THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of respiratory physiology in *Lactococcus lactis* for high-yield production of robust high-performance starter cultures

ANNA JOHANSON

Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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ANNA JOHANSON

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Division of Industrial Biotechnology
Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone: + 46 (0)31-772 1000

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Illustration of *Lactococcus lactis* in the form of fermentation broth, frozen and freeze-dried pellets. Images are copyright of Chr. Hansen A/S and printed with permission.

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ABSTRACT

Fermented food products are consumed world-wide, every day, and the demand is growing as intake increases. *Lactococcus lactis* is a lactic acid bacterium commonly used as a starter culture to produce fermented dairy products. The quality of starter cultures is linked to the conditions during its production process and affects the culture activity during its use in dairy product fermentation. Therefore, industrial manufacturers of starter cultures strive to not only optimize biomass yield, but also the cell robustness. *L. lactis* starter cultures are produced by anaerobic fermentation or by aerobic respiration when haemin is available in the cultivation medium.

The aim of this work was to investigate the response of *L. lactis* to respiration-permissive conditions, to enable redesign and optimization of the batch production process. The process parameters were carefully chosen to mimic the industrial process. The effects of the specific growth rate on respiratory metabolism, energetics and cell quality were quantified using chemostat cultivations. Compared to anaerobic metabolism, the respiratory metabolism of *L. lactis* was remarkably flexible, and could be modulated by controlling the dilution rate. The lowest dilution rate supported full respiratory metabolism, whereas the higher dilution rates caused a shift towards respiro-fermentative metabolism. The inoculation procedures were investigated in detail to gain an understanding of the occurrence of lag phases after inoculation. It was found that the length of the lag phase in subsequent main cultures was related to galactose excretion in lactose-grown pre-cultures. Furthermore, based on *lacS* gene expression measurements in lactose-grown cultures, it is suggested that LacS is responsible for the galactose excretion as a galactose-lactose antiporter.

The quality of frozen and freeze-dried products was investigated and sensitivity to freeze-drying was found to be associated with the physiological state of the cells during cultivation. Cells harvested under respiration-permissive conditions in batch and chemostat cultivations at low dilution rate were less robust to freeze-drying, whereas higher dilution rates led to robust cells performing equally well or better than anaerobic cells.

The findings of this work underline the importance of systematically studying the combination of upstream and downstream aspects of production processes. The results indicate that by controlling the specific growth rate and the haemin concentration during the aerobic growth of *L. lactis*, not only higher biomass yields, but also better cell robustness, can be achieved.

Keywords: Lactic acid bacteria, batch cultivations, continuous cultivations, aerobic growth, haemin/haem, respiration-permissive conditions, starter culture production, lag phase, acidification activity

Preface

This doctoral thesis partially fulfils the requirements for a PhD degree at the Department of Biology and Biology Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Sweden. The work presented in this thesis was performed between 2014 and 2020 under the supervision of Associate Professor Carl Johan Franzén, and in collaboration with Chr. Hansen A/S. The industrial PhD programme was funded by Chr. Hansen A/S and the Danish Agency for Science, Technology & Innovation, Innovation Fund Denmark, project no. 1355-00172B.

This thesis summarizes the most important parts of my research during these years, focusing on improving our understanding of the effects of cultivation conditions on *L. lactis* on its performance as a starter culture. The main part of the work, lactose cultivations in batch and chemostat mode with downstream processing, was carried out at the Upscaling Department, R&D Process Development at Chr. Hansen A/S, under the supervision of Dr Anisha Goel. Acidification analysis was performed at the Microbial Analytical Department at Chr. Hansen A/S. The galactose cultivations were performed at the Division of Industrial Biotechnology at Chalmers University of Technology, under the supervision of Dr Bettina Lorántfy, Professor Lisbeth Olsson and Associate Professor Carl Johan Franzén.

Anna Johanson
August 2020

List of publications

This thesis is based on the following papers, which will be referred to as **Papers I-IV** in the text. The papers are appended at the end of the thesis

Paper I **Johanson, A.**, Kovács J., Olsson, L., Goel, A., & Franzén, C. J. (2020)
Hemin-activated respiration in *Lactococcus lactis* for high-yield biomass production depends on hemin concentrations
Submitted.

Paper II **Johanson, A.**, Goel, A., Olsson, L., & Franzén, C. J. (2020)
Respiratory physiology of *Lactococcus lactis* in chemostat cultures and its effect on cellular robustness in frozen and freeze-dried starter cultures
Applied and Environmental Microbiology, 86:e02785-19.

Paper III Lorántfy, B., **Johanson, A.**, Faria-Oliveira, F., Franzén, C. J., Mapelli, V., & Olsson, L. (2019)
Presence of galactose in precultures induces *lacS* and leads to short lag phase in lactose-grown *Lactococcus lactis* cultures
Journal of Industrial Microbiology and Biotechnology, 46(1), 33–43.

Paper IV **Johanson, A.**, Olsson, L., Franzén, C. J. & Goel, A. (2020)
Respiratory growth of *Lactococcus lactis* depends on hemin concentration and leads to impaired performance after freeze-drying
Manuscript.

Author's contributions

My contributions to each of the papers included in this thesis are summarized below.

Paper I First author. I designed the study and performed the experimental work in collaboration with Jakub Kovács (MSc), I analysed and evaluated the data, and wrote the manuscript.

Paper II First author. I initiated the study, established a continuous cultivation platform, and planned and performed the experimental work. I collected and evaluated the data, and wrote the manuscript with the support of Associate Professor Carl J. Franzén.

Paper III Second author. I planned and designed the pre-culture study together with Dr Bettina Lorántfy, and we performed the experiments in collaboration. Dr Fábio Faria-Oliveira helped in the expression analysis of *lacS*. I participated in the evaluation of the data and writing the manuscript.

Paper IV First author. I designed the study and performed the experimental work. The acidification measurements were performed at the Analytical Department at Chr. Hansen A/S. I collected and analysed the data, and wrote the manuscript.

Abbreviations and Symbols

3PG	3-phosphoglycerate
ACKA	Acetate kinase
ADHA	Alcohol dehydrogenase
ADHE	Acetaldehyde
ADP	Adenosine diphosphate
AE	Aerobic
ALDB	Acetolactate decarboxylase
ALS	Acetolactate synthase
AMP	Adenosine monophosphate
AN	Anaerobic
ATP	Adenosine triphosphate
AXP	Adenine nucleotides ATP/ADP/AMP
CcpA	Carbon catabolite protein A
CCR	Carbon catabolite repression
cre	Catabolite responsive element
D	Dilution rate
DHAP	Dihydroxyacetone-phosphate
DNA	Deoxyribonucleic acid
Early	Early exponential growth phase, galactose not yet present
EC	Adenylate energy charge
EI	Enzyme I, general protein involved in the PTS
EII	Enzyme II, sugar-specific proteins involved in the PTS
ETC	Electron transport chain
FB	Fermentation broth
FBP	Fructose-1.6-bisphosphate bisphosphate
FDP	Freeze-dried product
FP	Frozen product
GalA	Galactose permease
gal-PTS	Galactose specific PTS
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-phosphate dehydrogenase
GS	60% glycerol-saline quenching solution
HPr	Phosphocarrier protein
LAB	Lactic acid bacteria
Lac	Lactose-grown anaerobic pre-cultures without pH control
Lac+Gal+pH	Lactose-grown and added galactose anaerobic pre-cultures with pH control, pH 6.0
Lac+pH	Lactose-grown anaerobic pre-cultures with pH control, pH 6.0
lac-PTS	Lactose specific PTS
LacS	Galactose-lactose antiporter
LacY	Lactose-proton symporter
Late	Late exponential growth phase, galactose is excreted while lactose is consumed
LDH	Lactate dehydrogenase
M	80% methanol quenching solution
MG	80% methanol/20% glycerol quenching solution

MQ	Menaquinones
MQH_2	Menaquinol, reduced MQ
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NOX	NADH oxidase
NT	No treatment
OD	Optical density
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PFL	Pyruvate formate lyase
P_i	Inorganic phosphate
PI	Propidium iodide
PK	Pyruvate kinase
PTA	Phosphotransacetylase
PTS	Phosphotransferase system
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Stat	Stationary growth phase, lactose is depleted and galactose remains
TCA	Tricarboxylic cycle

μ_{max}	Maximum specific growth rate with the unit [h^{-1}]
$r_{pH,max}$	Maximum acidification rate with the unit [$\Delta pH \cdot h^{-1}$]
t_{spe}	Specific acidification time with the unit [$min \cdot log(cells \cdot g^{-1})^{-1}$]

Contents

1. Introduction

Food processing is greatly facilitated by microorganisms that influence the taste, smell and functionality of food, in addition to helping food preservation. These organisms can be applied in the form of starter cultures, the production of which has become a global industry. In contrast to many other biotech processes in which metabolites are manufactured, starter cultures are viable cells used in the later production of food and beverages. This places special emphasis on the metabolic state of the cell, in order to ensure its survival and metabolic activity from the propagation step (upstream), through separation and freezing or freeze-drying (downstream).

In other words, cell robustness is essential, not only during the manufacture of starter cultures, but also in the specific application of the culture in the final production process. This robustness, e.g. the viability and milk acidification activity, of the cells after storage in frozen or freeze-dried state, determines the quality of the starter culture. A better understanding of the cellular mechanisms influencing cell robustness will bring added value to the customer by ensuring a product with a consistently high quality, and thus a higher profit margin. Moreover, increasing the yield of viable cells will have a direct impact on the cost of production, as well as the fermentation capacity in starter culture manufacturing.

Lactic acid bacteria (LAB) have a long history of use as starter cultures. The most important group of LAB is the group of dairy LAB used in the production of a variety of fermented milk products. LAB production has traditionally been performed in anaerobic batch fermentation. However, aerobic cultivation, in which respiratory metabolism is induced, has successfully been implemented for *L. lactis* starter cultures. This has resulted in increased biomass yield, although the factors affecting the robustness of cells grown under respiro-fermentative conditions are still not understood.

As the demand for starter cultures is constantly growing, it is highly desirable to develop a production process in which biomass yield and cell robustness are concomitantly

improved. A great deal of work has therefore been invested in the optimization of starter culture production. One of the most significant advances was the implementation of respiratory growth of *L. lactis* in large-scale production. Following a long period of research and strategic decisions, a €40 million factory dedicated to aerobic fermentation was built by Chr. Hansen A/S in 2008 (Pedersen et al., 2005, 2012). However, deeper insight is needed into the metabolism of *L. lactis* to understand the reasons for the differences in robustness of starter cultures produced by different processes. While most of the studies published so far have focused on unravelling the metabolic behaviour of *L. lactis* during anaerobic fermentation, less is known about the aerobic fermentative/respiratory and fully respiratory metabolism of *L. lactis*. Further optimization of the respiratory growth of starter cultures is therefore very important to fully exploit this considerable investment.

The general aim of this research was to describe and investigate how process design during the batch production process affects the metabolic state and robustness of starter cultures, with the goal of ensuring high yield, survival and metabolic activity, from the cultivation step, through separation, to freezing or freeze-drying.

The main focus was on the respiration-permissive production of *Lactococcus lactis*.

Cultivation conditions applied and discussed in this thesis work		
Respiration-permissive	–	Conditions with oxygen by aeration or shaken and haemin
Aerobic	–	Conditions with oxygen by aeration or shaken
Anaerobic	–	Conditions with no supply of oxygen

This lactic acid bacterium is of great industrial importance, being a major component in fermented dairy products (Pedersen et al., 2012). Respiration-permissive conditions are known to increase the biomass yield, the resistance to oxygen, and long-term cellular survival of *L. lactis* (Duwat et al., 2001; Gaudu et al., 2002; Rezaïki et al., 2004). However, studies using industrial-like conditions are scarce, and the cellular performance of final starter culture products, in the form of frozen and freeze-dried cells, has not been investigated. To address the general aim of this work, a number of specific aims were defined.

1. To identify the metabolic and physiological hallmarks of *L. lactis* CHCC2862 during anaerobic, aerobic and respiration-permissive batch cultivations. Parallel batch experiments were performed while monitoring substrate consumption, base addition, and the formation of products and biomass to characterize *L. lactis* CHCC2862, in order to obtain a solid basis for the description of its behaviour. Clear differences were observed in the behaviour of the cells under the three conditions, and further studies were based on these findings. (**Paper I, Chapter 4**)

2. To investigate the effect of specific growth rate on the respiratory growth of *L. lactis*. Respiration-permissive and anaerobic chemostat cultivations were performed to enable quantitative analysis of metabolism, and comparisons between homofermentative, mixed-acid, flavour-forming and respiratory growth. Under respiration-permissive conditions the metabolism could be modulated by controlling the specific growth rate. Low specific growth rate could eliminate lactate formation, however, no increase in biomass yield was obtained, compared to those observed at higher specific growth rates. (**Paper II, Chapter 4**)

3. To investigate the occurrence of lag phases in main cultivations. Several factors were investigated to identify the cause of the observed lag phases and their varying lengths. Two factors were found to affect the lag phase: the point of harvest of the pre-culture and the presence of haemin in the pre-culture (**Chapter 5**). Depending on the harvest point of lactose-based pre-cultures, galactose excretion was observed and the subsequent main cultures showed large variations in the length of the lag phase. These findings led to the fourth aim.

4. To elucidate the impact of the presence of galactose in pre-cultures on the lag phase in main cultivations of *L. lactis*. The metabolism of galactose, excreted and added, was characterized during pre-culture cultivation. Specific sugar uptake rates, β-galactosidase enzymatic activities, metabolic pathways and *lacS* expression were studied to shed light on the role of LacS in galactose excretion in *L. lactis* CHCC2862. (**Paper III, Chapter 5**)

5. To define the extent to which haemin supplementation supports respiratory growth and high-yield biomass production of *L. lactis.* Comparative batch experiments with different haemin concentrations were used to quantitatively analyse the metabolic fluxes. The essential role of haemin in respiratory growth was clear, however, beneficial effects were only observed in a certain haemin range under aerobic conditions. Suboptimal haemin concentrations and the addition of haemin to anaerobic cultures led to decreased maximum specific growth rate and oxygen uptake. (**Paper I, Chapter 6**)

6. To identify the impact of cultivation conditions on the final robustness of *L. lactis* as frozen and freeze-dried products. *L. lactis* was harvested from traditional dynamic batch cultivations and steady-state chemostat cultivations to investigate the effects of the physiological state. Respiration-permissive batch cultivation led to cells being more sensitive to freeze-drying and with a lower acidification activity than those cultivated under anaerobic conditions. However, the robustness of freeze-dried cells increased when using respiration-permissive chemostat cultures at high specific growth rate; performing equally well, or better than, anaerobically grown cells. (**Papers II and IV, Chapter 6**)

The long-term goal of this research was to enable the redesign and optimization of the *L. lactis* starter culture batch production process, thereby contributing to a reproducible production process providing both high final concentrations of biomass and excellent cell robustness. **Chapter 2** presents the background of starter cultures, and the reason why

lactic acid bacteria still remain of considerable industrial interest. **Chapter 3** describes the preparatory studies required to solve critical issues before starting the main experimental work, and the attempt to establish a quenching procedure for *L. lactis*, to enable metabolomic analysis. In **Chapter 4**, the respiratory physiology of *L. lactis* is presented, where the key differences between the anaerobic, aerobic and respiration-permissive conditions are defined. The impact of specific growth rate on respiratory growth is described, as well as the impact of galactose on the aerobic and respiratory physiology of *L. lactis*. **Chapter 5** discusses the lag phase in main cultures in relation to the inoculation procedure. Lag phases were only observed when pre-cultures were transfer to respiration-permissive conditions, and not to anaerobic conditions, which were reproducible and showed immediate growth. In **Chapter 6**, the implications of haemin addition to the medium are described and discussed. The performance of frozen and freeze-dried starter cultures is also described and potential improvements are presented, together with their associated challenges. Finally, the conclusions are presented in **Chapter 7**, and suggestions for future work are made in **Chapter 8**.

2. Starter Cultures – What They Are and Why We Use Them

Starter cultures play an essential role in the manufacture of fermented food products. Simply defined, starter cultures consist of microorganisms that are inoculated directly into food materials to drive the fermentation process. Starter cultures bring about desired and predictable changes in the product, which may include enhanced preservation, improved nutritional value or modified sensory qualities.

Traditionally fermented foods are produced without the use of commercial starter cultures, and this is still practiced at small-size facilities, as well as in the home. These procedures involve back-slopping, i.e., using a small amount of the final product, which contains the necessary microorganisms, to inoculate a new batch to start a new fermentation. However, such traditional procedures often lead to variation in the fermented products, and they are prone to slow or failed fermentation, contamination and inconsistent quality. Today, reliable product quality and predictable production schedules are required, as well as stringent quality control to ensure food safety. Commercial starter cultures ensure the manufacture of products at a predictable rate, with consistent product quality.

2.1 Industrial starter culture production

Commercial starter culture production had its beginning in the late 1880s. Initially, starter cultures were liquid cultures, prepared by cultivation of bacteria in sterilized milk. These cultures suffered from over-acidification, and thus lost their viability during storage. The loss of viability could be delayed by adding calcium carbonate as a buffer, but not completely avoided. Improvements in freezing and freeze-drying technologies, providing more stable preparations, allowed the problems of liquid cultures to be overcome (Speranza et al., 2017).

Today, starter culture development and production follow strict procedures with quality control to ensure reproducible robust processes with high biomass yield and high cellular performance. The industrial production of starter cultures involves several steps: the development and construction of a stock culture; preparation of a culture medium; propagation of the stock culture to final cell density in a bioreactor; harvesting and concentration of cells from the medium; and preservation of the culture, often by freezing or freeze-drying (as illustrated in Figure 2.1). Lactic acid bacteria are commonly freeze-dried as this ensures not only long-term preservation, but also lower storage and transportation costs compared to frozen cell suspensions. Bacterial tolerance to freeze-drying varies between strains and can have a considerable impact on the metabolic activity, e.g. the acidification rate, in the final application. Moreover, the robustness of the starter culture, in terms of cell survival and metabolic capacity after storage, is also affected by the conditions under which it is produced, e.g. the cultivation conditions and exposure to cold and heat shock (Broadbent & Lin, 1999; Velly et al., 2014).

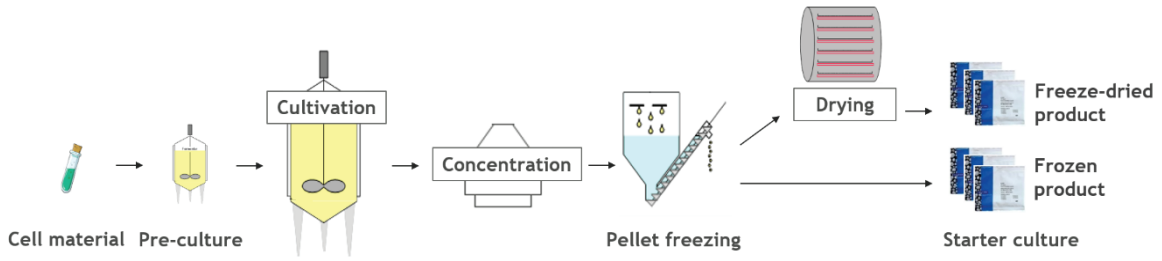


FIGURE 2.1 The industrial production of starter cultures starts with the cell material which is pre-cultured. This is transferred to the main cultivation in a bioreactor. After harvesting, the cells are concentrated by centrifugation, preserved by freezing or freeze-drying, and packed as final starter culture products.

In the commercial production of starter cultures efforts are made to maintain the viability of the cells during storage to ensure their function when later used. Therefore, starter cultures should be prepared under conditions that promote cell survival. Exposure to stress during the various production steps can result in altered growth and survival of the starter microbes (Speranza et al., 2017). In order to ensure sufficient functionality of cultures, it is thus essential to recognize and address stress-inducing factors in each step. Typical inducers of microbial stress during the cultivation phase are starvation, pH change and inhibitory metabolites (Tsakalidou & Papadimitriou, 2011). Also, oxidative, osmotic, mechanical and thermal stresses are often present, especially during the later phases of manufacturing, namely harvesting and preservation, and during storage (Speranza et al., 2017). The main microbial stress inducers during starter culture production are summarized in Table 2.1.

TABLE 2.1 Major microbial stress inducers in the production of starter cultures

Stress	Inducer	Process phase
Starvation	Lack of nutrients	Cultivation
pH	pH change	Cultivation
		Harvesting
Oxidative	Reactive oxygen species	Cultivation
		Product handling
Mechanical	Shear forces	Harvesting
Osmotic	Ionic strength	Cultivation
		Harvesting
		Freezing
		Freeze-drying
Thermal	Low temperatures	Freezing
	High temperatures	Freeze-drying
		Storage

2.2 Lactic acid bacteria

Lactic acid bacteria have a long history in the making of yogurt, cheese, cultured butter, sour cream, sausages, cucumber pickles, olives and sauerkraut. LAB strains produce lactic acid, which modifies the water binding capacity, thus contributing to the texture, moisture content, flavour and aroma of the products. LAB are generally considered beneficial microorganisms, some strains even as health-promoting, i.e., probiotic bacteria (Lahtinen et al., 2012).

LAB lack genuine catalase and are often devoid of cytochromes, and therefore obtain their energy by substrate-level phosphorylation, as they do not possess a functional respiratory system. They have limited biosynthetic ability, having evolved in environments that are rich in amino acids, vitamins, purines and pyrimidines, so they must be cultivated in complex media that fulfil all their nutritional requirements. The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation., Two broad metabolic categories of LAB can be defined based on their sugar fermentation patterns: homofermentative and heterofermentative (Cocaign-Bousquet et al., 1996). Homofermentative LAB catabolize hexoses by glycolysis, called the Embden-Meyerhof pathway, where one mole of glucose yields two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to mainly lactic acid. Overall, this process produces 2 moles of ATP per mole of glucose consumed (Figure 2.2A). Heterofermentative LAB utilize the phosphoketolase pathway, also known as the pentose phosphate pathway. One mole of glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole of glyceraldehyde phosphate (GAP) and one mole of acetyl phosphate. GAP is further metabolized to lactate, as in the homofermentative pathway, and the acetyl phosphate is reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Theoretically, the end

products, CO_2, lactate and ethanol, are produced in equimolar quantities by the catabolism of one mole of glucose, and one mole of ATP is produced (Figure 2.2B) (Cocaign-Bousquet et al., 1996; von Wright & Axelsson, 2012).

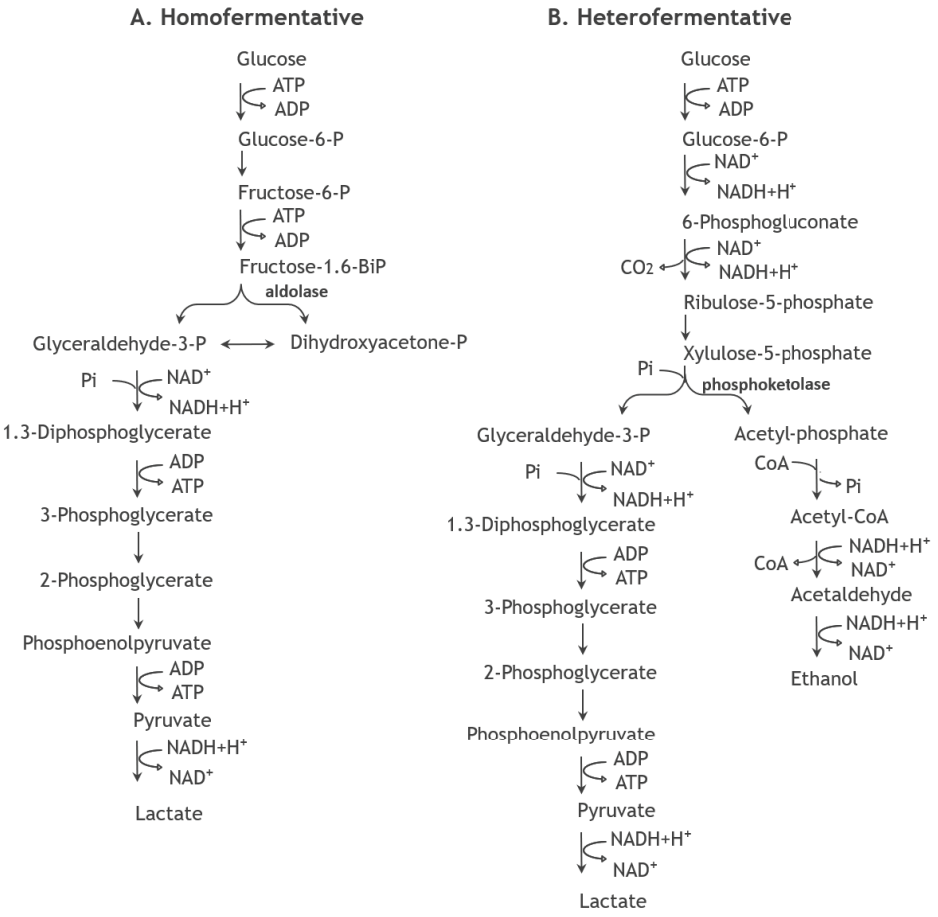


FIGURE 2.2 The two major fermentation patterns of sugar degradation by LAB, with glucose as an example. A) Homofermentative and B) Heterofermentative (Cocaign-Bousquet et al., 1996; von Wright & Axelsson, 2012).

LAB comprise a diverse group of Gram-positive bacteria united by certain morphological, metabolic and physiological characteristics. They are nonsporulating, non-respiring, but aerotolerant, cocci or rods, which produce lactic acid as one of their main fermentation products. According to the taxonomic classification, LAB belong to the phylum *Firmicutes*, class *Bacillus* in the order *Lactobacillales*, representing six families (*Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*), and about 40 genera.

LAB are commonly used as starter cultures in several areas of fermented food application. Their taxonomic status is given in Table 2.2 (Franz & Holzapfel, 2011; von Wright & Axelsson, 2012). The experimental work described in this thesis involved only the species *Lactococcus lactis* subsp. *lactis* CHCC2862, a preferred dairy starter culture for the production of hard and semi-hard cheeses. *L. lactis* has been the subject of numerous studies that have resulted in detailed knowledge of its molecular biology, metabolism and physiology, and it is considered the model organism of LAB. *L. lactis* is homofermentative and the dairy strains are typically quite fastidious and lack the ability to metabolize

pentoses, although *L. lactis* strains isolated from plant niches have also been shown to possess the ability to metabolize pentoses quite efficiently (Siezen et al., 2008).

TABLE 2.2 LAB genera used as starter cultures in various fermented food applications and their products (Franz & Holzapfel, 2011; von Wright & Axelsson, 2012; Wood & Warner, 2003)

Family	Genera	Fermentation pattern	Application	Product types
Streptococcaceae	*Lactococcus*	Homofermentative	Dairy	Cheese, butter,
	Streptococcus	Homofermentative	Dairy	Cheese, yoghurt
Enterococcaceae	*Enterococcus*	Homofermentative	Dairy	Cheese
Lactobacillaceae	*Pediococcus*	Homofermentative/ Heterofermentative	Dairy, Meat, Plants	Cheese, sausages, sauerkraut, soy sauce, pickles
	Lactobacillus,		Dairy, Meat,	Cheese, yoghurt,
	Group I	Homofermentative	Plants,	kefir, sausages,
	II	Homofermentative	Cereals	olives, sauerkraut,
	III	Heterofermentative		pickles, sourdough
Leuconostocaceae	*Leuconostoc*	Heterofermentative	Dairy, Plants	Cheese, butter, olives, pickles
	Oenococcus	Heterofermentative	Alcoholic beverages	Wine
	Weissella	Heterofermentative	Cereals	Sourdough

2.3 Sugar metabolism, transport and control in *L. lactis*

The extensive use of LAB in the production of fermented foods depends to a great extent on the unique features of the sugar metabolism in LAB. The initial step in the metabolism of carbohydrates is their transport across the cytoplasmic membrane. LAB have evolved different assimilation systems that differ in their phosphorylation states, intermediate metabolites and bioenergetics. Sugar assimilation takes place either by means of a specific permease or a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Figure 2.3). Through permease transport, sugar uptake can be coupled to proton/solute (symport) or solute/solute (antiport) translocation, followed by kinase-mediated phosphorylation of the free sugar within the cytosol. The PTS couples the transport and phosphorylation of the sugar and is often more bioenergetically efficient since it occurs in a single step at the expense of one phosphoenolpyruvate (PEP) molecule. Two general proteins are involved in the PTS, enzyme I (EI) and the phosphocarrier protein (HPr), and further the sugar-specific enzymes II (EII) (Cocaign-Bousquet et al., 2002; Neves et al., 2005).

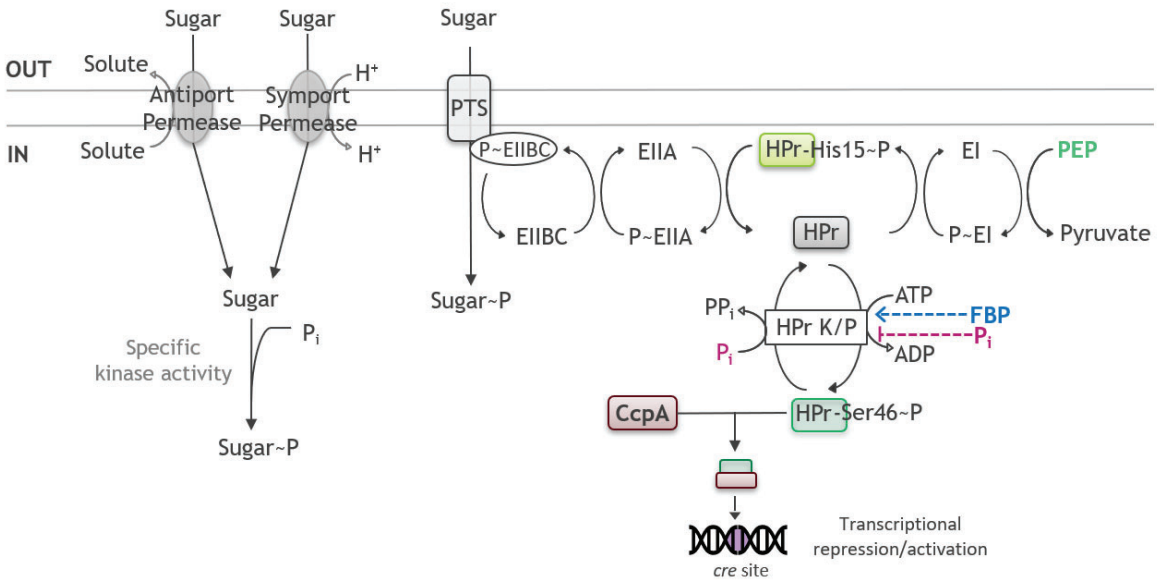


FIGURE 2.3 LAB have two alternative sugar uptake systems, passive transport via permease and active transport via the PTS. Through permease transport, the free sugar follows kinase-mediated phosphorylation within the cytosol. PTS transport involves coupled transport and phosphorylation, where the two general PTS components EI and HPr phosphorylate the sugar-specific EII complex. A phosphorylation cascade from PEP to the EII complex phosphorylates the sugar, which is subsequently released into the cytoplasm. PTS transport is linked to the carbon catabolic repression system by the phosphorylation status of HPr. HPr-Ser46-P together with carbon catabolite protein A (CcpA) binds to the specific DNA sequence, catabolite-responsive element (*cre*), which further acts by transcriptional repression/activation. The glycolytic intermediate, fructose-1.6-bisphosphate (FBP), affects the ATP-requiring phosphorylation of HPr at Ser-46, by increasing the kinase activity of the HPr kinase/phosphatase (HPr K/P) (Deutscher et al., 2014; Postma et al., 1993).

Due to the presence of lactose in milk, the metabolism of this disaccharide is of great interest in the dairy industry. In most lactococci, the lactose-specific components of the PTS, together with the following pathway related enzymes (the tagatose 6-phosphate pathway), are located on plasmids (Crow et al., 1983; de Vos, 1987). The PTS plasmid-linked *L. lactis* strains are often able to ferment lactose rapidly. Lactose translocation by lac-PTS results in lactose 6-phosphate, which is degraded to glucose and galactose-6-phosphate (Gal-6P) by phospho-β-galactosidase. Glucose is processed in the glycolytic pathway, while Gal-6P enters glycolysis at the level of triose phosphate after transformation in the tagatose pathway (Figure 2.4) (Cocaign-Bousquet et al., 2002; Crow et al., 1983; Neves et al., 2005). Alternatively, lactose uptake can take place through permease transport, where, after internalization, lactose is hydrolysed to glucose and galactose by cytosolic β-galactosidase. Glucose enters glycolysis, and galactose is metabolized via the Leloir pathway and then enters glycolysis at the level of glucose-6-phosphate (Glc-6P) (Figure 2.4) (Grossiord et al., 1998, 2003). A lactose-H^+ symport permease (LacY) has been described for *L. lactis* (Neves et al., 2005; Osumi & Saier, 1982), and in studies of *L. lactis* IL 1403, LacS has been described as a putative lactose-H^+ symport permease or galactose-lactose antiporter (Bolotin et al., 2001), with high affinity for galactose (Aleksandrzak-Piekarczyk et al., 2005; Thompson, 1980).

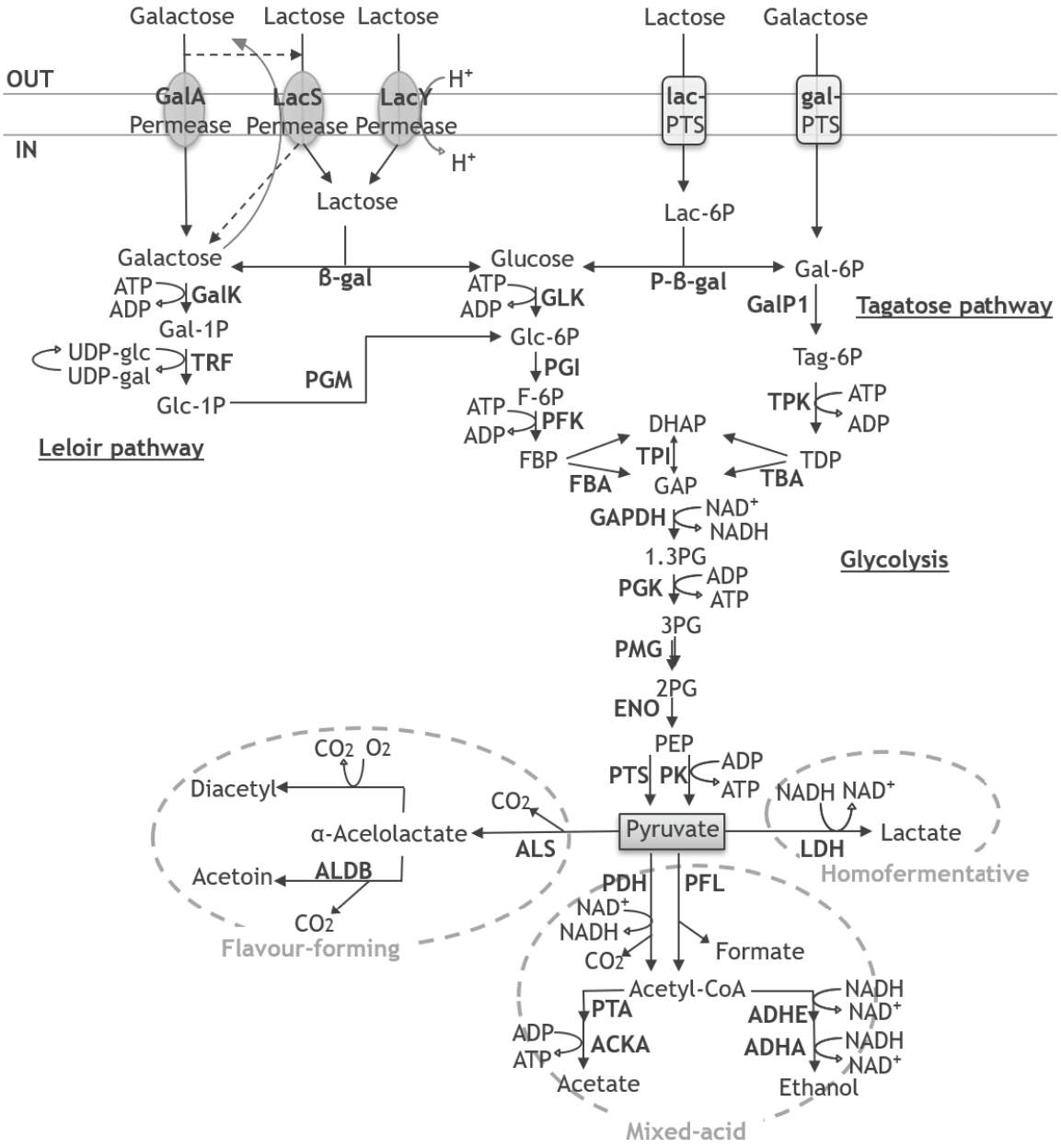


FIGURE 2.4 Lactose and galactose metabolism in *L. lactis*. Assimilation of lactose and galactose by either the PTS or permease follows the tagatose pathway or Leloir pathway, respectively. Sugar uptake: GalA galactose permease, LacS galactose-lactose antiporter, LacY lactose-proton symporter, lac-PTS lactose phosphotransferase system, gal-PTS galactose phosphotransferase system. Metabolites: Gal-1P galactose-1-phosphate, Glc-1P glucose-1-phosphate, Glc-6P glucose-6-phosphate, F-6P fructose-6-phosphate, FBP fructose-1.6-bisphosphate, DHAP dihydroxyacetone-phosphate, GAP glyceraldehyde-3-phosphate, 1.3PG 1.3-diphosphoglycerate, 3PG 3-phosphoglycerate, 2PG 2-phosphoglycerate, PEP phosphoenolpyruvate, Lac-6P lactose-6-phosphate, Gal-6P galactose-6-phosphate, Tag-6-P tagatose-6-phosphate, TDP tagatose-1.6-diphosphate. Enzymes: β-gal β-galactosidase, P-β-gal phospho-β-galactosidase, GalK galactokinase, TRF galactose/uridylyl transferase, PGM phosphoglucomutase, GLK glucokinase, PGI glucose-phosphate isomerase, PFK phosphofructokinase, FBA fructose-bisphosphate aldolase, TPI triose-phosphate isomerase, GAPDH glyceraldehyde-phosphate dehydrogenase, PGK phosphoglycerate kinase, PMG phosphoglycerate mutase, ENO enolase, PK pyruvate kinase, GalP1 galactose-phosphate isomerase, TPK tagatose-phosphate kinase, TBA tagatose-bisphosphate aldolase, LDH lactate dehydrogenase, PFL pyruvate formate lyase, PDH pyruvate dehydrogenase, PTA phosphotransacetylase, ACKA acetate kinase, ADHE acetaldehyde dehydrogenase, ADHA alcohol dehydrogenase, ALS acetolactate synthase, ALDB acetolactate decarboxylase. Adapted from (Cocaign-Bousquet et al., 2002; Johanson et al., 2020; Lorántfy et al., 2019).

In analogy with lactose metabolism, galactose uptake involves phosphorylation to Gal-6P and metabolism via the tagatose pathway when it occurs via the galactose-PTS (gal-PTS) (Neves et al., 2010). Galactose is assimilated and metabolized by the Leloir pathway through galactose permease (GalA) (Figure 2.4) (Grossiord et al., 1998; Thomas et al., 1980; Thompson, 1980). Based on the findings of the present work, a double role of the lactose permease LacS is proposed, where, in the presence of lactose, it functions as a galactose-lactose antiporter to facilitate fast lactose uptake by concomitant excretion of intracellular galactose, but also as a galactose permease when lactose is depleted or not present (**Paper III**).

2.4 Fermentation physiology of *Lactococcus lactis*

L. lactis strains have often been considered to be **homofermentative**, converting 90% of consumed sugars to lactate. However, conditions such as carbon limitation and low specific growth rate can change the metabolic flux towards so-called **mixed-acid** metabolism with formate, acetate and ethanol as additional products (Figure 2.4). Sugar intermediates enter glycolysis, resulting in pyruvate, a central branch point of the metabolic network. The oxidation-reduction balance is maintained in the cell when pyruvate is reduced to lactate via lactate dehydrogenase (LDH). Pyruvate can also be metabolized via pyruvate formate lyase (PFL) or pyruvate dehydrogenase (PDH) leading to formate, CO_2, acetate and ethanol. The significant difference between PDH and PFL is that PDH produces CO_2, rather than formate, while the PFL-catalysed reaction leads to the generation of additional NADH. Furthermore, PDH activity is induced by aeration, whereas PFL is inactivated. Irrespective of whether PDH or PFL is activated, the intermediate acetyl-CoA is further metabolized to either acetate or ethanol, via phosphotransacetylase (PTA) and acetate kinase (ACKA), or acetaldehyde (ADHE) and alcohol dehydrogenases (ADHA), respectively. Acetate formation generates additional ATP, while ethanol formation regenerates NAD^+ (Figure 2.4) (Cocaign-Bousquet et al., 2002; Duwat et al., 2001; Garrigues et al., 2001; Neves et al., 2005).

Nowadays, it is well recognized that *L. lactis* can undergo aerobic respiration in the presence of haem, i.e., **respiration-permissive conditions** (Duwat et al., 2001; Gaudu et al., 2002; Lechardeur et al., 2011; Pedersen et al., 2008). The first observations were reported in the early 1970s (Bryan-Jones & Whittenbury, 1969; Sijpesteijn, 1970), although they were not followed up until the late 1990s. Now, after several decades of studying the respiratory behaviour of *L. lactis*, it is generally accepted. Respiration-permissive conditions direct metabolism towards **flavour-forming metabolism**. The flux from pyruvate to lactate decreases significantly, and acetoin, diacetyl and CO_2 are formed (Figure 2.4). In the formation of acetoin and diacetyl, pyruvate undergoes decarboxylation by acetolactate synthase (ALS) into acetolactate. Acetolactate is unstable and can either undergo spontaneous conversion to diacetyl by chemical reaction with O_2, or it can be enzymatically converted to acetoin by acetolactate decarboxylase (ALDB) (Arioli et al., 2013). Under respiration-permissive conditions, the intracellular oxygen level is reduced, leading to metabolic changes and a reduction in acid production, and thus less stress on the

cells, as has been confirmed in both the literature and industrial practice (Gaudu et al., 2002; Pedersen et al., 2005, 2012). Together, respiration-permissive conditions lead to an increase in biomass yield, resistance to oxygen, and long-term cellular survival in liquid-form cultures stored at 4°C (Duwat et al., 2001; Gaudu et al., 2002; Rezaïki et al., 2004). Respiratory growth of *L. lactis* has only been studied in batch cultivations, and neither the potential relationship between the specific growth rate and the beneficial effects of respiration, nor the potential effects on the development of starter culture robustness, in terms of tolerance to downstream processing, has been considered.

Toxicity caused by the activity of reactive oxygen species that can attack proteins, lipids and nucleic acids, is often attributed to the presence of oxygen. It has been shown that oxygen toxicity leads to growth inhibition of *L. lactis*, leading to cell death and DNA degradation (Condon, 1987; Sanders et al., 1999). Defence systems involving superoxide dismutase (SOD) and catalase are activated in response to oxidative stress (Fridovich, 1998). These systems catalyse the dismutation of superoxide ($O_2^{\bullet}$) into oxygen and hydrogen peroxide, and the decomposition of hydrogen peroxide into water and oxygen. *L. lactis* possesses a single SOD and no catalase, and it is therefore not fully equipped to withstand the toxic effect of an oxidative environment. However, the addition of exogenous haem has been reported to have a good effect, as the cells were less susceptible to oxidative stress (Duwat et al., 2001). *L. lactis* can establish an electron transport chain (ETC) in the presence of haem. The beneficial respiratory growth of *L. lactis* is possible through the action of the ETC, which comprises three essential membrane-embedded components: an electron donor, an electron transporter and a terminal electron acceptor, as illustrated in Figure 2.5 (Brooijmans et al., 2009).

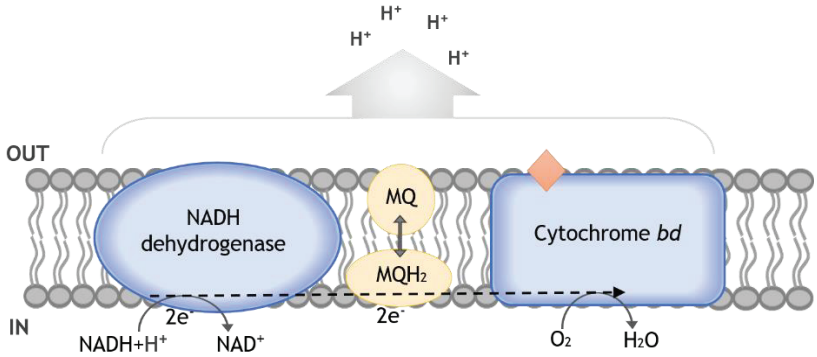


FIGURE 2.5 The electron transport chain (ETC) present in *L. lactis* is comprised of NADH dehydrogenase as electron donor, menaquinone (MQ) as electron transporter and cytochrome *bd* as the terminal electron acceptor. The ETC facilitates the oxidation of NADH to NAD^+, where two electrons (e^-) are translocated. The final electron receiver, the haem (orange diamond) requiring cytochrome *bd* oxidase, utilizes these electrons for the reduction of oxygen to water, together translocating protons to the extracellular space.

NADH dehydrogenase is an electron donor encoded by the *noxA* and *noxB* genes, and receives two electrons from NADH, which reduces the menaquinone (MQ) to menaquinol (MQH_2). The electrons are transported along the membrane to the haem-requiring terminal acceptor, cytochrome quinol oxidase, cytochrome *bd*. The cytochrome *bd*, encoded by *cydABCD*, reduces oxygen to water as it oxidizes the MQH_2 and thereby fulfils the proton extrusion function of the ETC. Since *L. lactis* does not synthesize haem, it must scavenge

haem or haem analogues from the environment (Lan et al., 2006; Lechardeur et al., 2011; Pedersen et al., 2012). With a functioning ETC, respiration-driven efflux of protons occurs across the membrane. A proton gradient across the membrane is often associated with oxidative phosphorylation, where ATP synthesis occurs by an ATP-synthase. The presence of such ATP-synthase has been confirmed in *L. lactis*, however, it is still not clear whether it is used for ATP synthesis (Blank et al., 2001; Koebmann et al., 2008). Initially, the ATP-synthase enzyme complex in *L. lactis* was reported to extrude protons to maintain intracellular pH at the expense of ATP (Blank et al., 2001; Koebmann et al., 2008). During anaerobic growth, high lactate levels occur and H^+-ATPase becomes essential for pH homeostasis (Koebmann et al., 2000). However, in the presence of haem, the ETC functions as a proton transport system causing respiration-driven efflux of protons, in addition to, or instead of, H^+-ATPase (Koebmann et al., 2002). *L. lactis* thus saves ATP through haem-activated respiration by diminishing the need to extrude protons, at no additional ATP cost. Some studies suggest that additional ATP generation may occur, however, this has not been fully verified (Blank et al., 2001; Koebmann et al., 2008; Pedersen et al., 2012).

2.5 Catabolic bottlenecks and regulation in *L. lactis*

Sugar uptake and metabolism in *L. lactis* is highly controlled by several mechanisms, where especially FBP has been suggested to play an important role (Thompson, 1987). High levels of FBP direct the flux towards the production of lactate by activation of PK and LDH during homofermentative fermentation (Figure 2.6). In starved cells, associated with sugar depletion, FBP levels are low, and intracellular inorganic phosphate (P_i) levels are high, which inhibit PK, and the cells accumulate 3PG and PEP (Neves et al., 2005; Thompson, 1987). It has been suggested that the shift to mixed-acid metabolism is caused by a reduction in the FBP pool, leading to LDH inactivation and relieving PFL (Thomas et al., 1979). However, this has been questioned as the intracellular FBP concentration has been shown to always be sufficiently high to ensure full activation of LDH. Instead, it has been proposed that inhibition or activation exerted by the $NADH/NAD^+$ ratio (redox state) on GAPDH or LDH activities could be the main factor regulating the metabolic shift in glycolysis (Garrigues et al., 1997, 2001). High $NADH/NAD^+$ ratios restrain GAPDH activity, but not LDH, leading to the accumulation of FBP and the triose phosphates (DHAP and GAP), which inhibit the activity of PFL, resulting in lactate production, i.e., homofermentative metabolism (Figure 2.6). Mixed-acid metabolism is then a consequence of lowered $NADH/NAD^+$ ratios, relieving GAPDH inhibition and limiting LDH activity (Garrigues et al., 1997).

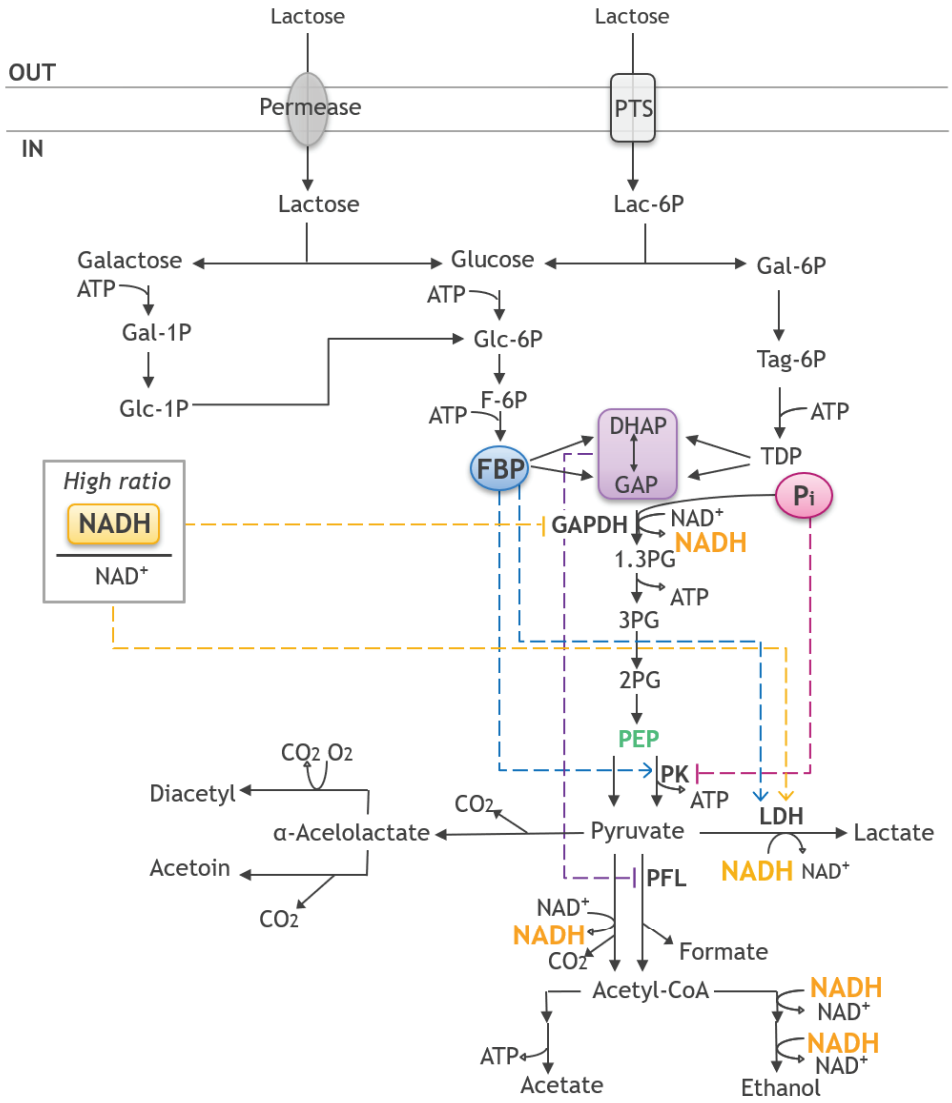


FIGURE 2.6 Schematic overview of the main metabolic regulation in lactose metabolism. Full lines show enzymatic reactions, coloured metabolites are effectors of enzyme activity and the dashed lines with either arrow or blockage indicate regulation by activation or inhibition, respectively. High levels of FBP activates PK and LDH directing the flux towards lactate. High levels of P_i drive the conversion of GAP to 1.3PG thereby reducing FBP and accumulating 3PG and PEP, but also inhibit PK thus relieving mixed-acid metabolism. High ratio of NADH/NAD^+ inhibits GAPDH but stimulates LDH leading to accumulation of FBP, DHAP and GAP, where the two latter inhibit PFL resulting in lactate formation.

The presence of oxygen influences the carbon metabolism of *L. lactis*, as it introduces greater NADH oxidase (NOX) activity and alters the redox state (Lopez De Felipe et al., 1997; Neves et al., 2002; Smart & Thomas, 1987). The catabolic carbon fluxes and the redox metabolism become uncoupled due to the extra pathway for NAD^+-regeneration involving NADH oxidases. Sugar catabolism is thus shifted towards mixed-acid/flavour-forming metabolism, with the formation of acetate and acetoin instead of lactate production (Lopez De Felipe et al., 1997, 1998). In the presence of oxygen, NOX provides an additional path for NADH oxidation, and the level of FBP is lower than under anaerobic conditions, whereas 3PG and PEP levels are higher. This is suggested to be a consequence of increased flux through GAPDH as the NADH concentration is lower. NADH consumption by NOX reduces the need to regenerate NAD^+ through LDH, causing

changes in the metabolite profile. Under respiration-permissive conditions, the NADH dehydrogenase introduces yet another point of NADH oxidation, which further amplifies the features of aerated conditions: low FBP level, high 3PG and PEP levels, and decreased flux towards lactate (Neves et al., 2005).

Interestingly, FBP and P_i provide a link between glycolytic activity and carbon catabolite repression, as FBP and P_i control the level of PEP via their function as activator and repressor of PK, respectively (Thompson, 1987). Together with the global transcriptional regulator, the carbon catabolite protein A (CcpA), FBP and P_i are major signalling molecules for the carbon catabolite repression (CCR) system, a central mechanism of carbohydrate metabolism in bacteria (Poncet et al., 2004). The CCR system is based on the *trans*-acting CcpA, which acts by binding to a *cis*-acting catabolite responsive element (*cre*), a specific DNA sequence present near the promoter of genes affected by CCR. A central role of the CCR system is to allow the consumption of rapidly metabolized sugars in the medium and repress the utilization of other sugars. The CCR system is linked to the sugar uptake by the PEP-dependent PTS, as it is controlled by the phosphorylation status of the HPr protein of the PTS (Figure 2.3) (Deutscher et al., 2014; Postma et al., 1993). If phosphorylation of HPr occurs at Ser46, instead of the standard site, His15, the result is the formation of a complex with the CcpA protein and the binding of this complex to the *cre* element. The phosphorylation status of HPr is regulated by FBP, ATP and P_i. High catabolic activity will increase the pool of FBP and ATP, which stimulates the kinase activity of HPr-kinase/phosphatase (HPr K/P), the enzyme that catalyses the phosphorylation of Ser46. At low catabolic activity the pools of P_i will increase, which inhibits the kinase activity of HPr K/P, freeing the HPr to function in the PTS transport (Poncet et al., 2004).

In *L. lactis* the CcpA is involved in the transcriptional activation of the *las* operon (comprising PFK, PK and LDH) and repression of the *gal* operon (encoding the proteins for galactose uptake and the Leloir pathway) (Luesink et al., 1998; Neves et al., 2005). Additionally, during respiratory growth, CcpA represses the haem uptake and the expression of cytosolic NOX (*noxE*) regulating the redox balance (Gaudu et al., 2003).

3. Preparatory Studies

Reliable experimental settings and analysis procedures were prerequisites for any attempt to investigate the physiological behaviour of lactic acid bacteria towards production of robust cells. To reach the goals, new methods had to be developed to challenge and broaden our understanding of the growth of *L. lactis* and its robustness as a starter culture. Ambitions were endless in the beginning, involving new approaches of the standard operation procedures, e.g. cultivation system and analysis assays, disturbing the usual everyday routines. Furthermore, the commercial production of *L. lactis* is subject to aspects of confidentiality that had to be addressed in presenting this work.

3.1 Medium selection

The choice of a suitable cultivation medium was the first question tackled. Using a complex cultivation medium with a composition similar to industrial media would result in a product similar to the industrial starter culture, but it would also limit the scope of the analytical methods. In contrast, the use of a chemically defined cultivation medium would make it easier to use existing analysis methods, and provide more comprehensive data (Novak et al., 1997), but the quality, performance and robustness of the biomass would most likely not be comparable to that produced in industrial processes.

As the goal was to improve our understanding of the interactions between cell growth, metabolism and cell performance, it was deemed important to retain the quality, performance and robustness of the biomass in the process. Efforts were therefore made to identify a medium that could sustain respiration-permissive as well as anaerobic growth, while retaining the industrial physiological features. Furthermore, the medium should be easily manageable and preferably related to literature studies. *Lactococcus lactis* is nutritionally fastidious and requires complex media for optimal growth. Standard M17 is a complex medium including yeast extract, peptone and beef extract, optimized in the 1970s (Terzaghi & Sandine, 1975), and frequently used since then (Duwat et al., 1995; Pedersen

et al., 2008; Rezaïki et al., 2004; Velly et al., 2014). It was thus deemed suitable for these studies. However, the standard M17 composition was based on 5 $g \cdot l^{-1}$ lactose, which is not compliant with the high sugar levels, 40 $g \cdot l^{-1}$, used in the industrial process. To create a medium supporting 40 $g \cdot l^{-1}$ lactose four media compositions were investigated. Each component was multiplied with a given factor where the greatest factor was based on the factor 8 increase of the lactose concentration, Table 3.1.

TABLE 3.1 The media compositions investigated to find the one best suited to imitate the industrial process, supporting respiratory growth while retaining the physiological features of *L. lactis*

Compound ($g \cdot l^{-1}$)	**Standard M17**	**1.4 x M17**	**2 x M17**	**3 x M17**	**8 x M17**
Lactose	5	40	40	40	40
Yeast extract	2.5	3.5	5	7.5	20
$MgSO_4 \cdot 7H_2O$	0.25	0.35	0.5	0.75	2
Tryptone	5	7	10	15	40
Peptone	5	7	10	15	40
Beef extract	5	7	10	15	40
Ascorbic acid	0.5	0.7	1	1.5	4

The four media compositions were tested in comparative batch experiments, and the results are shown in Figure 3.1. The concentrations of the compounds in the medium should not be too high, as was the case in the 8 x M17 composition, which led to inhibition of respiratory growth and higher lactate formation than in the respiration-permissive industrial process. On the other hand, the concentration should not be too low, as in the case of 1.4 x M17, as this led to growth limitation and incomplete lactose consumption. A factor of 1.4 has previously been used for anaerobic growth at 40 $g \cdot l^{-1}$ lactose (Boonmee et al., 2003), although this was reported not to be sufficient for respiratory growth. Using a factor of 2 improved respiratory growth, and the cells were able to consume all the lactose, however, the specific growth rate and biomass yield did not reach industrial levels. Applying a factor of 3 led to good respiratory growth and *L. lactis* with similar physiological features to those obtained in the industrial process.

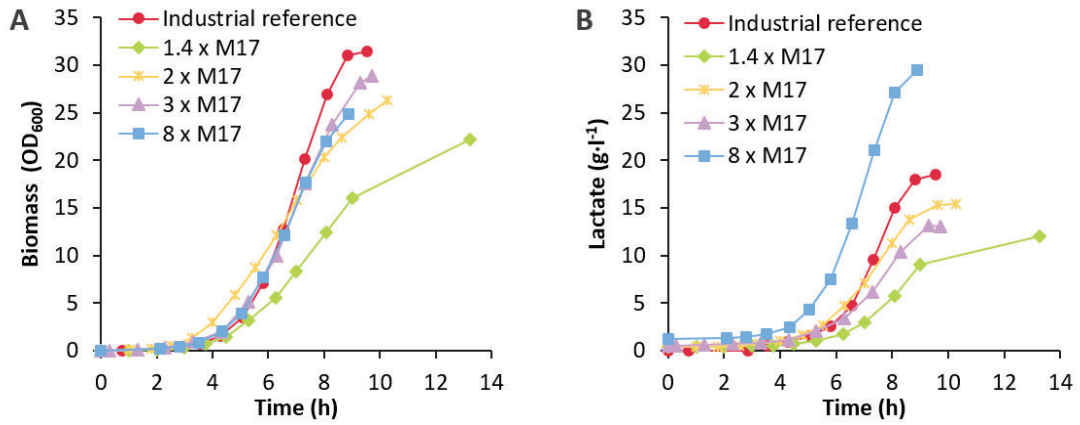


FIGURE 3.1 Results of batch cultivation of *L. lactis* in M17 with different compositions under respiration-permissive conditions. A) Growth curves (expressed as optical density, OD), B) Lactate formation (Johanson et al., unpublished data).

3.2 Batch and chemostat cultivation systems

Microbial growth kinetics are fundamental in all fields of microbiology, whether it is physiology, genetics, ecology, or biotechnology. In industry, starter cultures are usually cultivated in batch cultivations and harvested in the stationary phase. The physiology during a batch cultivation is dynamic as the substrate concentration and the specific growth rate changes throughout the process time. Early in the batch, the substrate concentration and the specific growth rate are high. Towards the end, substrate is depleted, product concentrations accumulate and the specific growth rate decreases. Observations made in batch cultivations can be obscured by the effect of growth rate-dependent factors (Hoskisson & Hobbs, 2005).

Cultivation of *L. lactis* under respiration-permissive conditions has only been conducted in batch cultivations (Duwat et al., 2001; Koebmann et al., 2008; Lan et al., 2006; Neves et al., 2005). The advantages of respiration-permissive conditions are well established in both the literature and in industrial practice, namely an increase in biomass yield, resistance to oxygen, and long-term cellular survival in liquid-form cultures (Duwat et al., 2001; Gaudu et al., 2002; Rezaïki et al., 2004). However, quantitative analyses of metabolic fluxes and energetics are scarce, and neither the potential relationship between the specific growth rate and the beneficial effects of respiration, nor the potential effects on the robustness in terms of tolerance to downstream processing, has been considered.

A continuous culture system provides the possibility to uncouple microbial growth from the transient conditions encountered in batch culture**.** Therefore, a chemostat cultivation system was established in order to mimic the cultivation conditions that prevail during different phases of industrial batch production (Hoskisson & Hobbs, 2005). Prior to a continuous culture a batch cultivation is conducted. Microbial growth proceeds to the point of fresh-feed addition while the removal of the fermentation broth at the rate of the fresh-feed addition takes places. This leads to a constant environment with a defined and controllable set of physical and chemical conditions and assumes a “steady-state”. In steady-state, growth occurs at constant specific growth rate, however, below the maximum specific growth rate (μ_{max}), allowing growth rate manipulation as a function of the feed addition (Hoskisson & Hobbs, 2005). This offers the possibility to examine the cell biology in a highly quantitative and reproducible manner compared to batch cultivations.

In this thesis work, chemostat cultivations were utilized to investigate how the physiological state of cells affected frozen and freeze-dried products by harvesting the cells at steady state (**Paper II**). To enable this a new chemostat culture system was implemented in a formerly traditional batch cultivation laboratory (Figure 3.2).

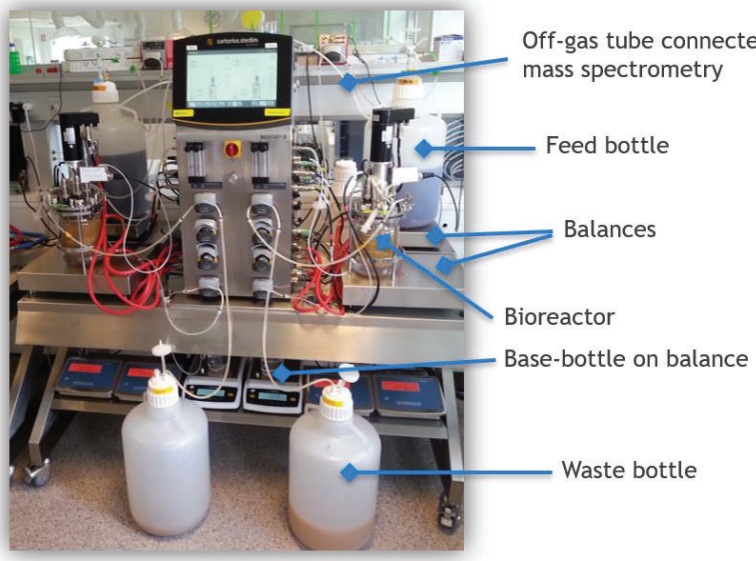


FIGURE 3.2 The implemented chemostat cultivation system, using a l-litre bioreactor connected to a Sartorius Biostat® B. The illustration shows the chemostat cultivation setup where the volume of the fermentation broth and feed-flow are controlled by monitoring the weight of the bioreactor and feed-bottle, respectively.

The greatest challenges of implementing the chemostat culture system were the handling of larger media volume needed and the sampling procedure of the fermentation broth. Compared to the traditional batch cultivation laboratory the standard sterilization procedures of media had to be revised and modulated to accommodate the larger volume media used for feed addition. Furthermore, a rapid sampling procedure is a prerequisite as measurements should reflect the conditions at the moment of sampling. Therefore, the use of pre-cooled stainless steel beads was introduced to immediately cool the sample broth, but not freeze it, allowing the samples to be quickly distributed to tubes for further analysis (Mashego et al., 2003).

3.3 Efforts to develop a quenching method for metabolome analysis

Microbial metabolomics has attracted considerable interest in recent years as it complements other "omics" research: genomics, transcriptomics and proteomics. The original aim was to perform a complete omics study to expand and deepen our knowledge of the metabolism of *Lactococcus lactis*. The general workflow of metabolomics consists of sample preparation, analysis and data processing (Figure 3.3). The parts are interdependent and each specific protocol must be aligned with the previous and following step. Furthermore, there may be considerable chemical diversity between the metabolites, which means that multiple analysis techniques may be required (Krastanov, 2010; Wittmann et al., 2004).

Several studies have been carried out on the metabolomic analysis of LAB, but many of the results are contradictory (Canelas et al., 2008; Chen et al., 2014; Jäpelt et al., 2015; Meyer et al., 2013). A prerequisite for an accurate and reproducible metabolomic method

is a functional sample preparation protocol. There is currently no validated protocol for the metabolomic analysis of *L. lactis*, and the first step undertaken here was to develop and optimize a suitable method. Sampling is critical in metabolomic analysis due to high exchange rates and the small pool sizes of the metabolites of interest. Sampling procedures for metabolomic analysis are often not given adequate consideration, and sampling protocols are often simply adapted from the literature without critical validation for the case and organism in question, leading to enormous errors (Bolten et al., 2007). The main purpose of the sample preparation methods is to quench the cells, i.e. stop the metabolism. Three preparation methods were considered in an attempt to develop a good sample preparation protocol for *L. lactis*: fast filtration, one-step quenching/extraction and two-step quenching and extraction (Figure 3.3).

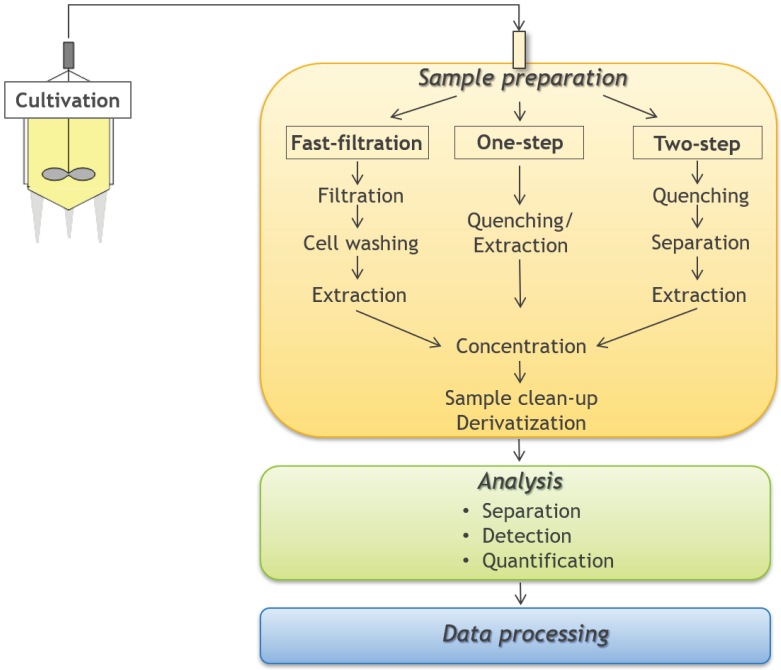


FIGURE 3.3. The potential procedure involved in metabolomic analysis involves withdrawal of the sample, sample preparation, sample analysis and data processing. Alignment of each step is crucial in achieving accurate and reproducible results. The three sample preparation methods considered in this work are illustrated.

The main objective was to analyse glycolytic sugar phosphates and adenine nucleotides, i.e., metabolites with a high turnover number. As discussed above, the experimental setup was based on a complex medium, which could not be filtered rapidly due to filter blockage. The one-step quenching and extraction method would require very demanding sample clean-up and concentration, and was not aligned with the available analytical instrumentation. Therefore, the two-step quenching and extraction method was chosen for further development and optimization.

Based on reports in the literature, three quenching solutions were tested: 80% methanol (M), 80% methanol/20% glycerol (MG), and 60% glycerol-saline (GS), and a sample with no treatment (NT) was included as a control (Canelas et al., 2008; Chen et al., 2014; Faijes et al., 2007; Schädel et al., 2011; Villas-Bôas & Bruheim, 2007). Cell samples taken

during cultivation were transferred to the quenching solution, mixed, centrifuged, washed, centrifuged again, and finally the cell pellet was frozen until further analysis. The control was centrifuged once and the pellet frozen. The effect of the quenching solutions to retain the integrity of the cell membrane and to immediately inactivate the metabolism was evaluated by propidium iodide (PI) staining (Jäpelt et al., 2015; Schädel et al., 2011) and quantification of adenine nucleotides (ATP/ADP/AMP) (Faijes et al., 2007; Meyer et al., 2013), respectively. Figure 3.4 shows a detailed illustration of the performed two-step sample preparation.

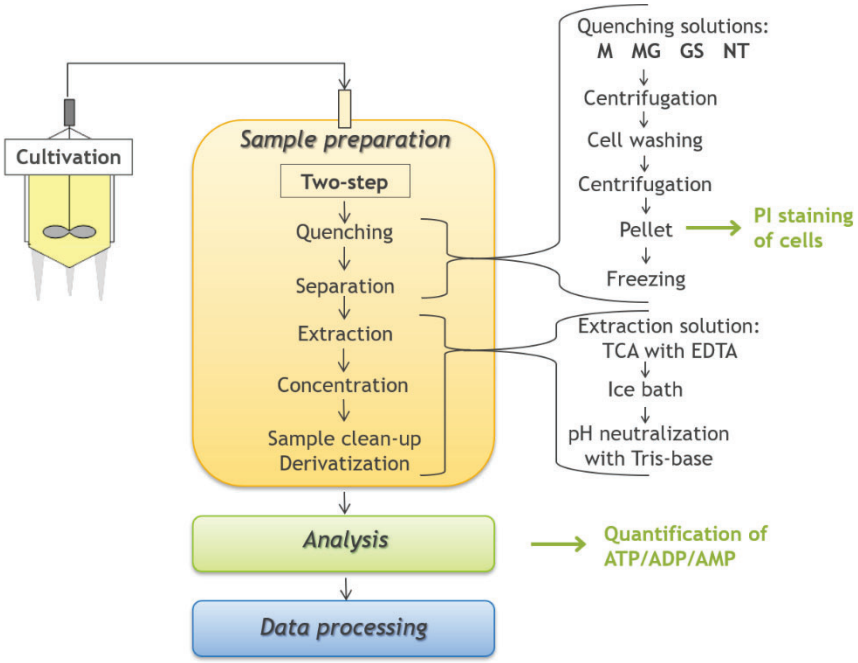


FIGURE 3.4 Illustration of the quenching protocol for a two-step sample preparation method for metabolomic analysis of *L. lactis*. Three quenching solutions were tested: 80% methanol (M), 80% methanol/20% glycerol (MG), and 60% glycerol-saline (GS), and as control a sample with no treatment (NT) was included. Propidium-iodide (PI) staining and ATP/ADP/AMP analysis were used to evaluate the tested procedures.

Two cell samples were taken during cultivation, in early and mid-exponential growth phase. PI staining was immediately performed on unfrozen cell pellets. If the cell membrane is damaged PI can enter the cell and react with DNA, leading to red fluorescence, which can be observed in a microscope. However, practical problems were encountered already during sample handling. The separation of the cells after quenching was not successful in all cases due to viscosity differences. Especially after quenching with GS, it was clear that the cell pellet was reduced in size compared with using the other solutions, or not present at all, and a higher centrifugation force was required. This resulted in no cells being available from early-exponential phase sample for PI staining. Tests with the other quenching solutions indicated that the use of solutions containing methanol (M and MG) resulted in a high degree of membrane damage (Figure 3.5). The glycerol-saline (GS) solution showed less damaged cells in the mid-exponential phase. There was no staining of the non-treated (NT) cells at either early or mid-exponential phase. These initial tests clearly indicated that the use of methanol in the quenching solution caused cell

membrane damage of *L. lactis*, as has also been reported in a previous study on *L. paracasei* (Jäpelt et al., 2015).

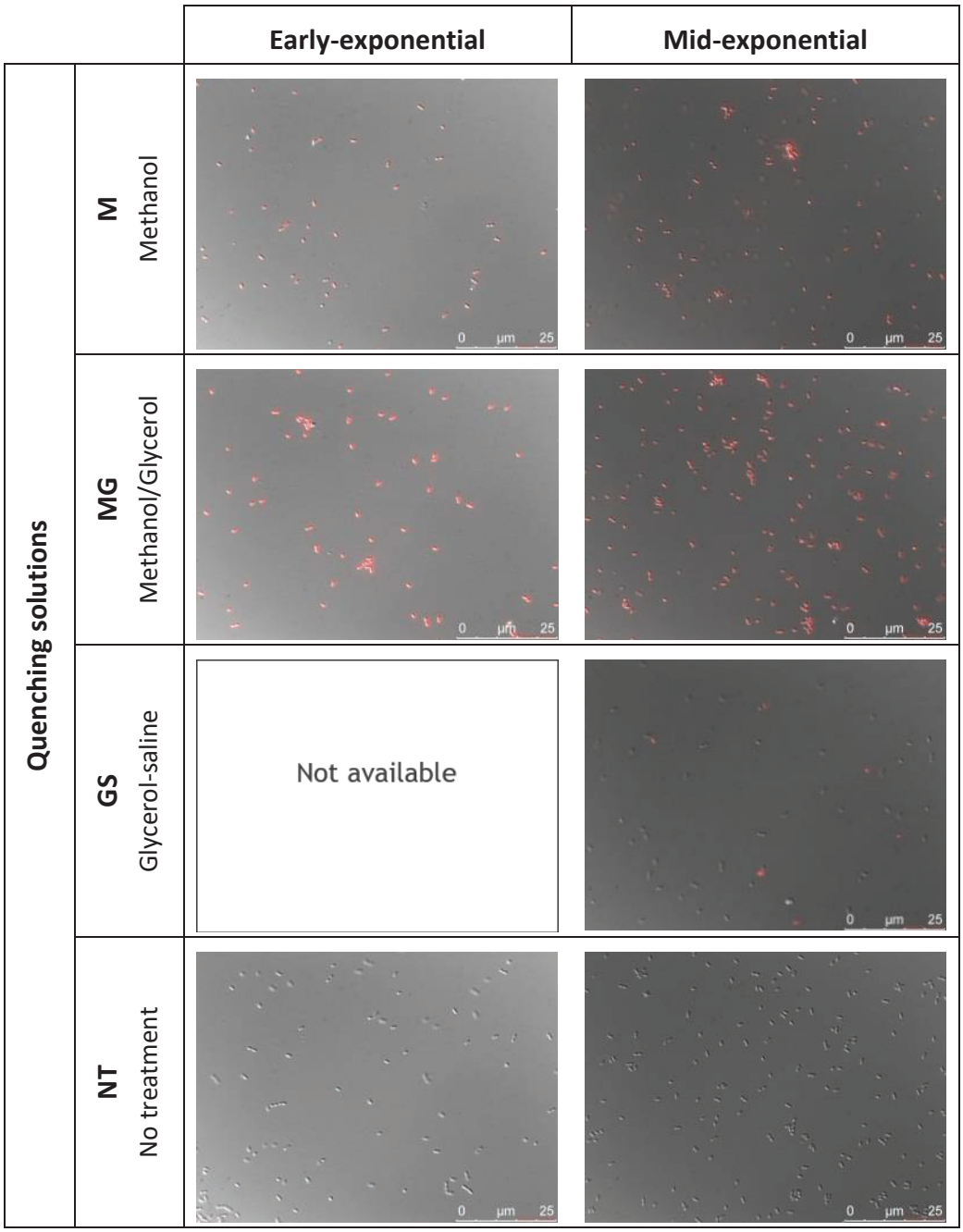


FIGURE 3.5 Microscope images showing the results of PI staining of quenched cells in the early and mid-exponential phase. Three quenching solutions were tested; 80% methanol (M), 80% methanol/20% glycerol (MG) and 60% glycerol-saline (GS), the control was subjected to no treatment (NT). Red staining indicates damage to the cell membrane (Johanson et al., unpublished data).

The adenine nucleotide (AXP) measurements of biological duplicates of early and mid-exponential phase samples revealed very different results for the different quenching solutions, as can be seen in Figure 3.6. Firstly, the quenching solutions containing glycerol (MG and GS) were difficult to separate, giving less final cell material. This resulted in a lower estimate of the total AXP as it was normalized to the cell level in the cultivation at

the point of sample. Surprisingly, the non-treated cells (NT) also exhibited a lower level of total AXP than cells quenched with 80% methanol (M), although, based on the PI staining, the methanol-quenched cells showed high degree of membrane disruption. The adenylate energy charge (EC), which provides an index of the energy state of a cell, is defined as (ATP+½ADP)/(AMP+ADP+ATP), and was calculated. The lowest EC was observed in GS quenched cells (0.68-0.75) and in the control NT (0.73-0.76), whereas quenching of cells using methanol (M and MG) led to the highest EC values (0.83-0.87). The EC for growing non-stressed cells is expected to be in the range 0.8-0.95 (Atkinson, 1968). The induction of stress during sampling would be reflected in metabolic changes; for example, a decrease in EC could be caused by either a decrease in ATP concentration and/or an increase in AMP concentration. For the NT cells no metabolic inactivation takes place which can explain the lower EC value. However, the low EC value of GS quenched cells could be an indication of either an insufficient metabolic arrest or an non-optimal quenching procedure resulting in inaccurate numbers. In contrast to the PI staining test, the adenine nucleotide analysis indicated that the use of methanol quenching achieved the best results to inactivate the metabolism.

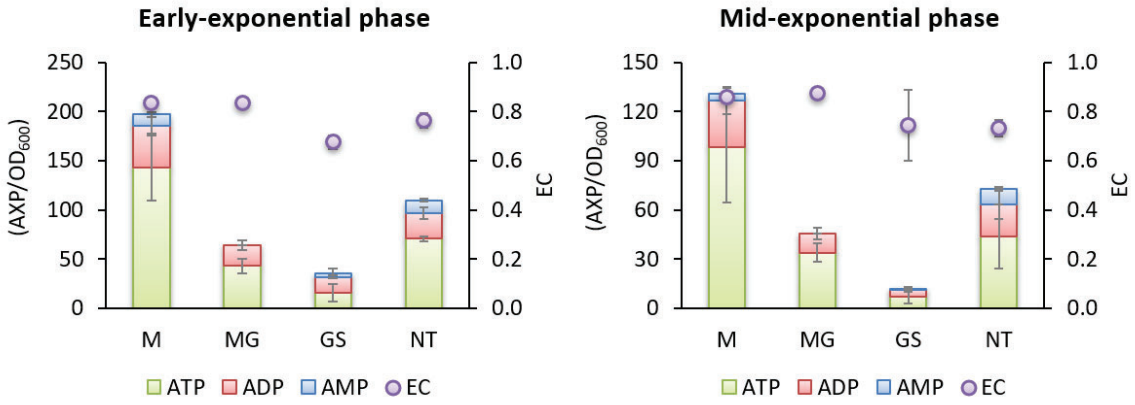


FIGURE 3.6 Measured amounts of ATP/ADP/AMP (AXP) normalized to OD_{600} at point of sample and calculated adenylate energy charge (EC) in samples collected in the early exponential phase and mid-exponential phase. Three quenching solutions were tested: 80% methanol (M), 80% methanol/20% glycerol (MG) and 60% glycerol-saline (GS), the control was subjected to no treatment (NT) (Johansson et al., unpublished data).

In conclusion, the results of PI staining indicated considerable membrane damage by methanol quenching, while glycerol-saline quenching had less effect on membrane integrity. The non-treated cells seemed to be intact. However, the energy charge of the AXP in the non-treated cells clearly indicated that cellular metabolism was not inactivated. The AXP measurements in the samples quenched with the GS solution were not successful due to the non-optimized sampling (separation) procedure. It has previously been reported that glycerol quenching maintains cell membrane integrity, but places restrictions on the analytical flow. Both a prolonged analysis time and the presence of glycerol remnants have been reported to lead to analytical problems in various methods (Chen et al., 2014; Jäpelt et al., 2015; Villas-Bôas & Bruheim, 2007).

Many different techniques are required in metabolomic studies, which makes the development of a standardized protocol for analysis impossible. Unfortunately, the development of a sampling method for metabolomic analysis of *L. lactis*, had to be terminated. The complexity of the problem was greater than anticipated, and fell outside the scope of this thesis. The question is, whether there is such a thing as the perfect metabolomic protocol. Compromises will undoubtedly be necessary, whether they are in the sampling time, the degree of cell damage or sample clean-up. Based on the results of the studies presented in this section, three important factors could be identified in sample preparation for metabolomic analysis: adequate separation of the cell material and quenching solution, monitoring biomass balance throughout all handling steps for accurate biomass normalization and potential membrane leakage. These will have to be addressed before an accurate and reproducible method can be developed.

4. Respiratory Physiology of *L. lactis*

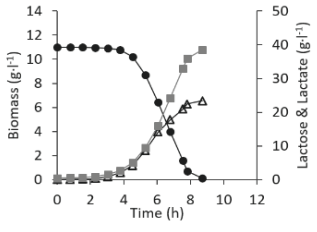
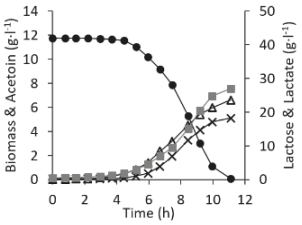
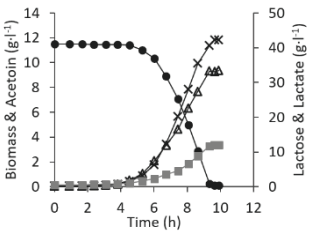
Aerobic cellular respiration is often associated with oxygen and the breakdown of a carbon source. Energy from sugars is converted into ATP, a high-energy molecule, and the by-products CO_2 and H_2O. ATP formation is essential for cellular growth and function, such as facilitation of metabolic reactions and transportation of solutes and compounds across the cellular membrane. Aerobic respiration occurs in both eukaryotic and prokaryotic cells, with most reactions taking place in the mitochondria of eukaryotes and in the cytoplasm of prokaryotes. Sugars first enter glycolysis, which does not require oxygen, from where the pathway continues to the tricarboxylic cycle (TCA). Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) are produced and proceed to the ETC for final oxidative phosphorylation. A membrane gradient is formed through electron translocation in the ETC, hydrogen ions are pumped out of the cell and finally ATP is produced (Sadava et al., 2008).

L. lactis belongs to the group of LAB capable of aerobic respiration when provided with exogenous sources of haem and menaquinone (MQ) (Lechardeur et al., 2011; Pedersen et al., 2012). *L. lactis* lacks the biosynthetic pathway of haem, an essential cofactor of the ETC (Duwat et al., 2001; Gaudu et al., 2002). The first observations of haem-induced respiration in LAB were made in the late 1960s (Bryan-Jones & Whittenbury, 1969; Sijpesteijn, 1970). However, the first in-depth studies on *L. lactis* were not carried out until 30 years later (Blank et al., 2001; Duwat et al., 2001). No functional TCA cycle is present in *L. lactis*, however, the established ETC significantly increases the biomass yield and some aspects of robustness of *L. lactis*, and aerobic respiration in LAB, especially *L. lactis*, has proven to be very promising for industrial applications.

4.1 Metabolic and physiological hallmarks of *L. lactis* under anaerobic, aerobic, and respiration-permissive conditions

L. lactis was traditionally classified as a facultative anaerobe on which oxygen has strong regulatory effects (Cocaign-Bousquet et al., 2002; Neves et al., 2005). As discussed in Section 2.4, *L. lactis* performs homofermentative fermentation under anaerobic conditions, using pyruvate as the electron receiver for the regeneration of NAD^+. Table 4.1 gives the typical time courses of cell growth, lactate and acetoin accumulation, and yields from lactose consumption in anaerobic, aerobic and respiration-permissive batch cultivations (**Paper I**). Under aerobic conditions, the metabolism switches to mixed-acid and flavour-forming fermentation in response to oxygen (Garrigues et al., 2001; Lopez De Felipe et al., 1997). Aeration introduces an additional point of NAD^+ regeneration by cytosolic NOX activity (Duwat et al., 2001; Lopez de Felipe & Hugenholtz, 2001). Pyruvate builds up, shifting the carbon flux, such that lactate formation is reduced and acetoin is formed instead (Cocaign-Bousquet et al., 1996; Neves et al., 2005). However, the presence of oxygen inhibits cellular growth by significantly decreasing the maximum specific growth rate (μ_{max}). The value for aerobic cultivation is 0.76 h^{-1} which can be compared to that in anaerobic cultivation, 1.03 h^{-1} (Table 4.1) (**Paper I**).

TABLE 4.1 Batch cultivations of *L. lactis* CHCC2862 under anaerobic, aerobic and respiration-permissive (aerobic+10 mg·l^{-1} haemin) conditions, including maximum specific growth rate (h^{-1}), and metabolite yields on consumed lactose (C-mol·C-mol^{-1}): biomass (△), lactate (■), acetoin (×) (**Paper I**) †

	ANAEROBIC	AEROBIC	RESPIRATION-PERMISSIVE
			
μ_{max}	1.03 ± 0.03	0.76 ± 0.01	0.93 ± 0.03
Biomass	0.19 ± 0.01	0.19 ± 0.01	0.27 ± 0.02
Lactate	0.93 ± 0.01	0.65 ± 0.02	0.31 ± 0.01
Acetoin	n.d.	0.17 ± 0.00	0.39 ± 0.00
Acetate	0.02 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
CO_2	n.m.	0.08 ± 0.00	0.17 ± 0.01
O_2	n.m.	0.04 ± 0.00	0.08 ± 0.00
Formate	0.03 ± 0.01	n.d.	n.d.
Ethanol	0.01 ± 0.00	t.a.	t.a.

† Initial lactose concentration (●) 40 g·l^{-1}, medium 3xM17, pH 6.0 and 30°C. Values given are the average of biological triplicate experiments with 95% confidence intervals (n.m. - not measured, n.d. - not detected, t.a. - trace amount)

Oxygen itself does not cause damage to the cell, but leads to the formation of reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{\bullet}$), the hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). Various defence mechanisms are triggered in response to oxidative stress (Condon, 1987; Sanders et al., 1999). The coupled NADH oxidase/NADH peroxidase system is a common oxidative stress resistance system in *L. lactis*. Intracellular O_2 can be eliminated by NOX activity, in which NADH is oxidized to NAD^+ and generates H_2O_2, which in turn is reduced to H_2O by NADH peroxidase (Table 4.2) (Condon, 1987). Other common oxygen resistance mechanisms are the action of SOD and catalase, which remove O_2 radicals and break down H_2O_2, respectively (Table 4.2) (Condon, 1987; Sanders et al., 1999). However, both NADH oxidase and SOD produce the highly toxic compound H_2O_2, and considering that *L. lactis* possesses low NADH peroxidase activity and lacks catalase activity, H_2O_2 accumulation is probably the main cause of the destruction of cell components under aerobic conditions (Duwat et al., 2001).

TABLE 4.2 Enzymatic oxygen resistance mechanisms and their respective catalytic enzymes

Enzymatic reaction	Catalytic enzyme
$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2$	NADH: H_2O_2 oxidase
$2NADH + 2H^+ + O_2 \rightarrow 2NAD^+ + 2H_2O$	NADH: H_2O oxidase
$NADH + H^+ + H_2O_2 \rightarrow NAD^+ + 2H_2O$	NADH peroxidase
$2\ O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$	Superoxide dismutase (SOD)
$2H_2O_2 \rightarrow 2H_2O + O_2$	Catalase

The final biomass yields of *L. lactis* under aerobic cultivation conditions are typically low due to significant oxygen stress (Table 4.1) (Niel et al., 2002). On the other hand, supplementation with haemin under aerated (respiration-permissive) conditions completes the non-functional ETC of *L. lactis* and provides respiration capacity. NADH acts as the primary electron donor for the respiratory chain, and oxygen is consumed concomitantly with NADH oxidation, relieving the cellular oxygen stress. The $NADH/NAD^+$ ratio decreases, reducing LDH activity and thereby lactate formation. As *L. lactis* does not possess a functional TCA, pyruvate cannot be completely oxidized to CO_2, and instead acetoin and acetate accumulate as organic sinks. Nevertheless, respiration-permissive conditions clearly benefit cellular growth, with a greater than 40% increase in overall biomass yield compared to aerobic and anaerobic cultivation, and a relatively high maximum specific growth rate (Table 4.1) (**Paper I**). Introducing respiration-permissive conditions decreased the intracellular oxygen level and reduced acid production, releasing more energy for biomass formation. Furthermore, as stated in Section 2.4, long-term cellular survival in liquid-form cultures stored at 4°C has been shown to improve significantly under respiration-permissive conditions, which is essential for starter culture quality (Duwat et al., 2001; Gaudu et al., 2002; Rezaïki et al., 2008), making the process attractive for starter culture production.

4.2 Metabolic changes during respiration

Respiration leads to higher production of flavour-forming metabolites and lower amounts of lactate (Table 4.1) (Duwat et al., 2001). However, despite the major changes in pyruvate metabolism, the expression of the *als* gene (encoding acetolactate synthase) and the *aldBC* gene (encoding acetolactate decarboxylase) was not significantly different between anaerobic and respiration-permissive conditions. Furthermore, the genes related to the conversion of acetyl-coenzyme A to acetate, *pta* (encoding phosphotransacetylase) and *acka* (encoding acetate kinase), were similarly expressed regardless of the growth conditions. Overall, gene expression related to carbon metabolism by mid-exponential phase cells did not reveal any respiration-specific changes (Gaudu et al., 2002; Pedersen et al., 2008; Rezaïki et al., 2004; Vido et al., 2004). The extensive changes found in the end product profile, and the lack of changes in expression levels of relevant genes, led to the proposal that the metabolic shift via respiration could be attributed to metabolic pool shifts engendered by the ETC, or changes in gene expression that occur later in growth (Pedersen et al., 2008). Pools of NADH are depleted by respiration activity, which decreases the $NADH/NAD^+$ ratio, determining the metabolic pathway (Blank et al., 2001; Garrigues et al., 1997). Under respiration-permissive conditions, low NADH correlates with lower LDH activity, reducing lactate formation and re-routing pyruvate to acetoin and acetate (Figure 2.4) (Duwat et al., 2001; Pedersen et al., 2008).

In a recent study, transcriptomic analysis of cells late in respiratory growth suggested that the acetate pathway plays a role in biomass formation, whereas the acetoin pathway is involved in the maintenance of metabolic activities by avoiding internal acidification (Cesselin et al., 2018). In contrast to earlier studies, it was revealed that respiration dramatically altered gene expression when the cells reached the stationary phase. Especially genes involved in pyruvate catabolism and the acetoin pathway were markedly changed. This led to the suggestion that the acetate pathway is needed in order for growth to benefit from respiratory conditions, as *pta* and *ack* mutants showed low biomass under respiration-permissive conditions. Biomass yield depends on the bacterial capacity to produce ATP, and acetate kinase is thus a likely source of ATP. In *L. lactis*, the ATP pool results from glycolysis, and part of this pool is consumed by ATPase for H^+ extrusion. With respiration, the ETC replaces ATPase as an H^+ extrusion pump, thus reducing ATP expenditure and making ATP more available. Interestingly, *L. lactis* MG1363 was found to require both PDH and, to a lesser extent, PFL (Cesselin et al., 2018). PDH activity is expected under aerobiosis, although it is remarkable that the oxygen-sensitive enzyme, PFL, is needed under aerobic respiration. In the present work on *L. lactis* CHCC2862, formate, a product of PFL, was not detected under aerated conditions. However, PFL activity has been shown to be attributed to the protective effect of the ETC against oxidative stress (Rezaïki et al., 2004).

Cesselin et al. (2018) also suggested that the acetoin pathway was linked to pH homeostasis, as respiration led to the induction of *als* and *aldB*, and deletion of *als* resulted in a reduction in pH and poorer survival. This could be explained by the channelling of pyruvic acid, being highly acidic with a pK_a of 2.4, towards the neutral acetoin. Low pH

has been shown to be responsible for growth arrest of *L. lactis* in sugar-rich media, and is a known cause of growth inhibition (Loubiere et al., 1997; Papadimitriou et al., 2016). The balance between the acetoin and acetate pathways may thus be a key factor in optimizing *L. lactis* growth and survival (Cesselin et al., 2018).

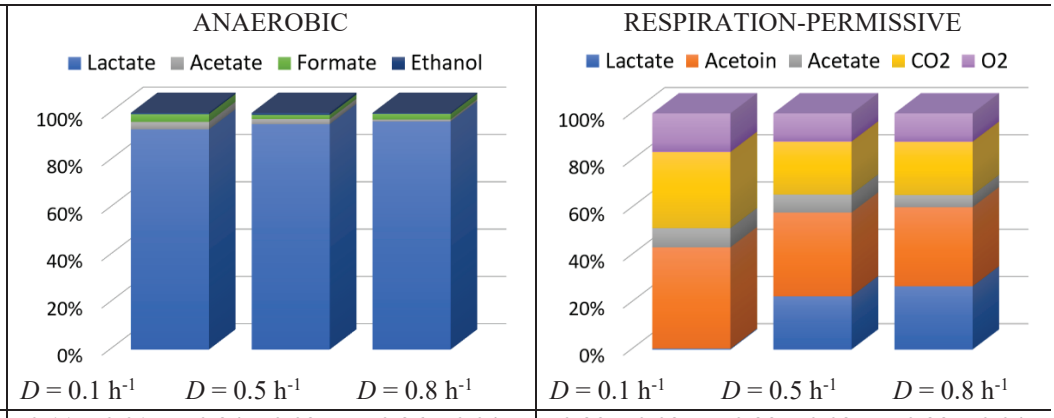
During aeration the cytoplasmic NOX gene (*noxE)*, implicated in the shift from homofermentative to mixed acid/flavour-forming fermentation, is among the most highly induced genes (Duwat et al., 2001; Lopez De Felipe et al., 1998). The aerobic life of *L. lactis* is stressful, several stress response genes encoding detoxification enzymes are affected, such as the superoxide dismutase gene, *soda*, which is required for survival under aerobic conditions, but not under respiration-permission conditions (Duwat et al., 2001). The different gene expression therefore indicates a different physiology under the two conditions. The greatly improved growth observed previously for *L. lactis* during respiration-permissive conditions, compared to aerobic cultivations, correlates with a markedly lower induction of stress response genes. Surprisingly, the expression of the genes involved in the respiratory metabolism, e.g., genes encoding cytochrome *bd* oxidase, menaquinone biosynthesis and haem uptake, were not specifically induced by respiration, and were expressed similarly regardless of the growth conditions (Pedersen et al., 2008). This indicates that *L. lactis* is always prepared to switch to respiration once haem and O_2 become available.

4.3 Impact of specific growth rate on respiratory growth

The respiratory capacity of *L. lactis* CHCC2862 was investigated using chemostat cultivation. In contrast to anaerobic chemostat cultivations, which remained homofermentative regardless of the dilution rate applied, the metabolic profile varied between the respiration-permissive chemostat cultivations depending on the dilution rate (Table 4.3) (**Paper II**). Pyruvate is a key point within *L. lactis* metabolism where LDH, ALS, PDH and PFL, compete for pyruvate as substrate, leading to homofermentative, mixed-acid and/or flavour-forming products. The flavour-forming and mixed-acid pathways were active at all dilution rates, while the greatest differences were observed in lactate formation. At the lowest dilution rate, no lactate was formed, while a mixture of lactate, acetoin, acetate, and CO_2 was produced at the higher dilution rates. At high dilution rates the product profile indicated that ALS and LDH were the most active enzymes, as the pyruvate flux was directed towards acetoin and lactate. Interestingly, the specific acetate formation rate did not continue to increase when the dilution rate was changed from 0.5 h^{-1} to 0.8 h^{-1}, and the acetate yield on lactose decreased. One explanation could be allosteric regulation of several enzymes by fructose-1.6-bisphosphate (FBP). The FBP level can be expected to increase at high specific growth rates (Lopez De Felipe & Gaudu, 2009; Neves et al., 2005; Thomas et al., 1979). FBP increases the affinity of LDH for both pyruvate and NADH (Crow & Pritchard, 1977), and it is known to inhibit PTA and ACKA, which are involved in acetate formation (Goel et al., 2012; Lopez De Felipe & Gaudu, 2009). Together, this would mean that carbon flux is redirected away from acetate towards lactate at higher dilution rates. It is, however, interesting that such a shift was not

clearly seen under anaerobic conditions in this strain, which showed similar yields at all anaerobic dilution rates (Table 4.3). The fact that no metabolic shift was observed during anaerobic chemostat cultivations, could be due to regulation via the NADH/NAD^+ ratio, which should generally be higher under anaerobic than under aerobic conditions. NADH has been suggested to be a key regulator in carbon metabolism, as a low NADH/NAD^+ ratio could allosterically redirect metabolism in favour of enzymes using NAD^+ or pyruvate as substrate, without necessarily changing the gene expression levels or enzyme concentrations (Garrigues et al., 1997; Lopez De Felipe et al., 1997).

TABLE 4.3 Metabolite yields on consumed lactose (C-mol·C-mol^{-1}) in chemostat cultivations of *L. lactis* CHCC2862 under anaerobic and respiration-permissive (10 mg·l^{-1} haemin) conditions at three dilution rates (*D*) (**Paper II**) †



	ANAEROBIC			RESPIRATION-PERMISSIVE		
	D = 0.1 h^{-1}	*D* = 0.5 h^{-1}	*D* = 0.8 h^{-1}	*D* = 0.1 h^{-1}	*D* = 0.5 h^{-1}	*D* = 0.8 h^{-1}
Biomass	0.11 ± 0.01	0.24 ± 0.03	0.26 ± 0.04	0.29 ± 0.03	0.32 ± 0.03	0.33 ± 0.04
Lactate	0.96 ± 0.03	0.99 ± 0.13	0.96 ± 0.09	0.00 ± 0.00	0.22 ± 0.01	0.23 ± 0.02
Acetoin	n.d.	n.d.	n.d.	0.39 ± 0.03	0.34 ± 0.03	0.29 ± 0.04
Acetate	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
CO_2	t.a.	t.a.	t.a.	0.30 ± 0.05	0.22 ± 0.02	0.20 ± 0.02
O_2	n.d.	n.d.	n.d.	0.15 ± 0.02	0.12 ± 0.00	0.11 ± 0.01
Formate	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	n.d.	n.d.	n.d.
Ethanol	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	t.a.	t.a.	t.a.

† Medium 3 x M17, initial lactose concentration in bioreactor and in feed 40 g·l^{-1}, pH 6.0 and 30°C. Values given are the average of biological duplicates and the 95% confidence intervals from measurements of two separate steady-state samples from each cultivation (n.m. - not measured, n.d. - not detected, t.a. - trace amount).

As mentioned above, it has been proposed that during respiratory growth, acetate production is necessary for the increased biomass yield, while the acetoin production alleviates acid stress and prolongs survival (Cesselin et al., 2018). The results of the present work imply that, in addition, the acetoin produced is required to compensate for the NADH consumed by respiration, as higher levels of acetoin were correlated to the net production of NADH from the catabolism of lactose in respiratory cultures (Figure 4.1). Glycolysis forms NADH, which must be re-oxidized. Under respiration-permissive conditions this can take place via LDH, ADHE/ADHA (ethanol formation), cytosolic NOX or the ETC. At the lowest dilution rate, almost no lactate was formed and only traces of ethanol were produced, which led to the conclusion that NADH is oxidized via the ETC (**Paper II**). However, comparison of aerobic and respiration-permissive batch cultivations

could suggest that part of the NADH consumed during respiration-permissive conditions takes places via the cytosolic NOX (Johanson et al. unpublished data). Nevertheless, the accumulated pyruvate must be consumed via NADH-producing or redox-neutral reactions, such as acetoin formation, to support respiration. It is notable that at low dilution rate, which resulted in high respiration capacity with no lactate formation, the biomass did not increase compared to conditions with the higher dilution rates where lactate was formed concomitantly with respiratory growth (**Paper II**). The biomass yield was instead lower, although not significantly. This could be related to the lack of increase in acetate concentration. The metabolic shift under respiration-permissive conditions is common, however, the ratio of acetoin to acetate formation differs between strains (Cesselin et al., 2018). Altogether, these observations support the conclusion that the balance between the acetoin and acetate pathways may be a key parameter in optimizing *L. lactis* growth.

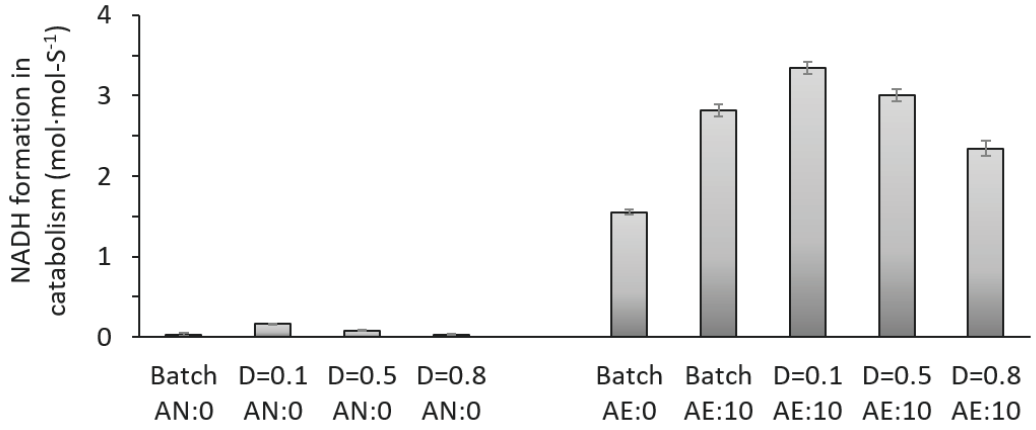


FIGURE 4.1 The estimated NADH formation through catabolism from the final metabolite profile. Anaerobic (AN) batch and chemostat cultivations at three dilution rates: D = 0.1, 0.5 and 0.8 h^{-1}without haemin. Aerobic (AE) batch cultivations without and with 10 $mg \cdot l^{-1}$ haemin and chemostat cultivations with 10 $mg \cdot l^{-1}$ haemin at three dilution rates: D = 0.1, 0.5 and 0.8 h^{-1}. Values given are the average of four biological replicates for the batch cultivations and duplicates of the chemostat cultivations. The error bars show the standard deviation (Calculated from data presented in **Paper I** and **Paper II**).

In both respiration-permissive and anaerobic chemostat cultivations, low dilution rate was associated with lower biomass yield (Table 4.3) (**Paper II)**. The maintenance energy demand is a likely reason for this. However, the maintenance coefficient obtained under respiration-permissive conditions was almost 90% lower than the value under anaerobic conditions. This explains the large difference between anaerobic and aerobic biomass yields at D = 0.1 h^{-1}, as a larger fraction of the lactose consumption would be used for maintenance energy requirements under anaerobic conditions, instead of providing energy for biomass formation. Respiration in *L. lactis* is different from common respiration, as NADH oxidation and ATP production are only weakly related. Most of the ATP is generated by substrate-level phosphorylation (Koebmann et al., 2008). The function of H^+-ATPase in relation to the ETC has not yet been fully elucidated, and the question is whether H^+-ATPase contributes to additional ATP formation apart from that produced in glycolysis and mixed-acid fermentation (Koebmann et al., 2008). Values of substrate-level phosphorylated ATP estimated from the metabolite yields in chemostat cultivations revealed higher values under anaerobic conditions than under respiration-permissive

conditions (**Paper II**). Nevertheless, the biomass yield was significantly increased under all respiration-permissive conditions studied. At the lowest dilution rate, the biomass yield increased by 165% compared to anaerobic conditions, whereas it increased by only 36% and 23% at $D = 0.5\ h^{-1}$ and $D = 0.8\ h^{-1}$, respectively.

These observations indicate that either additional ATP formation takes place from the available lactose due to respiration, or that respiration-permissive conditions lead to a lower energy requirement for maintenance. It was not possible to distinguish between these mechanisms in the present study, but both would lead to fulfilment of the energy demand for maintenance at a lower substrate consumption rate.

4.4 Aerobic and respiratory physiology of *L. lactis* on galactose

Several fermented dairy foods contain significant amounts of galactose as a result of incomplete lactose utilization (Alm, 1982). Galactose is a major contributor to the undesired browning that occurs when dairy products (e.g., yogurt and mozzarella, Swiss, and cheddar cheese) are cooked or heated in the manufacture of pizzas, sauces, or processed cheese. Hence, the presence of galactose has been associated with poor quality of the fermented products (Baskaran & Sivakumar, 2003; Hutkins et al., 1986; Michel & Martley, 2001). Studies aimed at understanding the galactose metabolism in LAB are therefore important if we are to improve the capacity of LAB to utilize galactose, and thus develop galactose-free products. Some studies of galactose metabolism in LAB under anaerobic conditions have been published (Grossiord et al., 1998; Thomas et al., 1980), but little information is available under aerobic conditions (Smart & Thomas, 1987).

During the course of the present work, galactose batch cultivations of *L. lactis* CHCC2862 were performed under aerobic and respiration-permissive conditions. The maximum specific growth rates under aerobic and respiration-permissive conditions were $0.63\ h^{-1}$ and $0.70\ h^{-1}$, respectively (Table 4.4) (**Paper III**), i.e., lower than the maximum specific growth rates on lactose under the same conditions. As in the cultivations on lactose, the metabolite profiles were a mixture of lactate, acetoin and acetate, however, the acetate yield was notably higher when galactose was used as the sugar source. The acetate yield in cultivations on lactose did not exceed 0.07 C-mol·C-mol^{-1}, which was observed in respiration-permissive chemostat culture at $D = 0.1\ h^{-1}$ (Table 4.3).

In addition, the lactate yield was lower during batch cultivation on galactose than on lactose. Furthermore, as in the lactose cultivations, the lactate yield during respiration-permissive growth on galactose was also lower than during aerobic growth. The higher acetate formation indicated a higher ATP yield, which could explain the increase in biomass yield observed during galactose batch cultivations. Together, these observations indicate that growth on galactose is energetically beneficial under aerobic and respiration-permissive conditions, and might play a positive role during aerobic growth, which may be interpreted as an increased aerotolerance.

TABLE 4.4 Maximum specific growth rate (h^{-1}) and metabolite yields on consumed galactose (C-mol·C-mol^{-1}) in batch cultivations of *L. lactis* CHCC2862 under aerobic and respiration-permissive conditions (**Paper III**)†

	AEROBIC	RESPIRATION-PERMISSIVE
μ_{max}	0.63 ± 0.15	0.70 ± 0.16
Biomass	0.33 ± 0.04	0.37 ± 0.07
Lactate	0.31 ± 0.02	0.19 ± 0.00
Acetoin	0.42 ± 0.00	0.43 ± 0.01
Acetate	0.35 ± 0.01	0.42 ± 0.13
CO_2	1.18 ± 0.07	2.17 ± 0.15
O_2	0.23 ± 0.03	0.24 ± 0.02

† Medium = standard M17, initial galactose concentration 5 g·l^{-1}, haemin concentration 5 mg·l^{-1}, pH 6.0 and 30°C. Values given are the average of biological duplicates ± standard deviation.

The catabolism of lactose and glucose by LAB is a tightly regulated mechanism, in which CcpA is the central regulator, assimilating glucose and lactose by expression control of the PTS and the tagatose pathway genes (Luesink et al., 1998). As described in Section 2.3, the lac-PTS and the concomitant ATP-dependent phosphorylation are prerequisites for the function of CcpA (Deutscher et al., 1995; Even et al., 2003). The metabolism of galactose is repressed by CcpA in the presence of lactose and glucose (Luesink et al., 1998), which challenges the galactose utilization in dairy products, where the concentration of lactose is normally high (Alm, 1982). The aerated batch cultivations on galactose showed excellent ability of the bacterium to metabolize galactose. An improvement in galactose utilization could relieve the CcpA repression, taking advantage of the increased energetics arising from galactose consumption.

5. The Inconsistent Lag Phase

A reproducible production process and high efficiency are very important in industrial production processes. A lag phase can occur as a result of environmental changes and/or instability of the inoculation material. However, lag phases must be minimized to achieve high productivity. The role of the pre-culture, before initiating the main cultivation step, is to create a smooth transition of the cells from the cell stock to the main cultivation and ensure the viability of the cells.

5.1 Lag phase in main cultivations

During the course of this experimental work, transferring inoculation material from static pre-culture without haemin to the main cultivation sometimes led to unexpected lag phases of varying length in the main cultivations. This was only observed with the transfer to respiration-permissive conditions (aeration + haemin supplementation) and not to anaerobic conditions, which were reproducible, and showed immediate growth. The reason for this had to be investigated/established before continuing the experimental work. There were several open questions: A. Was the haemin stock solution sufficiently stable? B. Were the experimental procedures reproducible? C. Were there any differences in how the pre-culture was cultivated? Several of these possible causes were investigated, as discussed below and summarized in Table 5.1 where the following capital letters will refer to the table.

A. Haemin stock solution. At the point of inoculation in respiration-permissive conditions the cells encountered the presence of haemin. Haemin consists of a porphyrin ring and a centred iron(III) atom which must be reduced to iron(II) in restoring the ETC. Thus, the lag phase could be due to a period during which haemin is converted into haem, before growth can proceed. The interaction of haemin with oxygen, possibly leading to disruption of the haemin molecule, could also affect cell growth. Iron porphyrins should be relatively stable complexes, although oxidation can lead to breakage of the porphyrin ring, thereby

removing the iron and its ability to react with cytochromes (Hogle et al., 2014). However, similar handling of haemin solutions previously resulted in successful experiments. Other factors investigated were the stability of the haemin powder supplied by the manufacturer and the impact of the sterilization procedure of the prepared haemin stock solution. A new supply of haemin was used (A1), and preparation of the haemin stock solutions was carefully controlled in each cultivation. Finally, the sterilization of the haemin stock solution was changed from autoclaving at 121°C for 15 minutes, to filtration (A2).

B. Experimental operation procedures. The way in which media are prepared can have considerable impact on cell growth. Close examination of the preparation process from balancing each component, water addition, sterilization, setting the pH and applying aeration prior to haemin addition revealed no obvious problems. In successful experiments resulting in high respiratory activity, a sticky black residual was observed on the inner surface of the bioreactor and baffles (Figure 5.1), which is believed to be associated with the high concentration of biomass and the presence of haemin. An extra cleaning step, with H_2O_2, was thus implemented to ensure that all cultivation residues were removed from the bioreactor (B1).

FIGURE 5.1 A black substance was observed after high respiratory activity was achieved under respiration-permissive conditions (Johanson et al., unpublished data).

C. Pre-culture. To obtain the desired cell growth, it is important that the pre-culture is of good quality, and can be reproducibly produced. The experimental process used in this work was an adaptation of an industrial procedure, involving some changes. Despite following the procedures described in the literature, lag phases sometimes occurred. The inoculation material (pre-culture) was therefore studied, including the amount transferred to the main culture (C1) and the cell stock material (C2). Furthermore, the impact of the metabolic state of cells harvested at different time points was investigated (C3-C5), and supplementation of haemin at the beginning of the pre-culture (C6).

Before testing different harvest time points prior to inoculation of the main culture, all the pre-cultures had been harvested in the mid-exponential phase. The investigation of the

haemin stability (A1, A2), the cleaning procedure (B1), increasing the inoculation amount (C1) and new cell stock material (C2) did not solve the lag phase problem (Table 5.1). On the other hand, introducing haemin into the static pre-cultures (C6) almost eliminated the lag phase in the subsequent main cultures, as did harvesting in the late-exponential phase (C4). However, harvesting earlier or later led to subsequent lag phase problems (C3, C5).

TABLE 5.1 The factors investigated to determine the cause of the unexpected lag phases upon transferring anaerobic static pre-cultures to respiration-permissive cultivation conditions. The effect of certain actions on the length of the lag phase is given (Data partially unpublished and partially presented in **Paper III**)

Factor considered	**Action**	**Lag phase in main culture (h)**
A. Haemin stability	A1. New haemin supply	7.7 ± 2.6
	A2. Sterilization of haemin by filtration	8.1 ± 2.2
B. Cleaning procedure	B1. Bioreactor cleaned with H_2O_2	5.9 ± 2.9
C. Pre-culture	C1. Increase in amount of pre-culture in main culture: OD 0.04 → 0.06	3.9 ± 1.6
	C2. New cell stock material	9.9 ± 4.3
	C3. Harvest point: Early-exponential phase	3.5 ± 2.0
	C4. Harvest point: Late-exponential phase	1.0 ± 0.5
	C5. Harvest point: Stationary phase	7.5 ± 4.0
	C6. Haemin addition	1.1 ± 0.5

The observation that harvesting the pre-culture in the late exponential phase eliminated the lag phase led to additional physiological studies on the static pre-culture. The extracellular metabolite profiles from the lactose-grown pre-cultures revealed the presence of galactose when lactose was almost completely consumed (Figure 5.2) (**Paper III**). The presence of galactose during lactose consumption correlated with the three harvest points of the pre-cultures:

1. Early-exponential phase: galactose not yet present
2. Late-exponential phase: galactose is excreted while lactose is consumed
3. Stationary phase: lactose is depleted and galactose remains

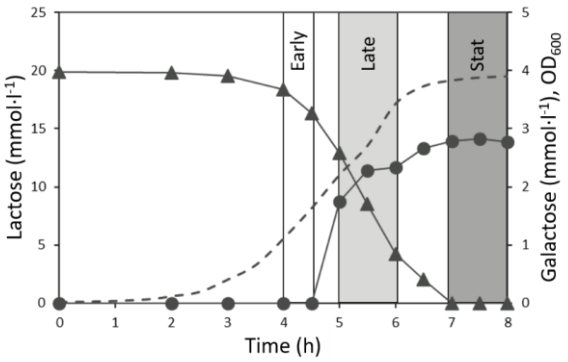


FIGURE 5.2 Galactose excretion in lactose-grown *L. lactis* static flask pre-cultures. Dotted line: growth curve; triangles: lactose concentration; circles: galactose concentration. The three harvest points of the pre-culture are denoted: Early - early exponential phase, Late - late exponential phase, and Stat - stationary phase (Adapted from **Paper III**).

The observations described above suggest that harvesting the pre-culture in the late exponential phase, leading to galactose excretion while lactose is consumed, was associated with short and reproducible lag phases in respiration-permissive main cultivations. In the following sections, the role of galactose in lactose-grown cultures and the effect of haemin addition to the pre-cultures are discussed.

5.2 Galactose excretion in lactose-grown pre-cultures

As mentioned in Section 2.2, the first step after lactose uptake is the hydrolysis of the molecule, resulting in glucose and galactose or galactose phosphate. While glucose readily enters glycolysis, further metabolism of galactose can be less efficient in some strains. The excretion of galactose into the medium during the growth of *L. lactis* on lactose can occur either after dephosphorylation of Gal-6P formed in the tagatose pathway, or after the hydrolysis of lactose by β-galactosidase in the Leloir pathway (Figure 2.4). Galactose excretion is usually explained as being the result of dephosphorylation, followed by an expulsion mechanism (Aleksandrzak-Piekarczyk et al., 2005; Benthin et al., 1994; Neves et al., 2010; Pool et al., 2006). However, a specific Gal-6P phosphatase has not yet been identified. Instead, it has been suggested that an unspecific phosphatase is involved (Neves et al., 2010).

A galactose-lactose antiporter system, which facilitates lactose uptake by concomitant excretion of intracellular galactose, has been described in *S. thermophilus*, which lacks the lac-PTS (Hols et al., 2005; Hutkins & Ponne, 1991). Based on sequence homology from *S. thermophilus*, it has been proposed that the lactose permease LacS in *L. lactis* IL1403 is a putative H^+-lactose symporter or galactose–lactose antiporter (Bolotin et al., 2001). Regardless of whether the excreted galactose originates from dephosphorylation of Gal-6P (tagatose pathway) or the hydrolysis of lactose taken up by the permease (Leloir pathway), the function of LacS as a galactose-lactose antiporter would be a possible explanation of galactose accumulation upon lactose depletion.

5.3 Impact of galactose on the lag phase in main cultures

The observations in the pre-cultures, that both lactose and galactose were present when the inoculum was harvested in the late exponential phase, led to the hypothesis that the length of the lag phase in the main culture is related to the presence of galactose in the pre-culture.

To investigate this hypothesis, controlled bioreactors were used for pre-cultivation, and cells were again harvested at the Early, Late, and Stat time points. The presence of galactose in the pre-cultures and the effect on subsequent main cultures were investigated in two parallel anaerobic cultivations: lactose-grown pre-cultures without pH control (Lac), and cultivation with lactose and added galactose with pH control (pH 6.0) (Lac+Gal+pH). Cells harvested from the Lac pre-cultures showed a similar lag phase pattern to those in the

static pre-cultures described above; the duration of the lag phase depending on the harvest time. Early- and Stat-harvested cells resulted in lag phases of 2.4-4.5 h, whereas Late harvesting led to reproducible and consistently short 1 h lag phases. When galactose was added to the pre-culture and the pH was controlled (Lac+Gal+pH), the lag phases in the main cultures were about 1 h, regardless of the time of harvest of the pre-culture (Table 5.2) (**Paper III**).

TABLE 5.2 The length of the lag phase in main cultivations using inoculum from the different anaerobic pre-cultures at different harvest times (Early - Early exponential phase, Late - Late exponential phase, Stat - Stationary phase) (**Paper III**)

	Lactose-grown		**Lactose-grown with galactose and pH control**	**Lactose-grown with pH control**
	(Lac)		**(Lac+Gal+pH)**	**(Lac+pH)**
Pre-culture harvest point	Inoculum pH	Lag phase (h)	Lag phase (h)	Lag phase (h)
Early (4 - 4.5 h)	6.24 - 6.45	4.5 ± 3.4	1 ± 0.0	5.5 ± 4.2
Late (5.5 - 6 h)	4.97	1.0 ± 0.4	1 ± 0.0	9.8 ± 0.8
Stat (7 - 8 h)	4.72 - 4.77	2.4 ± 2.7	0.8 ± 0.4	6.5 ± 3.5

During Lac+Gal+pH pre-cultures, galactose was consumed after lactose depletion, whereas in the Lac pre-cultures without pH control the galactose remained in the medium (Figure 5.3a and c). In the Lac pre-cultures, galactose excretion was observed as the specific lactose uptake rate decreased, although lactose was still available in the medium (Figure 5.3b). This could suggest a limitation in either the lactose uptake or in lactose metabolism. *L. lactis* CHCC2862 is a lac-PTS-positive strain, and thus exhibits high affinity to lactose (Wood & Warner, 2003), and a limitation in the lactose uptake would thus not be a plausible explanation of the decrease in the specific lactose uptake rate. A possible reason for limitations on lactose metabolism could be a shortage of the glycolytic derivate PEP, which is needed to drive the PTS for lactose assimilation. Galactose excretion started when the extracellular lactose concentration reached 13 mM, and reached a final concentration of 3.3 mM (Figure 5.3a). Assuming equimolarity of the galactose-lactose antiporter mechanism, the excretion of 3.3 mM galactose corresponds to the uptake of 3.3 mM lactose. This suggests that in that Late phase of the pre-culture, when galactose is excreted, lactose permease is responsible for 25% of the residual lactose, while the rest is still internalized via the PEP-dependent lac-PTS transporter.

In Lac+Gal+pH pre-cultures, the specific lactose uptake rate was maintained until the lactose was completely depleted (Figure 5.3d). In order to rule out a possible effect of pH, lactose-grown anaerobic pre-cultures were performed with pH control (Lac+pH) (Figure 5.3e and f). Despite the fact that the pH was controlled, the Early and Stat harvest points of Lac+pH still resulted in long lag phases in the main cultivations (Table 5.2), as was also the case for the Late harvest point. Compared with Lac pre-cultures, the amount of galactose excreted was lower and was rapidly metabolized, and furthermore the lactose uptake rate did not decrease. The above findings show that lower pH, acidic conditions,

have a major effect on cellular physiology; supporting galactose excretion and decreasing lactose uptake.

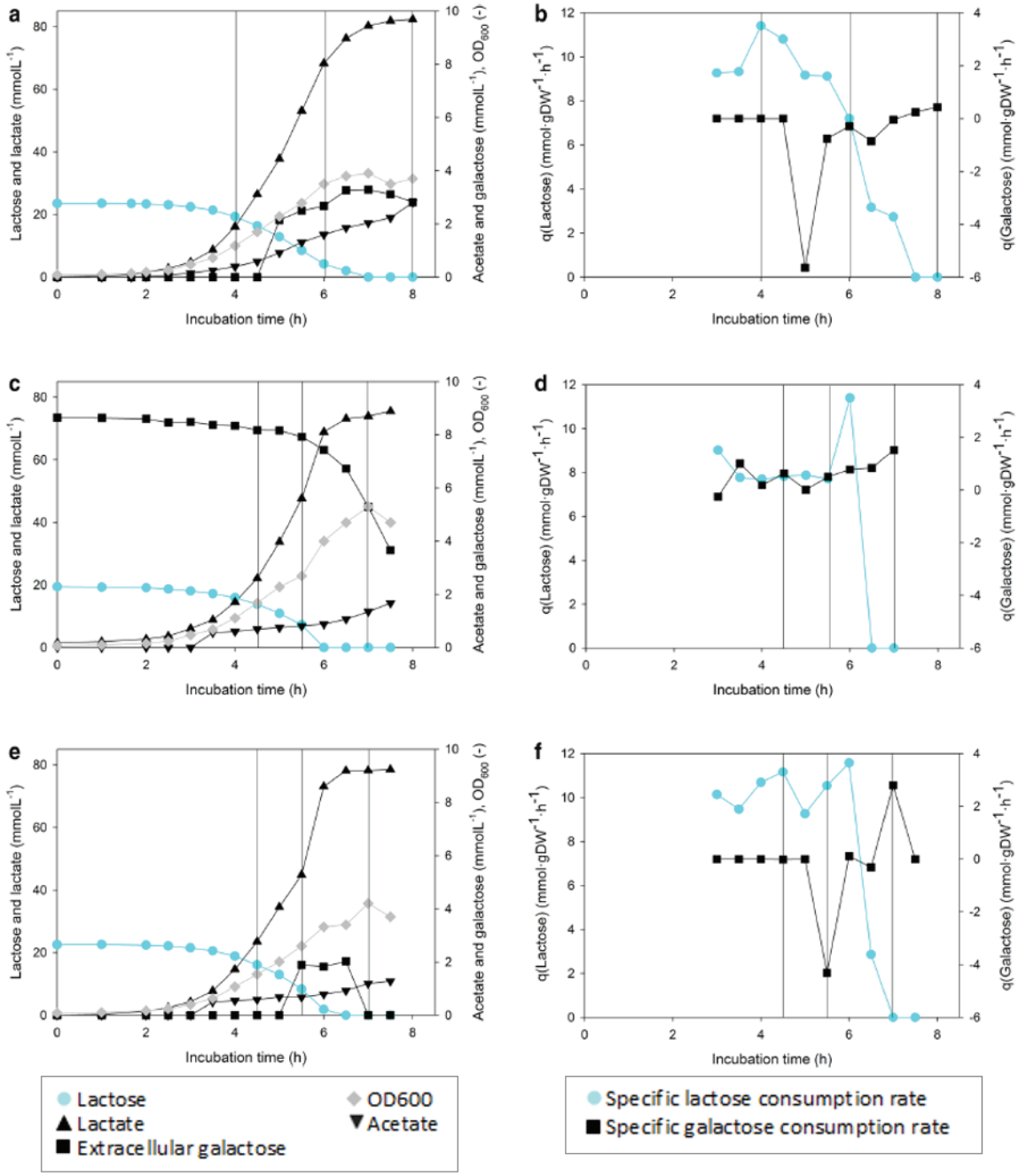


FIGURE 5.3 Pre-culture cultivations performed in controlled bioreactors. Concentration profiles (**a, c, e**) and specific sugar consumption rates (**b, d, f**) in lactose-grown anaerobic pre-cultures without pH control (Lac) (**a, b**), pH-controlled pre-cultures on lactose with the initial addition of galactose (Lac+Gal+pH) (**c, d**), and pH-controlled pre-cultures on lactose (Lac+pH) (**e, f**). The vertical black lines indicate the time of pre-culture harvest (from **Paper III**).

In a previous sequence analysis the galactose permease (*galA*) of *L. lactis* subsp. *cremoris* was found to have 96% identity with the *lacS* of *L. lactis* IL1403, indicating the potential of galactose permease function in LacS (Grossiord et al., 1998). Furthermore, LacS in *L. lactis* IL1403 has been shown to have a higher affinity for galactose than for lactose

(Aleksandrzak-Piekarczyk et al., 2005; Thompson, 1980). The role of LacS in galactose excretion in *L. lactis* CHCC2862 was investigated by gene expression, and together with the observations of galactose and lactose flux in the pre-cultures, the results supported the putative galactose-lactose antiporter function in LacS (**Paper III**).

Gene expression of *lacS* in the Lac pre-cultures differed from that in the Lac+Gal+pH pre-cultures (Figure 5.4). In the Lac pre-cultures *lacS* was expressed at the harvest points Early and Late, where extracellular galactose accumulation also occurs (Figure 5.3a). Stat-harvested cells had a lower *lacS* expression, presumably caused by limitation on galactose uptake due to the decrease in pH after lactose depletion. When galactose was added to the pre-cultures (Lac+Gal+pH) the gene expression of *lacS* increased in cells harvested at all points, compared to the Lac pre-cultures (Figure 5.4). The Stat-harvested cells had an especially high expression of *lacS*. The cells harvested Early and Late were still consuming lactose in the presence of the added galactose, whereas in Stat-harvested cells the lactose was depleted and the added galactose was being consumed (Figure 5.3c). The presence of galactose, together with *lacS* expression, suggests that the extracellular galactose is related to the enhanced *lacS* expression. Furthermore, the high *lacS* expression in the Stat-harvested cells in the Lac+Gal+pH pre-cultures could very well be due to galactose consumption. Examination of the *lacS* gene expression in galactose-grown cultures (aerobic and respiration-permissive conditions) also showed high *lacS* expression, significantly higher than in the cells consuming lactose in the Lac and Lac+Gal+pH pre-cultures (Figure 5.4) (**Paper III**). The strong correlation between *lacS* gene expression and galactose consumption in galactose-grown cultures indicates that LacS plays a role in galactose uptake, and it was therefore proposed that *lacS* also functions as a galactose permease. Furthermore, the short reproducible lag phases together with the presence of galactose and *lacS* gene expression, suggest the potential of extracellular galactose as a biomarker for the transfer of cells to obtain an optimal starter culture production.

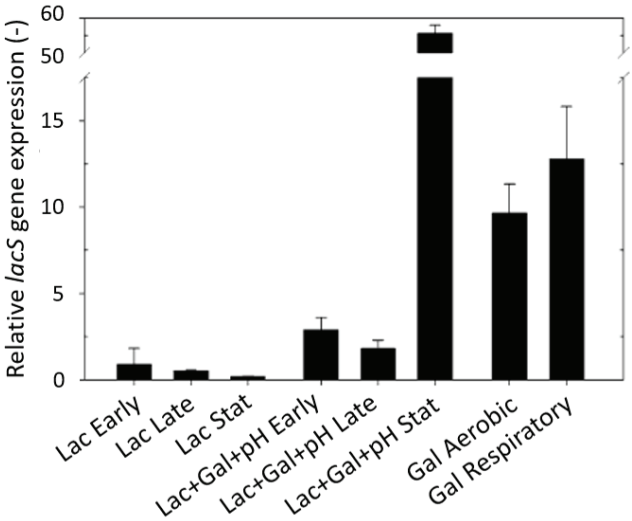


FIGURE 5.4 Relative *lacS* gene expression in samples from lactose-grown anaerobic pre-cultures (Lac), lactose-grown pH-controlled anaerobic pre-cultures initially supplied with galactose (Lac+Gal+pH), at Early, Late and Stat harvest points, and galactose-grown aerobic (Gal Aerobic) and respiration-permissive (Gal Respiratory) cultures (Adapted from **Paper III**).

5.4 Impact of haemin supplementation in pre-culture on lag phase in main cultures

As discussed above, the time of harvest of static lactose-based pre-cultures was found to determine the lag phase in subsequent main cultivations. However, it was also observed that adding haemin at the beginning of pre-culture also led to short and consistent lag phases. This was further investigated in static overnight pre-cultures with haemin, and shaken pre-cultures with haemin, the latter creating respiration-permissive conditions. Both mid-exponential and overnight harvested cells from the static pre-cultures with haemin resulted in a short lag phase in the subsequent main cultures. However, the lag phase in the main culture when using inoculum from the respiration-permissive pre-cultures varied depending on the time of harvest; mid-exponentially harvested cells leading to a short lag phase and overnight cultures to very long lag phase (Table 5.3).

The beneficial effect of haemin in the static pre-culture could be explained by a pre-adaptation feature, preparing the cells for later exposure, and making them ready for respiratory growth in the presence of oxygen. A similar explanation could be applied to mid-exponentially harvested cells in the respiration-permissive pre-cultures, as the cells are transferred to similar conditions, and can continue their respiratory growth. However, overnight cultured cells in respiration-permissive pre-cultures might have been exposed to too much stress, resulting from starvation, aeration and haemin, thereby leading to lower viability and thus fewer cells to continue cell growth.

TABLE 5.3 The length of the lag phase in main cultivations using inoculum from static pre-cultures with haemin and respiration-permissive pre-cultures. Cells harvested at different times mid-exponential phase (Mid) and very late stationary phase, i.e. after overnight cultivations (Overnight) (Johanson et al., unpublished data).

	Static lactose-grown with haemin addition		**Respiration-permissive lactose-grown**	
Pre-culture harvest point	Inoculum pH	Lag phase (h)	Inoculum pH	Lag phase (h)
Mid (6 - 6.5 h)	6.24 - 6.35	1.1 ± 0.5	6.37 - 6.45	0.73 ± 0.9
Overnight (15 h)	4.45 - 4.58	0.8 ± 0.2	5.05 - 5.19	19.8 ± 2.8

To summarize the findings presented in this chapter, microbial growth can be affected by several parameters, and changes in established procedures and conditions may lead to major changes in the kinetics of metabolism and growth. Undesired results, such as long and inconsistent lag phases, lead to practical problems, as well as difficulties in obtaining reproducible data. Possible reasons for these inconsistent lag phases were identified in this work, and two solutions proposed for transferring inoculum to respiration-permissive conditions. The first is to use galactose as a biomarker for when to transfer the cells from the pre-culture, and the second is to add haemin at the beginning of the pre-culture. The main aim of this part of the work was to obtain reproducible results and a short lag phase. The galactose monitoring approach would require either online metabolite measurements, or a very reproducible process, whereas the addition of haemin is a very simple modification of the established process. It was therefore concluded that haemin should be

added to static pre-cultures to ensure short and reproducible lag times in subsequent main cultures.

6. The Production Process, the Metabolic State and Performance of *L. lactis*

The industrialization of fermented foods has had a tremendous effect on the economic importance of lactic acid bacteria as they play a central role in sensory and safety properties. Thus, the reliability of starter cultures is important in terms of both quality, i.e., functional properties, and in terms of growth performance and the robustness of strains during production, i.e., cultivation, preservation (freezing, lyophilization or spray-drying), storage and product handling. Several critical stages during starter culture production can have serious effects on cellular physiology and the performance of the starter culture produced. The production of starter cultures is not only about achieving high biomass yields, as cellular robustness throughout the whole production process is crucial for the final performance in the respective application.

Batch cultivation is often the choice in production, as it has been used traditionally and is a relatively simple setup. At the time of harvest the biomass is separated from the medium by means of centrifugation, resulting in a 10-20 times concentrated slurry. To retain the viability and activity of the bacterial cells, they are frozen or freeze-dried before storage. Both preservation techniques are used in commercial starter culture production, each having advantages and disadvantages (Santivarangkna et al., 2011). Frozen cultures are often prepared by dispersing the concentrated slurry in liquid nitrogen which is a simple process, although it can increase transportation costs, as frozen cultures must be prevented from thawing. Freeze-drying involves freezing followed by drying at sub-atmospheric pressure, and transportation and storage can take place at ambient temperatures. However, the drying step is highly energy-demanding. The choice of preservation process step depends on the desired product formulation and activity, as cellular robustness can vary depending on the process procedures chosen. Not all strains can withstand freeze-drying, and some strains can be very sensitive to storage in one or the other form.

During production, the cells will be subjected to several kinds of stress conditions, which can be chemical, physical or biological in nature. Such stress factors can be of environmental origin, e.g. temperature, pH, substrate concentrations, osmotic pressure, oxygen, antimicrobial components, while others can be self-generated, such as acidity, product inhibition, low nutrient availability or the generation of ROS (Franz & Holzapfel, 2011; Miyoshi et al., 2003; Spano & Massa, 2006; van de Guchte et al., 2002). One of the aims of this work was to evaluate the robustness of *L. lactis* as a starter culture in the small-scale simulation of a production process, to produce frozen and freeze-dried products. The main focus was on the cultivation step, where the cells were propagated under different conditions. The cells were processed according to standardized downstream processing to obtain a final product. The viability and performance of the product was then evaluated.

6.1 Haemin – relief and cause of oxidative stress

As discussed in Chapter 4, *L. lactis* becomes highly stressed by oxygen under aerated conditions. However, in the presence of haemin, i.e., under respiration-permissive conditions, the ETC thus established reduces the intracellular oxygen level, and acid production is reduced. This reduction in cellular stress leads to an increase in biomass yield, and a resistance to oxygen is seen (Duwat et al., 1995, 2001; Gaudu et al., 2002). Haem is an iron-containing porphyrin, facilitating iron solubility and optimizing iron catalytic activity (Kraemer, 2004). The properties of iron as a transition metal, that can cycle between redox states, are maintained in haem, and it is thus a valuable cofactor for biological processes. Respiration of *L. lactis* depends on haem availability in the extracellular environment as *L. lactis* does not possess a complete haem biosynthetic pathway. Other aerobic bacteria, such as *E. coli* and *B. subtilis*, capable of haem biosynthesis, maintain haem at low levels to prevent intracellular toxicity, as haem/haemin can form toxic free radicals that can be deleterious to lipids, proteins and nucleic acids (Alayash et al., 2001; Anzaldi & Skaar, 2010; Gaudu et al., 2003; Rezaïki et al., 2004).

The impact of haemin supplementation was investigated in batch cultivations with different haemin concentrations: anaerobic with 0-5 $mg \cdot l^{-1}$ haemin and aerobic with 0-20 $mg \cdot l^{-1}$ haemin (**Paper I**). Quantitative assessment of the metabolism showed that the metabolic profile was unchanged under anaerobic conditions, but varied under aerobic conditions depending on the amount of haemin (Table 6.1).

TABLE 6.1 Metabolic assessment of batch cultivations under aerobic and anaerobic conditions with different initial haemin concentrations including maximum specific growth rate (μ_{max}), maximum specific oxygen uptake rate (qO_2), and final yields of biomass, acetoin and lactate (Adapted from **Paper I**) †

	Haemin level (mg·l^{-1})	μ_{max} (h^{-1})	$q(O_2)$ (mmol·gDW^{-1}·h^{-1})	Biomass	Acetoin	Lactate
				(C-mol·C-mol^{-1})		
AEROBIC						
Scenario 1	**0**	0.77 ± 0.01	4.7 ± 0.3	0.19 ± 0.00	0.17 ± 0.00	0.17 ± 0.00
Scenario 2	**1**	1.09 ± 0.03	10.0 ± 0.6	0.29 ± 0.01	0.40 ± 0.01	0.40 ± 0.01
	2.5	1.08 ± 0.03	8.8 ± 0.4	0.29 ± 0.01	0.40 ± 0.01	0.40 ± 0.01
	5	1.05 ± 0.01	9.4 ± 0.8	0.29 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
	10	0.94 ± 0.02	8.3 ± 0.2	0.27 ± 0.01	0.39 ± 0.01	0.39 ± 0.01
Scenario 3	**15**	0.83 ± 0.01	7.2 ± 0.6	0.26 ± 0.00	0.31 ± 0.01	0.31 ± 0.01
Scenario 4	**20**	0.90 ± 0.02	5.5 ± 0.6	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
ANAEROBIC						
	0	1.05 ± 0.03	n.d	0.19 ± 0.00	n.d.	0.93 ± 0.01
	2.5	0.95 ± 0.01	n.d	0.18 ± 0.01	n.d.	0.93 ± 0.01
	5	0.85 ± 0.01	n.d	0.19 ± 0.00	n.d.	0.91 ± 0.01

† Values given are the average of 2-4 biological replicate experiments with standard deviation (n.d. - not detected)

Scenario 2 represents the culture conditions with most respiratory behaviour, i.e., high oxygen uptake rate, high acetoin yields and low lactate yields (**Paper I**). Increasing the haemin concentration, as in Scenarios 3 and 4, caused an increase in lactate formation and a decrease in acetoin. Surprisingly, in Scenario 4, a clear metabolic shift was seen from acetoin towards lactate, with a drastic decrease in final biomass yield, almost comparable to Scenario 1, without haemin. In contrast to the metabolic profile, the maximum specific growth rate was affected under both anaerobic and aerobic conditions. Haemin supplementation reduced μ_{max}: in anaerobic cultivations this occurred already at 2.5 mg·l^{-1} haemin, whereas in aerobic cultivations the reduction occurred above 10 mg·l^{-1} haemin.

Haem homeostasis is likely to be the reason for the metabolic and growth changes. However, haem/haemin regulation within *L. lactis* is not completely understood. It has been suggested that haemin diffuses freely across the cell membrane, while haem is more likely to accumulate within the membrane (Anzaldi & Skaar, 2010; Joubert et al., 2014; Lechardeur et al., 2012; Tiburzi et al., 2009). Two competing but independent mechanisms, with opposite effects, have been proposed to be responsible for the regulation of membrane-bound and intracellular haem homeostasis in *L. lactis.* Haem sequestration into membranes is favoured by menaquinone (MQ) and excretion is governed by a haem-specific ATP-permease efflux pump, HrtBA, to prevent toxic build-up in both the membrane and the intracellular compartment (Joubert et al., 2014; Lechardeur et al., 2012; Pedersen et al., 2008).

Menaquinones are essential for respiration as their reducing activity facilitates electron transport within the ETC. However, MQ can also participate in many other redox

reactions, being mediators of metal reduction and generators of ROS (Nohl et al., 2003; Rezaïki et al., 2008; Yamamoto et al., 2006). Under aerated conditions without haemin, ROS can originate from redox reactions involving MQ, ($MQ + O_2 \rightarrow MQ^{\bullet} + O_2^{\bullet}$), which could partly explain the growth inhibition observed during oxygenation (Figure 6.1A). MQ can also reduce haemin to haem, ($MQ + Haemin(Fe^{3+}) \rightarrow MQ^{\bullet} + Haem(Fe^{2+})$), where the MQ becomes reactive and the haem becomes membrane-bound. Reducing conditions have been shown to increase the membrane-bound haem level, which could be the case under anaerobic conditions with haemin. The reduction of MQ-mediated haemin would lead to membrane-bound haem (Figure 6.1B), presumably increasing with rising haemin concentrations. Under respiration-permissive conditions, the reducing activity of MQ is carried out in the ETC preventing haemin reduction by MQ and less membrane-bound haem is accumulated (Figure 6.1C). However, haemin can diffuse into the cytosol, and the counter transport via the efflux pump, HrtBA, then becomes essential to prevent intracellular toxicity. When the haemin concentration crosses a certain level, haem homeostasis seems to be challenged. High haemin concentrations would probably lead to increased intracellular haemin and membrane-bound haem from higher activity with MQ. This would further increase reactive $MQ^{\bullet}$ and decrease MQ available for the electron shuttle in the ETC, thus reducing the oxygen uptake. As a result the $NADH/NAD^+$ ratio would increase, changing the metabolic flux towards lactate and increasing the ATP demand for necessary proton efflux, subsequently reducing growth (Figure 6.1D) (Joubert et al., 2014; Rezaïki et al., 2004; Wakeman et al., 2012).

From the experimental work presented in this thesis, it can be concluded that aerobic conditions make cells more tolerant to haemin addition than anaerobic (**Paper I**). This could be explained by the inhibitory effect of accumulated membrane-bound haem. In Scenario 3 (aerobic with 15 $mg \cdot l^{-1}$ haemin) μ_{max} is 20% lower than in Scenario 2 (haemin 1-10 $mg \cdot l^{-1}$). This is probably simply the consequence of the increased level of haemin, leading to an increase in membrane-bound haem and intracellular haemin. However, the observed increase in lactate and decrease in oxygen uptake could indicate impairment of the ETC, e.g. less NADH oxidation and less oxygen reduction. This could be explained by excess reactivity between MQ and haemin, reducing the availability of MQ to shuttle the electrons in the ETC. At the highest haemin level investigated, 20 $mg \cdot l^{-1}$, Scenario 4, the decrease in μ_{max} was only 13% compared to Scenario 2 (haemin 1-10 $mg \cdot l^{-1}$). This was somewhat surprising, although the metabolic shift towards lactate formation, suggests this could be a strategy to overcome highly stressed environments (Goel et al., 2015).

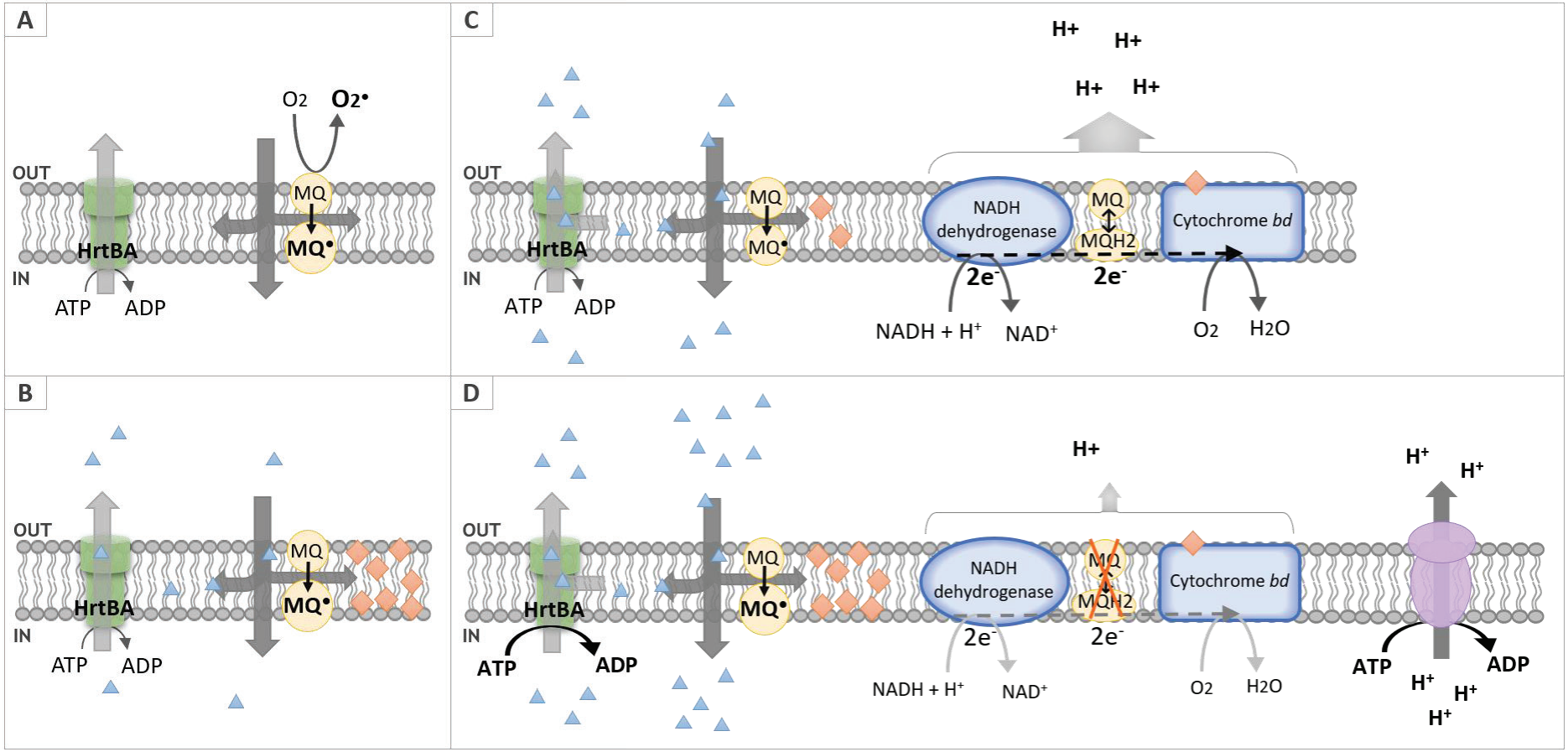


FIGURE 6.1 Suggested haem/haemin regulation in four cases. Generally, added haemin (blue triangles) diffuses into the cytosol. Haemin is either captured by the efflux system, HrtBA, and led back to the medium, or reacts with menaquinone (MQ), and is reduced to haem (orange diamonds) which becomes membrane-bound. A) Aeration without haemin: MQ reacts with oxygen, forming inhibiting reactive species. B) Anaerobic conditions with haemin: MQ reacts with haemin which accumulates. C) Aeration with moderate haemin levels: the reducing activity of MQ is used in the ETC to transport electrons ($2e^-$), less membrane-bound haem is accumulated, and intracellular haemin is counterbalanced by the HrtBA. D) Aeration with high haemin levels: increased reaction between MQ and haemin would result in reactive MQ• and less MQ being available for the electron shuttle in the ETC, reducing the oxygen uptake, increasing the NADH/NAD^+ ratio, changing the metabolic flux towards lactate, and increasing ATP demand for proton efflux (Adapted from **Paper I**).

6.2 Performance of *L. lactis* originating from anaerobic, aerobic and respiration-permissive cultivations

Resistance to freezing and freeze-drying varies between strains, and not all strains are capable of maintaining activity or surviving such treatment (Carvalho et al., 2004; Pyar & Peh, 2011; Savini et al., 2010). In this work, the resistance of *L. lactis* CHCC2862 was evaluated in terms of its ability to acidify standardized milk (**Paper II** and **Paper IV**). Acidification activity was expressed as the ability of the cells to reduce pH, using the specific acidification time, t_{spe} ($min \cdot log(cells \cdot g^{-1})^{-1}$): the higher the value, the lower the acidification activity. *L. lactis* CHCC2862 was found to be robust to both freezing and freeze-drying when cultivated under anaerobic conditions; cells in fermentation broth, frozen product and freeze-dried product showing similar acidification activity (Figure 6.2). Furthermore, the presence of haemin during cultivation had no significant effects on the acidification performance (**Paper IV**). However, when produced under respiration-permissive conditions, frozen and freeze-dried *L. lactis* CHCC2862 differed in their behaviour. Freezing did not affect the performance significantly, and the acidification activities were maintained, and were very similar to, or even better than, under the comparable anaerobic conditions. Freeze-drying, on the other hand, was detrimental to the performance of *L. lactis* CHCC2862 when it had been cultured under respiration-permissive conditions (**Paper II** and **Paper IV**). The freeze-dried products obtained from respiration-permissive batch cultivations with 2.5 and 10 $mg \cdot l^{--1}$ haemin and chemostat cultivations at D =0.1 h^{-1} had significantly lower acidification activity than the corresponding frozen products. These conditions led to a high degree of respiratory growth, and it was therefore speculated that the sensitivity to freeze-drying is related to respiratory growth. Interestingly, in chemostat cultivations at high dilution rates, when the metabolism shifted away from acetoin and towards lactate formation, while maintaining a high biomass yield, the sensitivity of the freeze-dried product was eliminated (**Paper II**). The freeze-dried product actually performed even better than its anaerobic counterpart, and similarly to the freeze-dried product produced under anaerobic batch conditions (Figure 6.2).

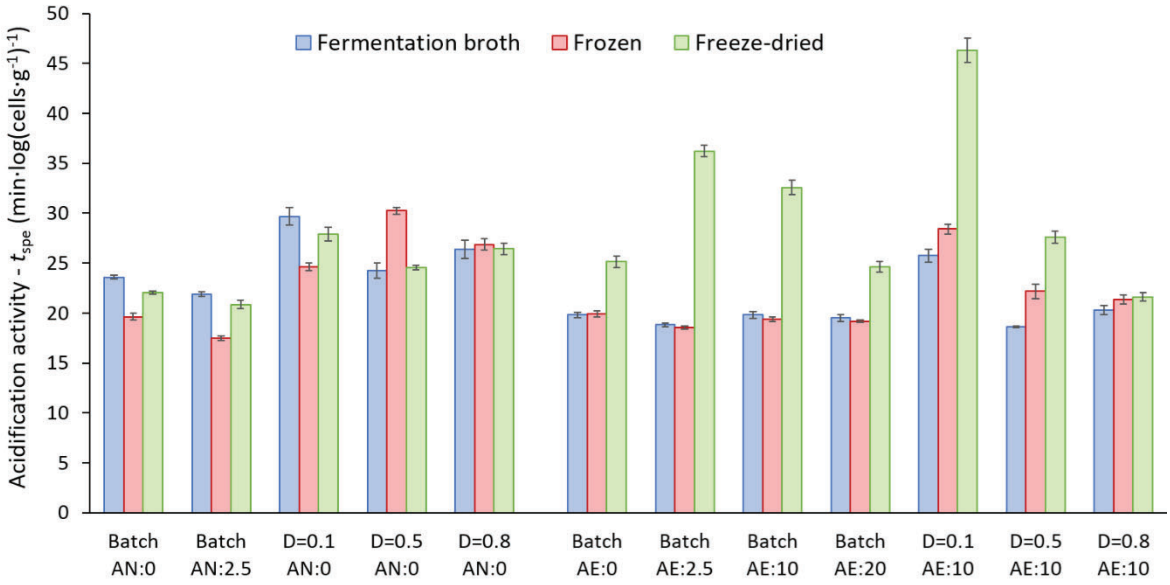


FIGURE 6.2 Acidification activity of cells originating from fermentation broth, frozen product and freeze-dried product, expressed as the specific acidification time (t_{spe}). Anaerobic (AN) batch cultivations without and with 2.5 mg·l^{-1} haemin and chemostat cultivations at three dilution rates: D = 0.1, 0.5 and 0.8 h^{-1}. Aerobic (AE) batch cultivations without and with 2.5-20 mg·l^{-1} haemin and chemostat cultivations with 10 mg·l^{-1} haemin at three dilution rates: D = 0.1, 0.5 and 0.8 h^{-1}. Error bars represent standard deviation, calculated from three and two biological replicates in batch and chemostat cultivations, respectively (Combined figure from **Paper II** and **Paper IV**).

The freezing process converts the liquid cell concentrate into a mixture of ice and solutes, and water is removed from the solution as ice crystals are formed. Extracellular ice crystals are usually formed first, while the cytoplasm remains unfrozen down to -10 to -15°C. Consequently, intracellular water will migrate out of the cell due to the higher extracellular osmotic pressure, dehydrating the cell (Morgan & Vesey, 2009). In the present work, the concentrate of *L. lactis* was frozen rapidly by distributing the cell concentrate drop-wise into liquid nitrogen, presumably avoiding intracellular water loss by rapid freezing of intracellular water (Santivarangkna et al., 2011). Freeze-drying involves two main steps; freezing, as just described, and drying at a pressure below 0.3 mbar. During drying, ice crystals are removed by sublimation, and unfrozen cellular bound water is partly removed by desorption. The low water content leads to a scenario where the suspending medium becomes a "glassy state", which leads to high storage stability (Morgan & Vesey, 2009).

The results of the present study showed that *L. lactis* CHCC2862 cells were robust to freezing. This could be the result of the rapid freezing procedure, as the extra- and intracellular water formed ice crystals, and the water availability is immediately restored after thawing, allowing the cells to become fully functional. In contrast, the cells cultivated under respiratory conditions become sensitive to freeze-drying. It was interesting that in the batch cultivations of *L. lactis* CHCC2862, proline expulsion was observed, i.e. free proline was measured in the fermentation broth at the beginning and end of cultivation (Figure 6.3) (**Paper IV**). The specific proline increase was significantly higher for cells cultivated under respiratory growth conditions, i.e., aeration with 2.5 mg·l^{-1} and 10 mg·l^{-1} haemin, than in the cells cultivated in less respiratory conditions, i.e. aeration with 20 mg·l^{-}

[1] haemin, and aerobic and anaerobic cultivations. No explanation could be found for these observations. However, in a previous study, proline expulsion in stationary phase from *L. lactis* MG1363 was also reported to be associated with respiratory growth (Vido et al., 2004), and it is therefore reasonable to assume that proline expulsion is associated with the respiratory physiology of *L. lactis*.

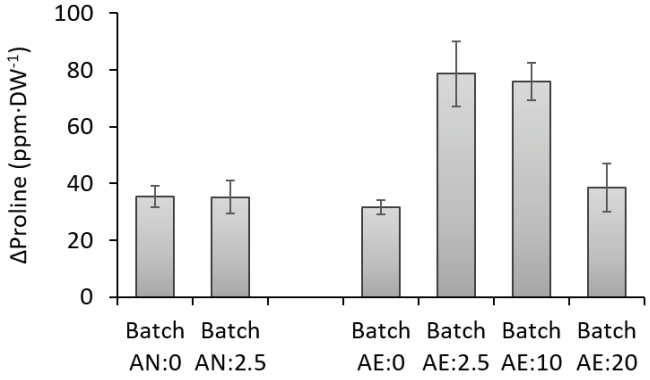


FIGURE 6.3 Increase in proline in fermentation broth during batch cultivations under anaerobic (AN) and aerobic (AE) conditions, with and without haemin addition. The error bars represent the 95% confidence interval obtained from measurements of two biological replicates (**Paper IV**).

Proline is known to be a compatible solute (Molenaar et al., 1993; van de Guchte et al., 2002), and in order for cells to maintain constant turgor pressure, they must respond to environmental changes in the water activity and solute concentration (Morbach & Krämer, 2002).

The freeze-drying sensitivity of the respiratory cells compared with non-respiratory or anaerobic cells may be explained by the difference in exposure to acid stress before the harvest point. Cells exhibiting increased lactate-forming metabolism are exposed to elevated levels of acid stress, which induce cellular robustness against heat, oxidative and osmotic stresses (O'Sullivan & Condon, 1999). Cross-protection by stress responses have been previously employed to design preadaptation approaches for production of robust LAB with improved cell survival and functional properties for later industrial applications (Papadimitriou et al., 2016; van de Guchte et al., 2002). This could explain why the cultures with higher lactate formation are more robust towards freeze-drying.

6.3 High-yield production of robust high-performance starter cultures

The process currently used for the production of *L. lactis* starter cultures involves either anaerobic or respiration-permissive cultivation conditions (Pedersen et al., 2005). Batch cultivations are characterized by dynamic changes during the process, including high substrate concentration and declining specific growth rate towards the end of the cultivation, when the substrate is depleted, and products have accumulated. The cells are often harvested either in the late log phase or early stationary phase. Waiting until the stationary phase improves the tolerance of many microorganism to various kinds of stresses (Morgan et al., 2006).

Exposure to a mild stress may allow the cells to improve their tolerance to an otherwise lethal level of the same kind of stress, as well as providing protection against other forms of stress (Desmond et al., 2002; Leyer & Johnson, 1993; Zhang et al., 2011). The main difference between anaerobic and respiration-permissive growth of *L. lactis* is clearly the metabolic profile, being homofermentative fermentation in the former, and mixed-acid/flavour-forming metabolism in the latter (**Paper I** and **Paper II**). Stress responses are related to metabolic activity and the energy state of the cells. Furthermore, certain intracellular regulators, such as the cytoplasmic pH, and several decarboxylation reactions, favour stress tolerance (Zhang et al., 2011). It is possible that different stress responses are induced depending on the cultivation conditions, and can therefore affect the robustness of the cell to later processing.

L. lactis showed a clear relation between the physiological state of the harvested cells and sensitivity to freeze-drying. The high amount of lactate formed in anaerobic cultivation, leading to high acid stress, could explain the high robustness of the cells to freeze-drying, observed during both batch and chemostat cultivation (Figure 6.2) (**Paper II** and **Paper IV**). The respiration-permissive batch cultivations (with 1-10 $mg \cdot l^{-1}$ haemin) provided a higher biomass yield, but the freeze-dried products showed relatively poor performance (**Paper IV**). This might be explained by lower acid stress, as less lactate was formed. This is also supported by the results of the chemostat cultivations at the lowest dilution rate (D = 0.1 h^{-1}), in which no lactate was formed and the robustness of the freeze-dried product was the poorest of all the conditions tested (**Paper II**). A higher dilution rate resulted in cells that were more robust to freeze-drying; a dilution rate of 0.8 h^{-1} produced the best freeze-dried cells (Figure 6.2). The metabolism at higher dilution rates was similar to the metabolism of aerated batch cultivations with 1-10 $mg \cdot l^{-1}$ haemin, which was partly mixed-acid and partly flavour-forming metabolism. This indicates that the metabolic state at the time of harvest might affect the cell survival later in the production process.

A considerable difference was seen in the physiological state of the cells between those cultivated in batch cultures and in chemostat cultures. At the end of batch culturing, the sugar will have been depleted, and the cells will be in a very slow or non-growing state, whereas at D = 0.8 h^{-1} in chemostat cultures, the cells are growing actively at a high rate. In relation to the before mentioned proline expulsion observation, Vido and co-workers did also report proline expulsion in stationary cells, however, they did not observe it in exponentially growing cells (Vido et al., 2004). This could indicate that proline expulsion does not occur from cells harvested while growing at a high specific growth rate and could lead to a high yield from respiration-permissive cultivation of cells, that are robust also to freeze-drying.

7. Conclusions

There is considerable industrial interest in the respiratory behaviour of lactic acid bacteria as high yield biomass production could potentially be achieved with retained quality of the cells. The research presented in this thesis was undertaken to thoroughly characterize the respiratory behaviour of the model strain *L. lactis* CHCC2862 and its performance as a starter culture. The physiology of the strain and the performance of cells cultivated in both batch and chemostat cultivations, under anaerobic and aerobic conditions, with and without haemin addition, were investigated.

The results of the studies on the respiratory physiology of *L. lactis* provided an important basis for understanding its growth and metabolic behaviour. In batch cultures, the haemin concentration providing the largest beneficial effects of respiratory growth with high-yield biomass production was found to be in the range of 1-5 $mg \cdot l^{-1}$. Haemin addition affected the cellular physiology. At moderate levels, the metabolism remained respiratory with high acetoin and biomass formation and low lactate levels, whereas high haemin levels induced a shift in flux from acetoin towards lactate and lower biomass yield (**Paper I**).

Respiration-permissive conditions in chemostat cultivations led to very low maintenance energy requirements compared to anaerobic conditions. This is probably the main reason for the higher biomass yield in respiratory cultures. Respiration-permissive conditions have also been reported to have several advantages in previous studies, and the present findings confirm the superior performance at high dilution rates, but not necessarily of freeze-dried products obtained at lower dilution rates (**Paper II**).

Two solutions were suggested to solve the problem of inconsistent and long lag phases in respiration-permissive batch cultivation: adding haemin at the beginning of pre-cultivation or harvesting the pre-culture in the late exponential phase. Haemin addition to static pre-cultures almost eliminated the subsequent lag phase in respiration-permissive cultivations, regardless of the pre-culture harvest time (**Chapter 5**). In lactose-grown cultures, galactose was excreted during growth on lactose, and re-consumed after lactose depletion, as long as

there was no inhibition due to low pH. Galactose, internally excreted or added to the medium, resulted in a short and reproducible lag phase in the main cultivation (**Paper III**). The start of galactose excretion and the decrease in the specific lactose uptake rate coincided. Based on this observation, gene sequence homology, and the upregulation of *lacS*, it is suggested that LacS is responsible for the excretion of galactose in *L. lactis* CHCC2862, acting as a galactose-lactose antiporter. Furthermore, higher *lacS* expression during galactose-grown culture indicated that LacS has an additional galactose permease function (**Paper III**).

The performance, in terms of the specific acidification time, of frozen *L. lactis* CHCC2862 was demonstrated to be the same regardless of whether the cultivation conditions were aerobic or anaerobic, and with or without the addition of haemin. The respiration-permissive process was deemed to be the best performing production process for frozen starter cultures due to its higher biomass yield. However, sensitivity to freeze-drying was highly related to respiratory growth, and this was not compensated for by the increase in biomass yield during the cultivation step. Thus, the advantage of respiration-permissive conditions was lost, and anaerobic culture conditions were more favourable for freeze-dried products (**Paper IV**). Harvesting cells at high specific growth rate in respiration-permissive chemostat cultures also led to high performance of freeze-dried cells (**Paper II**), demonstrating that high biomass yield and robustness could be achieved under respiratory conditions.

The findings of the studies presented in this thesis will provide further support in the development of processes for the high-yield production of robust, high-performance starter cultures. This work underlines the importance of systematically studying the upstream and downstream aspects of production processes, as well as potential interactions. An improved understanding of cell physiology and its impact on the final starter culture product is important, as it will provide a deeper insight into the starter culture production process.

8. Future perspectives

These studies were focused on investigating the relation between cultivation conditions and the biomass yield and final performance of starter cultures. In the case of *L. lactis*, respiratory growth was found to improve the biomass yield, but also made the cells sensitive to freeze-drying. Interestingly, the results showed that cells harvested during active growth, i.e., at a high specific growth rate, when cultured under respiration-permissive conditions, led to more robust cells that were better able to withstand freezing and freeze-drying than cells harvested after the exponential growth phase. Further investigations into these findings and the underlying mechanisms may enable the development of process conditions that allow both a high biomass yield and high robustness to freeze-drying. In this regard, it may be interesting to investigate the expression profile during different growth phases and harvest times. Especially the expression of stress response proteins and chaperones that can protect the cells from environmental stress, and the expression of aquaporins that may be essential for the cellular water balance. Improved tolerance to freezing under rapid-freezing conditions has been reported to be related to the presence of aquaporins, and other factors could be at play. The selection of robust (spontaneous) mutants following extended growth and freeze-thaw cycles could also result in more freeze-resistant strain variants better able to survive the freeze-drying process (Tanghe et al., 2006).

Investigation of the biochemical composition of the cell membrane could be another interesting approach, as it has been reported to be correlated to freeze-drying tolerance (Velly et al., 2015). Modifying the cultivation conditions led to changes in the physiological responses at the membrane and cytosolic levels, and further studies could lead to opportunities to improve the robustness of respiratory grown cells by altering the membrane lipid composition, intracellular antioxidants, energy charge and redox level.

The importance of haemin in aerated cultivations was clear from the increase in biomass yield, although too high concentrations had a negative impact. Since no haemin assay was available during the course of this work, the fate of haemin was not empirically

determined, and the mechanisms proposed were instead based on the observed metabolic behaviour. Therefore, analysis of the levels of haem analogues during cultivation, both intracellular and extracellular, could help further our understanding of haem homeostasis and its physiological impact. A deeper understanding of the mechanism of haem homeostasis may, for instance, lead to an addition strategy that could further improve biomass yield and cell robustness.

This work focused on the cultivation conditions. However, it may be useful to reconsider the downstream process parameters and the cryo-additives applied. The choice of cryo-additives in the experimental work was based on suggestions from experienced colleagues and on studies described in the literature. However, the differences observed in metabolism and proline expulsion could imply the need for different cryo-additives applied depending on the cultivation conditions. Cells originating from respiration-permissive conditions may need other combinations to compensate for the decreased lactate-forming metabolism.

Continuous production of starter cultures is unusual. The advantages of continuous culture include high biomass yield and volumetric productivity. Moreover, cells grown in a continuous culture are in a controlled physiological state that can be manipulated via the process parameters. Disadvantages include a higher risk of contamination and an increased risk of changes being introduced into plasmid-carrying cell cultures. A fed-batch cultivation approach that combines the feasibility of batch mode and the cellular robustness of cells growing in a respiration-permissive chemostat might therefore be more appealing. The feeding profile could be controlled by adjusting the growth rate using a carbon-limited feed medium, or by the dissolved oxygen level to obtain the optimal growth. Another approach could be to add haemin continuously to relieve the toxicity of haemin. However, major process changes such as changing the cultivation mode would require significant reconstruction of existing bioreactors and new on-line analytical systems, both of which would be costly. The implementation of a novel process in industrial production is therefore far from straightforward, and the benefits must be quantitatively demonstrated before attempting implementation.

The lack of a comprehensive understanding of the mechanisms behind tolerance to freeze-drying and the reason why respiratory cells are more sensitive remain an obstacle for future process development. The results of this work did not answer the mechanistic questions. However, they showed that cells harvested during high specific growth under respiration-permissive conditions became tolerant to freeze-drying. This provides a good starting point for further investigations into continuous cultivation processes and possible modifications to the traditional batch process. This could pave the way for the development of novel processes with considerable benefits in the industrial production of *L. lactis* starter cultures.

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