by addition of a pyrophosphate group from ATP to ribose-5-phosphate. The reaction is catalyzed by the PRPP synthase, encoded by the *prsA* and *prsB* genes. PRPP is utilized in both biosynthesis and salvage of purines and pyrimidines, biosynthesis of the amino acids histidine and tryptophane, and production of nicotinamides. Finally, PRPP is an effector molecule in PurR-mediated gene regulation (Kilstrup *et al.*, 2005).

5.1 Purine Metabolism

In general 10 enzymatic steps are required for *de novo* synthesis of IMP (Figure 13), which can function as a precursor for both AMP and GMP nucleotides (Zalkin *et al.*, 1992; Kilstrup *et al.*, 2005). Purine nucleotides can also be formed by salvage reactions from purine nucleosides and bases (Neuhard *et al.*, 1987; Nygaard, 1993a; Nygaard, 1993b). The *de novo* synthesis seems to be conserved among various organisms, whereas the salvage pathways seems to vary more between the organisms (Nygaard, 1993b).

ATP   GTP
↑ *pyk*   ↑ *pyk*
ADP   GDP
↑ *adk*   *purB*   *purA*   ↑ *gmk*
AMP ← sAMP ← IMP —*guaB*→ XMP —*guaA*→ GMP

                              ↑ *purH*

                         FAICAR
                         ↑ *purH*

PRPP - - - -*his*- - - → AICAR
↓ *purF*   ↑ *purB*
PRA   SAICAR
↓ *purD*   ↑ *purC*
GAR   CAIR
↓ *purN*   ↑ *purEK*
FGAR → FGAM → AIR
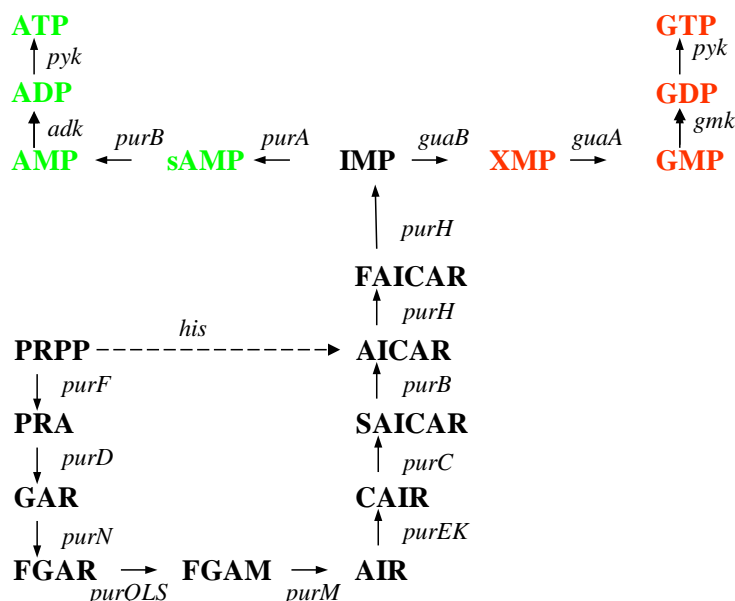   *purQLS*   *purM*

Figure 13. IMP is formed *de novo* from PRPP through several steps. IMP can be converted into ATP or GTP. The following abbreviations are used: PRPP, 5-Phosphoribosyl 1-pyrophosphate; PRA, phosphoribosyl amine; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, phosphoribosyl carboxyaminomidazole; SAICAR, succinocarboxyamide carboxyaminoimidazole ribonucleotide; AICAR, aminoimidazole carboxyamide ribonucleotide; FAICAR, formaminoimidazole carboxyamide ribonucleotide; sAMP, adenylsuccinate; IMP, inosine monophosphate; XMP, xanthosine monophosphate. Enzymes encoded by the genes in alphabetical order, *adk*, adenylate kinase (AMP kinase); *gmk*, guanylate kinase (GMP kinase); *guaA*, GMP synthase; *guaB*, IMP dehydrogenase; *purA*, adenylosuccinate synthase; *purB*, adenylosuccinate lyase; *purC*, SAICAR synthase; *purD*, GAR synthase; *purEK*, CAIR synthase; *purF* PRPP amidotransferase; *purH* bifunctional AICAR transformylase/IMP cyclohydrolase; *purM*, AIR synthase; *purN*, GAR transformylase; *purQSL*, FGAM synthase; *pyk*, pyruvate kinase. Modified from Kilstrup *et al.* (2005).

In gram-positive bacteria only one of the genes in the de novo synthesis of purines (*purF*) has been studied in details (Kilstrup *et al.*, 2005). PurF catalyzes the formation of phosphoribosyl amine (PRA) by the displacement of the pyrophosphate group of PRPP with an amide group (Figure 13). Ruppen and Switzer (1983) showed that the stringent response and accumulation of 3'-pyrophosphorylguanosine-5'-pyrophosphate (ppGpp) and 3'-pyrophosphorylguanosine-5'-triphosphate (pppGpp) play a role in the degradation of PurF. The stringent response is a regulatory response induced by starvation for amino acids or carbon sources. When the ribosome

encounters uncharged tRNA's, the stringent factor (RelA) become activated and further activate the synthesis of the two nucleotides, ppGpp and pppGpp (Haseltine *et al.*, 1973; Wendrich *et al.*, 2002). This high level of (p)ppGpp accumulation that is induced under amino acid starvation (Ruppen *et al.*, 1983), is known to specifically inhibit the IMP dehydrogenase enzyme, resulting in limited synthesis of XMP and GMP (Gallant *et al.*, 1971; Ochi *et al.*, 1982; Inaoka *et al.*, 2002; Budin-Verneuil *et al.*, 2007). Some mutants in the *relA* gene have shown stress resistant, as described more in the next section.

L. lactis rely on phosphoribosylation of bases in purine salvage since they cannot directly phosphorylate purine ribonucleosides. PRPP is essential in these organisms because it is used in the biosynthesis of nucleotides and the salvage of nucleobases (Kilstrup *et al.*, 2005).

Kilstrup and Martinussen (1998) showed, as previously found in *Bacillus subtilis* (Weng *et al.*, 1995), that PRPP in *L. lactis* is not only a substrate in the biosynthetic pathway, but also induce gene expression of genes in the *de novo* synthesis of purines. The expression of the purine biosynthesis genes in *Lactococcus* is dependent on an activator (PurR), which stimulates transcription under high PRPP concentrations. The regulation of the *de novo* pathway is therefore feed-forward by its precursor PRPP, which ensures that the pathway is only fully active when the substrate is found in excess. PRPP is under allosteric regulation from ADP, which has the effect that a high concentration of ADP will inhibit the catalytic effect of PRPP (Eriksen *et al.*, 2000) and prevent activation of PurR regulated genes. Martinussen *et al*. (2003) showed that in the presence of exogenous purines the PRPP pool was low. This again indicates that PRPP functions as a reporter of the purine nucleoside status in the cell.

Purine-requiring mutants of *L. lactis* cannot grow in milk, as the wild type strain (Dickely *et al.*, 1995). This means *de novo* synthesis of purine nucleotides are required for *L. lactis* when growing in milk (Nilsson *et al.*, 1998). Martinussen *et al.* (2003) showed that addition of exogenous purines in GSA media led to an increase in both growth rate and size of the purine nucleotide (triphosphate) pools in *L. lactis* subsp. *cremoris* MG1363, which implies that the strain has a partial purine requirement. It appears that the capacity of *de novo* synthesis of both purines and pyrimidine in *L. lactis* are limited (Kilstrup *et al.*, 2005). Reduced growth rates were obtained by growing strains with mutations in nucleotide biosynthesis genes under conditions

where the availability of nucleotide precursors was limited (Martinussen *et al.*, 2003) (Appendix 1).

Two different nucleoside transporters have been found in *L. lactis*. One enables the uptake of uridine and the other is responsible for the uptake of different purine nucleosides and cytidine (Martinussen *et al.*, 2003). This means that adenosine (AR), guanosine (GR), inosine (IR) and cytidine will be competing for the same transporter and that high concentration of one nucleoside will diminish the uptake of the other nucleosides. In this way it is possible to modulate the intracellular nucleotide pools. This is a principle we have used in Appendix 2.

Purines and Stress Resistance

Rallu *et al*. (2000) obtained 21 different insertional mutants of *L. lactis* subsp. *cremoris* MG1363 that are resistant to acid stress. Some of these mutants were only resistant to acid stress and some were multi stress resistant. Analysing these mutant indicated that intracellular pools of phosphate and guanine nucleotides (both GMP/GTP and (p)ppGpp) could be involved in the regulation of acid stress resistance. Some of the mutants examined by Rallu *et al*. (2000) was *guaA*, *hpt, deoB*, *pstS* and *relA**. It is important to know the function of these genes in relation to the purine metabolism, in order to understand how they can lead to stress resistance, therefore a short description of these genes follows (also see Figure 14):

guaA

The GuaA enzyme catalyzes the formation of GMP from XMP, and inactivation of the *guaA* gene will block GMP synthesis and render GMP formation dependent upon the salvage of GR or G (guanine) from the medium.

Hpt and *hprT*

The genes *hpt* and *hprT* encode guanine and hypoxantine phosphoribosyl tranferases that catalyses the formation of GMP and IMP from G and Hx (hypoxanthine), respectively (Nilsson *et al.*, 1992) (Appendix 2). Inactivation of *hpt* and *hprT* will block GMP formation by salvage and render GMP formation dependent upon de novo synthesis.

deoB/pup

The gene *deo*B is part of en operon containing *deo*B, *orf*C (unidentified open reading frame) and *deo*D (purine-nucleoside phosphorylase (*pup*)). Insertion in *deo*B is believed to inactivate the whole operon and therefore also the *pup* gene (Duwat *et al.*, 1999). The Pup enzyme catalyzed the convertion of GR, IR and AR to G, Hx and A, respectively (Martinussen *et al.*, 1995). An inactivation of the (*deoB* or *orfC* or) *pup* gene will therefore prevent salvage of purines from nucleosides.

pstS

*pst*S encode the substrate binding component of the high affinity ABC transporter of phosphate. Inactivation of *pstS* leads to a reduction in the intracellular phosphate concentration. Phosphate is needed for salvage of purines and a lowering of the phosphate concentration might therefore lead to a lowering of the GMP/GTP pool.

relA

The *relA* mutant, which has been found to be multi-stress resistant (Rallu *et al.*, 2000) has by a more complicated pathway to stress resistance. The wild type RelA protein is closely bound to the ribosome and produces (p)ppGpp, from GTP and GDP, when the ribosome encounters uncharged tRNA's during the stringent response (Haseltine *et al.*, 1973; Wendrich *et al.*, 2002). It was found that the multi-stress resistant mutation resulted in truncated RelA proteins, called RelA*, which synthesized (p)ppGpp in an uncontrolled fashion (Rallu *et al.*, 2000; Budin-Verneuil *et al.*, 2007). Since high concentrations of (p)ppGpp is known to specifically inhibit the IMP dehydrogenase enzyme, the synthesis of XMP and thereby GTP was proposed to be limited in the *relA** mutant (Ochi *et al.*, 1982; Rallu *et al.*, 2000; Inaoka *et al.*, 2002; Budin-Verneuil *et al.*, 2007). Besides limiting the production of GTP the high production of (p)ppGpp drains the GDP and GTP pools and results in even lower levels. In either case a RelA* mutant is expected to have low levels of GMP and GTP.
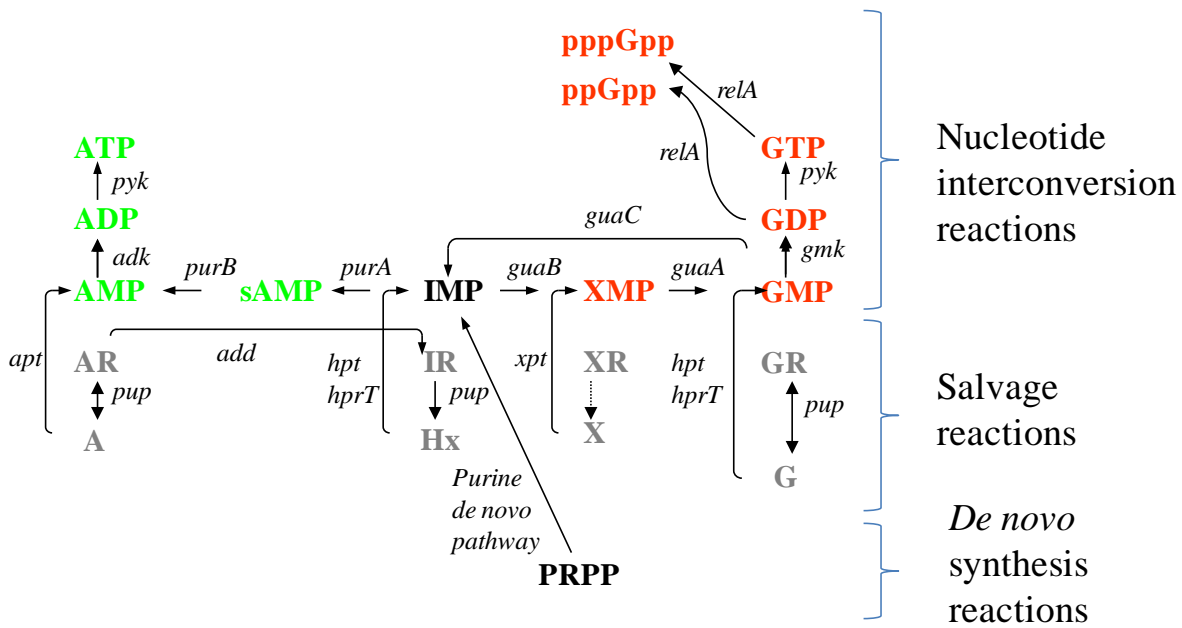
Figure 14. Purine metabolic network in *L. lactis*. The metabolic network is devided into three types of reactions, *de* novo synthesis reactions, nucleotide interconversion reactions, and salvage reactions. IMP is formed *de novo* from PRPP through several steps. Only bases and nucleosides, the substrates for the salvage reactions can be supplied from the medium. The following abbreviations are used: PRPP, 5-Phosphoribosyl 1-pyrophosphate; A, adenine; Hx, hypoxanthine; X, xanthine; G, guanine; AR, adenosine; IR, inosine; XR, xanthosine; GR, guanosine; sAMP, adenylsuccinate; IMP, inosine monophosphate; XMP, xanthosine monophosphate. Enzymes encoded by the genes in alphabetical order, *add*, adenosine deaminase; *adk*, adenylate kinase (AMP kinase); *apt*, adenine phosphoribosyltransferase; *gmk*, guanylate kinase (GMP kinase); *guaA*, GMP synthase; *guaB*, IMP dehydrogenase; *guaC*, GMP reductase; *hpt*, hypoxanthine/guanine phosphoribosyltransferase; *hprT*, hypoxanthine/guanine phosphoribosyltransferase; *pup*, purine nucleoside phosphorylase; *purA*, adenylosuccinate synthase; *purB*, adenylosuccinate lyase; *pyk*, pyruvate kinase; *relA*, (p)ppGpp synthase and *xpt*, xanthine phosphoribosyltransferase. Modified from Kilstrup *et al.* (2005).

Rallu *et al* (2000) showed that addition of GR/G did not have any influence on the survival of the *hpt* mutant in acid stress experiments, but addition of Hx made the mutant die out at the same rate as the wild type. The *gua*A mutant stayed resistant with the addition of Hx, but was not resistant when GR or G was added. The results for the *gua*A mutant are consistent with the result we got (see Appendix 2) and with the hypothesis that a low GMP/GTP pool induces stress resistance. Multi-stress resistant *guaA* mutants have been shown to grow slower than the wild type (Rallu *et al.*, 2000), which indicates that the supply of GMP from purine salvage was suboptimal. The *gua*A gene is shown to be inhibited by the addition of decoyinine, and addition of decoyinine to the wild type before acid challenge gives the cells 100 fold better survival than

the non treated cells (Rallu *et al.*, 2000). This again indicates that the intracellular nucleotide pool have to be low for the cell to be resistant to acid stress.

Three of the acid resistant mutant from Rallu *et al* (2000) were further examined by Budin-Verneuil *et al.* (2007) for their protein expression compared to the wild type. The three mutants had insertions in the *gua*A, *rel*A or *pst*S gene (see Figure 14). When Budin-Verneuil *et al.* (2007) examined the expression of proteins in the mutants compared to the wild type they found significantly different patterns. Of a total of 58 upregulated proteins in the three mutants, but few of these genes were upregulated in all three mutants. The three mutants had similar effects on the concentration of GTP. As mentioned, insertion in *gua*A leads to a decrease in GMP/GTP because the formation of GMP from XMP is inhibited. The truncated RelA* protein promotes the accumulation of (p)ppGpp, which leads to inhibition GuaB (IMP dehydrogenase) and this again leads to a decrease in GMP/GTP. The lower intracellular phosphate concentration, as a result of the insertion in *pst*S gene, probably affects the concentration of GMP/GTP by decreasing it (Budin-Verneuil *et al.*, 2007). This means that these acid resistant mutants have a low concentration of GMP/GTP, this is consistent with our result from Appendix 2. Here we also show a better survival at acid challenging by cells with low intracellular GMP/GTP concentration.

Duwat *et al.* (1999) examined temperature resistant mutants found in a *rec*A strain of *L. lactis* subsp. *cremoris* MG1363. Out of 18 obtained mutants 10 were involved in guanine metabolism. They were affected in the genes *deo*B, *gua*A and *tkt*A. The function of *deo*B and *gua*A were explained above. *tkt*A encodes a transketolase that catalyses the transformation of xylose-5-phosphate to ribose-5-phosphate (a precursor in the nucleotide biosynthesis). All these three genes are therefore likely to affect the guanine nucleotide pools in the cell by lowering it. The temperature resistance of the *rec*A-*gua*A mutant could be lost by addition of GR or G to the media, which would properly increase the guanine nucleotide pool. For the *rec*A-*deo*B mutant the resistance was lost by addition of G and Hx and for the *rec*A-*tkt*A mutant it was the addition of GR, G, Hx and IR that made the strain sensitive (Duwat *et al.*, 1999). These results indicate that the guanine nucleotide pools also have an impact on the temperature sensitivity in *L. lactis* subsp. *cremoris* MG1363, this was also the case for acid resistance.

In appendix 1 it is shown that when lowering the expression of *guaB* the generation time in GSA is increased. This tendency is illustrated in Figure 11. *L. lactis* subsp. *lactis* IL1403 is the wild type and *L. lactis* subsp. *lactis* SGJ126 is a mutant that expresses only 41% of *guaB* compared to IL1403. The cell death of SGJ126 is much lower than IL1403, which could be explained by the low level of guanine nucleotides in SGJ126. Starvation for guanine nucleotides in *Lactococcus* makes it more stress resistance (appendix 2). All mutants examined in appendix 1 with a lower expression of *guaB* compared to the wild type dies in a lower rate (Figure 11). Again this points out the significance of the GMP/GTP level on the survival and stress resistance of the cells.

It is shown in Appendix 2 and also shown by Rallu *et al* (2000) that the GMP pools may have an important influence on the acid stress resistance of MG1363. Also for temperature resistance the GMP pools was shown to have an influence (Duwat *et al.*, 1999). Of special interest for studying stress resistance are the genes *guaA, guaB, hpt, hprT, pup* and *relA* and the way their gene products are related to purine nucleotide metabolism (Figure 14). These mutants are able to prevent *de novo* synthesis of purine nucleotides (*guaA, guaB* and *relA*) or salvages of purine bases and nucleosides (*hpt, hprT* and *pup*) and lead to low GMP/GTP pools, which again lead to stress resistance as shown Appendix 2. The *relA** mutant was not examined in Appendix 2, but we are convinced that it is the low GMP/GTP pool in the mutant that gives it its stress resistance phenotype.

6 Conclusion and Perspectives

Results from Appendix 1 show that large variations are seen when examining growth of *Lactococcus lactis* on a solid surface. We showed it was necessary to make multiple observations in order to lower the standard error means, so that differences in growth between strains could be determined. The developed method could be further developed to examine growth and death of cells on other solid surfaces, for example in cheese. This could provide us with a greater knowledge of how cells behave in microcolonies on solid surfaces or within a food matrix.

The mutants used in Appendix 1 had different levels of *guaB* expression. The level of gene expression influenced the growth rates as well as the death rates of the mutants. Both the wild type and the mutant with wild type expression of *guaB*, grew faster and to a higher number compared to the mutants with a lower expression of *guaB*. Furthermore, they had the highest ratio of dead cells. A higher expression of *guaB* would probably lead to a higher amount of GMP/GTP and this leads to stress sensitivity as shown in Appendix 2. However, additional studies on mutants with higher expressions of *guaB* would be needed to confirm this. Since stress sensitive strains have a higher level of autolysis, this could accelerate the ripening of cheese. However, further studies are needed in order to shed light on this theory.

In Appendix 1 we also observed that cells from the same strain stopped growing at different stages, visualized as microcolonies of different sizes. As mentioned in the Appendix, we do not have an explanation for this. Further studies are needed to examine the background for this phenomenon, and could be a subject for future studies.

In Appendix 2 we have shown that *Lactococcus lactis* were intrinsically resistant to acid stress, but became sensitive when grown in media containing purines and where conversion in *Lactococcus lactis* to GMP/GTP was possible. We were puzzled by the fact that the double deletion mutant Δ*hpt* Δ*hprT* was stress sensitive in the GSAM17 medium that we had designed. The double mutant was not expected to be able to convert nucleobases (Hx, G) into nucleosides (IMP, GMP), but it appeared to retain this ability under certain conditions. The results in Appendix 2 suggest that the *apt* gene product could be responsible for conversion of Hx and G

into IMP and GMP, and for future studies a $\Delta apt \, \Delta hpt \, \Delta hprT$ triple mutant could be used to elucidate this issue.

It is possible to measure the intracellular levels of GMP/GTP, and by combining these measurements with the stress experiments, it might provide us with even deeper insight into stress sensitivity. The addition of phosphate appeared to increase stress sensitivity in the experiments from Appendix 2. The role of phosphate is not quite clear, but we do consider that phosphate addition could speed up the conversion of nucleosides and thereby increase the level of nucleotides. By determining the GMP/GTP levels before and after phosphate addition, the effect of phosphate addition on the level of GMP/GTP could be determined.

In the future, further investigation on the role of guanine nucleotides in the acid stress sensitivity of *Lactococcus lactis* (and maybe other LAB) is required. In my view, it is of the utmost importance to the dairy industry to widen their knowledge about the background for stress resistance and sensitivity in LAB. If acidification to a low pH is desired, the *Lactococcus* should grow with a low amount of G-nucleotides, whereas adding precursors for the G-nucleotide will inhibit the *Lactococcus* because of stress sensitivity. More knowledge of this balance would be of great importance in regulating the process and creating high quality products.

The results from Appendix 3 show, that we are able to fractionate cells from a cheese (casein) matrix by the use of guanidinium chloride. The cells remained intact, we could subsequently purify RNA from the cells that can be used further for transcriptome analysis. Proteome analysis of cells after guanidinium chloride extraction from acidified milk and from a Cheddar cheese was performed and showed promising results. Until now it has been rather difficult to examine the proteome and transcriptome expression of LAB as soon as they are embedded in the coagulated milk or cheese. The ability to fractionate the cells from a casein matrix permits us to follow the behaviour of the LAB during the whole cheese production and thereby enabling us to regulate and control the process more elaborately.

The method developed in Appendix 3, might be used for other food matrixes, and would be a great advantage in allowing examination of microbial development, proteome and transcriptome analysis in many kinds of food matrixes. We know that cells behave differently in a synthetic

laboratory medium compared to a complex food matrix, and by extracting cells from these complex matrixes we will get a better understanding of microbial behaviour in the actual products.

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