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Evolutionary and Metabolic Engineering of *Lactococcus lactis* for Improved Food and Flavor Compound Production

PhD Thesis

Robin Dorau

May, 2020

Thesis Information:

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Submission date:	31.05.2020

Preface

The present thesis entitled “Evolutionary and Metabolic Engineering of *Lactococcus lactis* for Improved Food and Flavor Compound Production” is submitted as part of the requirements for obtaining a PhD degree at the Technical University of Denmark (DTU), National Food Institute (DTU Food). The experimental work was performed in the period June 2017 to May 2020 in the Research Group Microbial Biotechnology and Biorefinery, located at the DTU campus in Kongens Lyngby, Denmark. The work was supervised by Associate Professor Christian Solem and Professor Peter Ruhdal Jensen. During the PhD project, one month was spent at the Research and Development Department of the biotechnology and starter culture company Sacco S.r.l. in Cadorago, Italy. This project was funded by the Danish Dairy Research Foundation and the Innovation Foundation Denmark, as well as DTU Food.

Frederiksberg, May 2020

Robin Dorau

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Summary

Soon after the beginning of agriculture and animal husbandry, lactic acid bacteria (LAB) were used for fermenting milk or other food materials to improve storability and ensure safe consumption. One of these products, which is still of immense importance, is cheese. Nowadays, the fermentation with LAB not only provides food with long lifetimes but also results in delicious products, which are valued throughout the world. Many cheese varieties owe their unique flavors to mesophilic LAB, especially *Lactococcus lactis* (*L. lactis*), which thrive at temperatures below 40°C. For producing hard cheeses, often temperatures higher than 40°C are required, suitable only for thermophilic LAB, which have different flavor forming properties. In this project, the possibility of using the mesophilic *L. lactis* for making hard cheeses was explored. For this, thermotolerant mutants of an industrial *L. lactis* strain (SD96) were created by using a natural selection method, namely adaptive laboratory evolution (ALE). Three mutants were characterized in detail using genomics and transcriptomics analysis. By comparison with the parent strain SD96, mechanisms for overcoming the temperature limitation were identified. Unexpectedly, the mutants were found to be more autolytic, which is beneficial for cheese ripening, and this observation was further investigated. Overall, the thermotolerant mutants are promising candidates for producing hard cheese variants with the unique flavor profile provided by mesophilic LAB. Generally, this project shows that it is possible to obtain thermotolerant mutants of mesophilic LAB with superior industrially relevant properties.

Besides applications for food fermentations, LAB have become increasingly important for the industrial biotechnology sector. Due to their long-term and safe application in food products, many LAB are have Generally Recognized As Safe (GRAS) status and are therefore promising candidates for overproducing ingredients for food or cosmetic products. Moreover, due to their simple metabolism and comparably high robustness, LAB are rising candidates as biocatalysts for producing valuable chemicals using waste streams from the food industry as starting material. Metabolic engineering, by genome editing, facilitates the streamlining of metabolic pathways for converting carbohydrates into desired chemicals. For several decades, such applications had been restricted to a handful of laboratory strains due to the limited availability of genome editing tools. More recent progress in the field, which allowed metabolic engineering of wild LAB, was reviewed in this thesis and provides a comprehensive overview of recent developments in the field. Building on these advances, in the current project, metabolically engineered *L. lactis* were constructed and used for producing large amounts of α-acetolactate from a dairy waste stream. This compound quickly degrades to diacetyl, which provides a rich butter flavor. A two-step bioprocess was developed, combining fermentation and whole-cell biocatalysis, which turned out to be superior to one-step processes solely relying on fermentation. Furthermore, the optimization of this process using permeabilized cells and initial experiments towards using another low-value waste stream from the dairy industry for α-acetolactate production are reported.

Dansk Resume

Kort tid efter at mennesket begyndte at drive landbrug og holde dyr, blev mælkesyrebakterier (LAB, Lactic Acid Bacteria) brugt til fermentering af mælk og andre fødevarer for at forbedre holdbarheden og fødevaresikkerheden. Et af de produkter som blev udviklet, og som stadig er af enorm betydning i dag, er ost. I dag giver syrningen med LAB ikke blot produkter med lang levetid, men resulterer også i smagfulde og delikate produkter, der værdsættes overalt i verden. Mange ostesorter har deres unikke smag pga. mesofile LAB, især *Lactococcus lactis* (*L. lactis*), der trives ved temperaturer under 40°C. Til fremstilling af hårdere ostevarianter kræves ofte højere temperaturer end 40°C, og til dette formål benyttes termofile LAB, hvilket resulterer i oste med en anderledes smag. I dette projekt er det blevet undersøgt om den mesofile *L. lactis* kan benyttes til fremstilling af hårdere ostevarianter. Dertil blev termotolerante mutanter af en industriel *L. lactis*-stamme (SD96) genereret ved hjælp af en naturlig selektionsmetode, nemlig adaptiv laboratorieudvikling (ALE). Tre mutanter blev karakteriseret i detaljer vha. genom-undersøgelser og på transkriptomniveau. Når de temperaturadapterede stammer blev sammenlignet med forældrestammen SD96, resulterede dette i identificering af mekanismer der kan forklare den øgede tolerance for høje temperaturer. I forbindelse med karakteriseringen, blev det fundet, at mutanterne var mere autolytiske, hvilket er en fordel i forbindelse med ostemodning, og denne observation blev yderligere undersøgt. Generelt viser dette projekt, at det er muligt at generere termotolerante mutanter af mesofile LAB, som har overlegne industrielt relevante egenskaber.
Udover fødevareanvendelser så har LAB mange potentielle anvendelser indenfor industriel bioteknologi. LAB er i tusindvis af år blevet anvendt til fødevarefermenteringer, og mange anses derfor som sikre, og er derfor lovende kandidater til produktion af fødevareingredienser og andre stoffer med følsomme anvendelser. På grund af deres enkle metabolisme og høje robusthed er LAB desuden gode kandidater som biokatalysatorer til produktion af værdifulde kemikalier, f.eks. ud fra affaldsstrømme fra fødevareindustrien. Ved at anvende redskaber til genetisk manipulation kan disse mikroorganismer optimeres på en hensigtsmæssig måde således at de bliver mere effektive til at omdanne kulhydrater til de ønskede kemikalier. I mange år har sådanne manipulationer dog været begrænset til en håndfuld af laboratoriestammer, men nutildags er det muligt at optimere på industrielle stammer samt stammer isoleret fra naturen, og nogle af disse teknologier bliver gennemgået i denne afhandling. I nærværende projekt er genetiske redskaber blevet benyttet til at konstruere *L. lactis* stammer til fremstilling af store mængder α–acetolaktat. Denne forbindelse nedbrydes let til diacetyl, som giver en rig smørsmag. En to-trins bioproces er blevet udviklet, der kombinerer fermentering og cellebaseret biokatalyse, og denne proces viste sig at være bedre end en enkelt-trins proces, der udelukkende er baseret på fermentering. Endvidere beskrives optimering af denne proces ved anvendelse af permeabiliserede celler og indledende eksperimenter til anvendelse af en anden affaldsstrøm fra mejeriindustrien til α-acetolaktatproduktion.

Acknowledgments

I would like to begin here by thanking my two supervisors Christian Solem and Peter Ruhdal Jensen, for accepting me as a PhD student into their research group and supporting me in all possible ways throughout the last three years. With their confidence, guidance, and expertise, I could carry out my research projects freely, and thereby explore my scientific interests. They also greatly helped me to identify my goals for the coming years. I feel fortunate that such a well-functioning and balanced team supervised me during this PhD project.

In particular, I would like to thank my principal supervisor, Christian Solem. He has not only been helpful during scheduled project meetings but was available for questions and discussions whenever needed. Christian always saw the best in my results, which was a great motivation.

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List of Publications

Publications in peer-reviewed journals:

1) Dorau R, Chen J, Jensen PR, Solem C. Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis SD96. *Microb Resour Announc* **2020,** 9(3):1–2.

2) Dorau R, Chen J, Liu J, Jensen PR, Solem C. Systems biology approach for understanding thermotolerant *Lactococcus lactis* mutants. (*Manuscript in preparation*)

3) Dorau R, Liu J, Jensen PR, Solem C. Towards single strain starter cultures – characterization of a robust *Lactococcus lactis* subsp. *lactis* strain with superior autolytic properties. (*Manuscript in preparation*)

4) Dorau R, Chen L, Liu J, Jensen PR, Solem C. Efficient production of α-acetolactate by whole cell catalytic transformation of fermentation-derived pyruvate. *Microb Cell Fact* **2019,**18(1):1–11.

Published not peer-reviewed contributions:

5) Dorau R, Liu J, Solem C, Jensen PR. Metabolic Engineering of Lactic Acid Bacteria. In: Metabolic Engineering (Advanced Biotechnology Series). Editors: Lee SY, Nielsen J, Stephanopoulos G. Wiley-VCH Verlag GmbH & Co. (*Manuscript accepted for publication*)

Research articles published during the PhD project but not as part of this PhD thesis:

6) Dorau R, Görbe T, Svedendahl Humble M. Improved Enantioselectivity of Subtilisin Carlsberg towards Secondary Alcohols by Protein Engineering. *ChemBioChem* **2018**, 19(4):338–46.

7) Liu J, Chen L, Dorau R, Lillevang SK, Jensen PR, Solem C. From waste to taste – Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) *Lactococcus lactis* dairy isolate. *J Agric Food Chem* **2020**, 68(21):5891-9.

Outline of the Thesis

This thesis covers the optimization of *Lactococcus lactis* (*L. lactis*) for both food and flavor production using different approaches and therefore comprises two parts.

In the first part, the characterization and optimization of an industrial *L. lactis* strain, which is used for cheese production, are described. Relevant background information is provided in **Chapter 1**, where lactic acid bacteria (LAB), and especially *L. lactis,* are introduced. Special attention was paid to stress response mechanisms and strategies for optimizing LAB for food production. In **Chapter 2**, de novo genome sequencing of the industrial strain *L. lactis* subsp. *lactis* biovar diacetylactis SD96 (SD96 in short) is reported, which serves as a basis for the next two chapters. The optimization of SD96 for growth at high temperatures using adaptive laboratory evolution and the characterization of three improved mutant strains is described in **Chapter 3**. Here a system-wide analysis using a genomics and transcriptomics analysis is presented. Next, further characterization of SD96 and the thermotolerant mutants regarding their autolytic properties is summarized in **Chapter 4**. Part I is completed with summary and conclusion in **Chapter 5**.

Chapter 6 serves as an introduction to the second part. Here, a literature review was conducted describing important recent methods and achievements using LAB as biocatalysts for producing chemicals or food ingredients with a focus on metabolically engineering and genome editing techniques. In line with this topic, in **Chapter 7**, the optimization and application of metabolically engineered *L. lactis* strains for high-level production of α-acetolactate from lactose in dairy waste are described. α-Acetolactate serves as a precursor of the flavor compound diacetyl. The developed two-step process was subjected to further optimization, which is summarized in **Chapter 8**. Therefore, permeabilization was shown to enhance the conversion rates of the whole-cell catalyst even further. Lastly, steps towards the utilization of a citrate-rich waste stream from the dairy industry for producing α-acetolactate using whole-cell biocatalysis are shown. The results and conclusion of Part II are summarized in **Chapter 9**.

An overview of the thesis is shown on the next page.

Overview

Part I – Evolutionary Engineering of *Lactococcus lactis* for Improved Food Production

Chapter 1 Lactic Acid Bacteria as Starter Cultures – Challenges and Opportunities

Chapter 2 Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis SD96

Chapter 3 Systems Biology Approach for Understanding Thermotolerant *Lactococcus lactis* Mutants

Chapter 4 Towards Single Strain Starter Cultures – Characterization of a Robust *Lactococcus lactis* subsp. *lactis* Strain with Superior Autolytic Properties

Chapter 5 Summary and Conclusion of Part I

Part II – Metabolic Engineering of *Lactococcus lactis* for Flavor Compound Production

Chapter 6 Metabolic Engineering of Lactic Acid Bacteria

Chapter 7 Efficient Production of α-Acetolactate by Whole-Cell Catalytic Transformation of Fermentation-Derived Pyruvate

Chapter 8 Boosting α-Acetolactate Production from Pyruvate and Waste-Derived Citrate Using Permeabilized Cells

Chapter 9 Summary and Conclusion of Part II

Part I – Evolutionary Engineering of *Lactococcus lactis* for Improved Food Production

1. Lactic Acid Bacteria as Starter Cultures – Challenges and Opportunities

Parts of this chapter are copied from ***Manuscript 5****: "Metabolic Engineering of Lactic Acid Bacteria."*

1.1 Abstract

Scientific progress is often driven by its technological implications. Microbial biotechnology is one example. For millennia, cheese production has been carried out, relying on spontaneous and uncontrolled fermentation by naturally occurring lactic acid bacteria (LAB). With an increasing demand for consistent high-quality products, the need for understanding and controlling food fermentations involving LAB arose. During the last decades, this need has led to numerous scientific discoveries, and this knowledge has been transferred to industry and has thus benefited companies and scientists alike. The most intensively used LAB, *Lactococcus lactis,* is now well-understood and has become a model organism for LAB. Today, still many open questions exist, which concern, for instance, the response and adaptation to environmental stress, the development of complex bacterial communities during cheese ripening, or how to channel the metabolic flux towards overproducing desired metabolites. This chapter serves as an introduction to LAB and highlights their role in cheese production. Special focus is paid to stress response mechanisms and their relevance during cheese production. Furthermore, methods and examples of how to create superior LAB using natural selection or evolutionary strategies are explained.

1.2 Overview of LAB

(From ***Manuscript 5****: "Metabolic Engineering of Lactic Acid Bacteria")*

Lactic acid bacteria (LAB) are probably the group of microorganisms having the longest history of human use, and one of the main reasons for this is the preservative effect they can impart on various foods fermented with them. A good example is milk, where the preservative effects of LAB have been harnessed for at least 9000 years (1). Most likely, LAB were used unintentionally in the beginning as fermentation occurred spontaneously due to their omnipresence in many ecological niches, ranging from food-related habitats such as fruits, vegetables or milk to mucosal surfaces of higher organisms, where they amongst other places inhabit the gastrointestinal tract

(GIT) and the vagina (2). Their roles and functions are as diverse as the habitats they reside in. Nowadays, they are abundantly found in fermented foods, in particular in dairy products. Of the approximately 850 million tons of milk produced globally in 2018 (3), 400 million tons were fermented using LAB, resulting in products like cheese, yogurt, and fermented butter. In vegetable or cereal fermentations, LAB also play crucial roles and are found in popular products such as sauerkraut, kimchi, or sourdough. In Africa, approximately 800 million people consume cassava daily, and here processing via LAB fermentation also plays an important role in making it safe for human consumption (4). In addition to their industrial importance within the food industry, LAB are important members within the human microbiota, and health benefits have been associated with their presence in various mucosal surfaces. Bacterial counts increase from 10^1 – 10^3 cfu/ml in the stomach or duodenum to 10^4 – 10^7 cfu/ml in the ileum, to ultimately 10^{11} - 10^{12} cfu/ml in the colon (5). Of these bacteria, various fractions are reported to belong to LAB, particularly to the lactobacilli, ranging from 0.5% in the colon to 6% in the small intestine (6,7). In the vagina, the presence of 10^7-10^8 cfu/ml lactobacilli in the mucosal surface is associated with a healthy microflora (6). Driven by the immense relevance for the food and health industry, many efforts towards understanding and optimizing LAB have been undertaken. This has paved the way for more modern synthetic applications within white biotechnology, for example, where LAB are used as production hosts for functional proteins, bio-based chemicals, flavor compounds, and other food ingredients. Many of these novel applications have only been possible due to the development of efficient molecular tools and screening technologies, which have facilitated the basic and applied research necessary for developing LAB that are better suitable for the conditions they encounter in an industrial setting.

1.2.1 General Features and Phylogeny of LAB

LAB is a diverse group of bacteria, with the characteristic feature of producing lactic acid as their main product. Various LAB have a long history of use in food fermentations, particularly within the dairy industry, and these strains have acquired GRAS (generally recognized as safe) status and are considered safe for human consumption. These features make them ideal candidates for producing industrially relevant compounds with sensitive applications such as food ingredients or pharmaceuticals. Furthermore, because of their harmless and in some cases even beneficial nature, LAB have gained attention, as they can be used as delivery vehicles for vaccines or therapeutic proteins for humans via oral administration (8). Industrially relevant genera include *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc,* and *Lactobacillus*, which are phylogenetically related to various extent (**Figure 1.1**). Interestingly, some *Lactobacillus* species only show a quite low similarity, for example, *Lactobacillus plantarum* and *Lactobacillus delbrueckii*. The bacteria in these genera have in common that they are Gram-positive, non-sporulating, low-GC and microaerophilic, and they have comparably small genomes ranging from approximately 1.23 (*Lactobacillus sanfranciscensis*) to 4.91 Mpb (*Lactobacillus*

parakefir). Another common trait among LAB is their fastidious nature, which is most likely a consequence of their niche adaptation (9). Many LAB are auxotrophic for several amino acids, often including cysteine, methionine, histidine, glutamic acid, and the branched-chain amino acids (10,11).

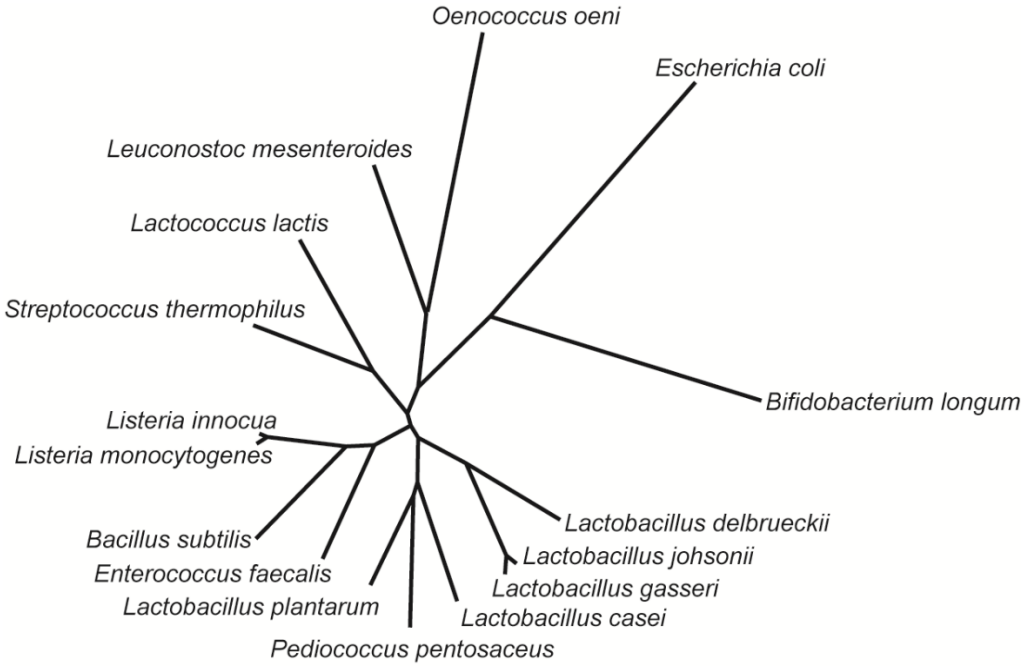

Figure 1.1 16s rRNA-based unrooted phylogenetic tree of representative lactic acid bacteria (LAB) and other relevant bacterial species. This figure is adapted with permission from (12).

1.2.2 Metabolism of LAB

LAB have a relatively simple central metabolism (**Figure 1.2**) with a high glycolytic flux (13). They possess a fermentative metabolism and mainly rely on different sugars to obtain energy through substrate phosphorylation. There are two main ways in which LAB can metabolize sugars: homofermentatively or heterofermentatively (14,15). Homofermentative LAB, represented by lactococci, streptococci, and some lactobacilli, rely on glycolysis (Embden-Meyerhof-Parnas pathway) and almost exclusively produce lactic acid when readily fermentable sugars are metabolized. Heterofermentative LAB, represented amongst others by *Leuconostoc* ssp., most pediococci, and some lactobacilli, to various extents rely on the oxidative pentose phosphate pathway (PPP) in conjunction with a phosphoketolase. In addition to glycolysis, they produce a mixture of lactic acid, acetic acid, ethanol, and CO_2 (16). Obligate heterofermentative LAB lack glycolytic enzymes and fully rely on the oxidative PPP. Under some conditions, especially when metabolizing pentose sugars (for example, arabinose, ribose, and xylose), some LAB rely on the non-oxidative PPP as an alternative to using a phosphoketolase. Surprisingly the latter has also been found for plant-associated *Lactococcus lactis* (*L. lactis*) IO-1 and KF147 strains (17–19) when growing on pentoses, and these *L. lactis* strains also possess a phosphoketolase in contrast to most dairy isolates.

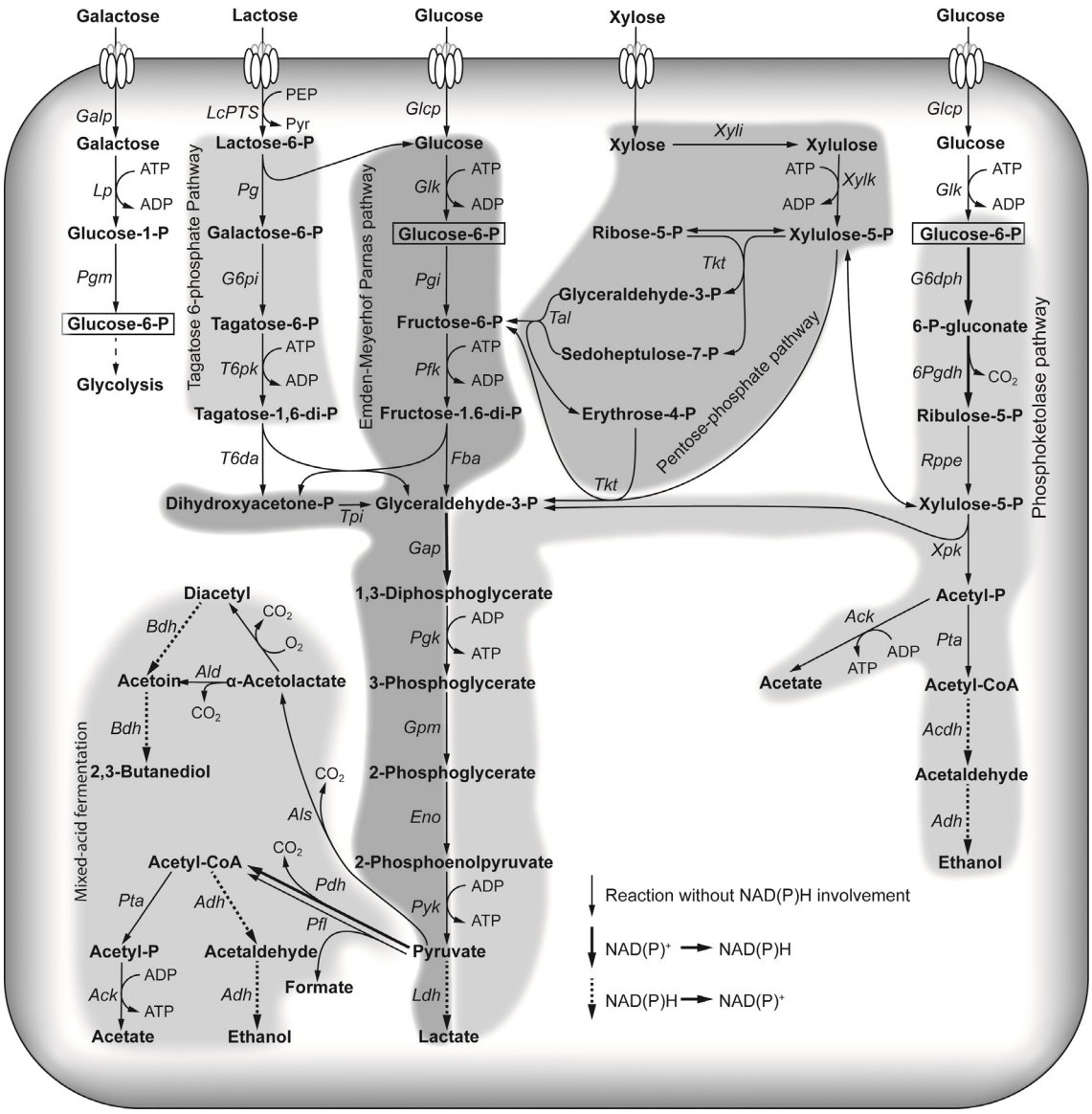

Figure 1.2 Sugar metabolism of lactic acid bacteria. Stoichiometry is not considered for simplification. The following pathways are depicted: Tagatose 6-phosphate-Pathway (TP) for lactose utilization (top-left), Emden-Meyerhof Parnas pathway (EMP) for homofermentative glucose consumption (middle - dark grey) and mixed-acid fermentation (bottom-left), Phosphoketolase pathway (PKP) for heterofermentative glucose consumption (middle - light grey) and the pentose-phosphate pathway (PPP) for pentose utilization (top-right – medium grey). ATP production or consumption is indicated at the respective enzymatic reaction. Redox-cofactor (NAD(P)) involvement is indicated by arrow-type (see legend bottom-right): thin arrow: no redox co-factor involvement, thick arrow: NAD(P)H generation, dashed arrow: NAD(P)H consumption. Enzyme abbreviations are as follows: LcPTS: lactose-PTS, Pg: phospho-β-galactosidase, G6pi: galactose-6-phosphate isomerase, T6pk: tagatose-6-phosphate kinase, T6da: tagatose-1,6-diphosphate aldolase, Glcp: glucose permease, Glk: glucokinase, Pgi: glucose-6-phosphate isomerase, Pfk: phosphofructokinase, Fba: fructose-bisphosphate aldolase, Tpi: triosephosphate isomerase, Gap: glyceraldehyde 3-phosphate dehydrogenase, Pgk: phosphoglycerate kinase, Gpm: phosphoglyceromutase, Eno: enolase, Pyk: pyruvate kinase, Ldh: lactate dehydrogenase, Pfl: pyruvate formate lyase, Pdh: pyruvate dehydrogenase, Pta:

phosphotransacetylase, Ack: acetate kinase, Acdh: acetaldehyde dehydrogenase, Adh: bifunctional alcohol and aldehyde dehydrogenase, Als: α-acetolactate synthase, Ald: α-acetolactate decarboxylase, Bdh: butanediol dehydrogenase, Galp: galactose permease, Lp: Leloir pathway (galMKTE), Pgm: α-phosphoglucomutase, G6dph: glucose-6-phosphate dehydrogenase, 6Pgdh: 6-phosphogluconate dehydrogenase, Rpe: ribulose-5-phosphate 3-epimerase, Xpk: D-xylulose-5-phosphate phosphoketolase, Xyli: xylose isomerase, Xylk: xylulokinase, Tkt: transketolase, Tal: transaldolase.

When growing on slowly fermentable sugars, for some LAB, the metabolism can shift to the "mixed-acid" mode, where a mixture of lactic acid, formic acid, acetic acid, ethanol, and CO_2 are formed, and this fermentation mode also provides a higher ATP yield. The conditions for shifting between the two modes can vary among species or subspecies, and although much research has gone into finding an explanation for the shift, it is still not fully understood (20–22).

In principle, LAB are also able to utilize disaccharides, such as lactose, maltose, sucrose or cellobiose, as carbon and energy sources, but in some cases, the genes required for this ability are located on mobile genetic elements or plasmids, as was demonstrated for the dairy *L. lactis* strains NCDO712 and IL594 (23,24). Consequently, the ability to utilize certain disaccharides is highly strain-dependent. Disaccharides are taken up via permeases or phosphotransferase systems (PTS) and split intracellularly into monosaccharides. As an example, in S*treptococcus thermophilus*, one of the most widely used bacteria in yogurt fermentation, lactose is taken up via lactose permease and split into glucose and galactose. Depending on the LAB, glucose is metabolized either through EMP or phosphoketolase pathway (PKP). Galactose is metabolized via the tagatose-6-phosphate pathway or the Leloir pathway. The former pathway is usually plasmid-encoded, and the latter is located on the chromosome (25).

*(End of the part taken from **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

1.3 Cheese Production

1.3.1 Overview

An abundant variety of cheese exists; however, nearly all types of cheese are made by using similar starting materials and processing steps. Regarding the starting materials, nearly all cheeses are made using four ingredients: milk, LAB, NaCl, and coagulating enzymes (coagulant). The wide variety is introduced by variations during the production process, choice of LAB, coagulant, or milk. Several classification schemes have been suggested during the last decades, all focusing on different aspects of the cheese. For example, cheese can be classified according to the milk used, differentiating mainly between cow's, sheep's goat's, or buffalo's milk. Classification according to the texture, coagulation method, or ripening process has also been suggested, all having their benefits and limitations (26).

All cheeses have in common that the liquid milk is transformed into a solid cheese curd by coagulation, which will then be further processed to become the final cheese product (**Figure 1.3**).

The initial cheese curd is formed due to protein aggregation induced either by protease activity conferred by the coagulant or by denaturation caused through acidification. Protein aggregation is achieved by interrupting the protein emulsion in milk, the casein micelles, resulting in a stable protein gel. The protease activity has traditionally been introduced by the addition of rennet, which is an enzyme preparation extracted from calfs' or cows' stomachs, but nowadays, microbial or recombinant enzymes are used for many products (27). The cheese curd is processed according to the desired final product and usually involves cooking, salting, molding, and ripening in different modes of action. In the next section, the focus will be out on the production of semi-hard or hard cheese variants, including for example Gouda, Cheddar or similar Scandinavian cheese variants, which are all cheeses made from cows milk coagulated by the action of coagulating enzyme, fermented by mesophilic starter cultures, and ripened by residual bacterial activity (26).

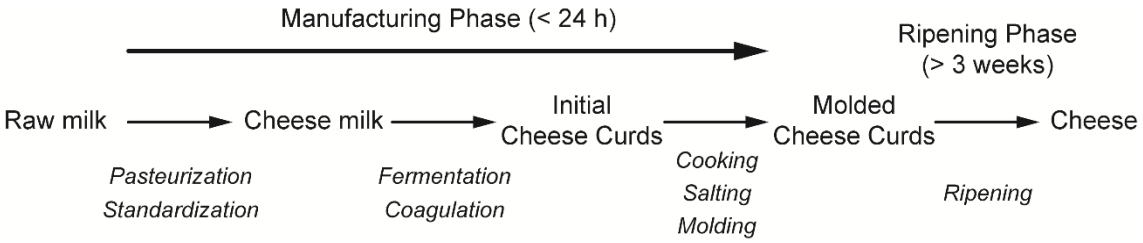

Figure 1.3 Overview of the processes during cheese production.

1.3.2 Production Steps for Producing Renneted Cheese

Preparation of Milk for Cheese Production

As a first step for making cheese, raw milk is standardized and heat-treated to assure consistent quality and microbial safety. Raw milk contains large numbers of microorganisms and is an excellent substrate for supporting microbial growth. This is one of the reasons why cheese making became popular: fermentation with LAB made it possible to obtain a safe and durable product (1). Nowadays, due to the development of pasteurization technologies, milk is a rather safe product (28). In particular, ultra-high-temperature (UHT) treatment efficiently inactivates most pathogens or spoiling microorganisms, but at the same time has a negative effect on bacterial growth, so that UHT treatment is not applied for producing cheese (29). Low-temperature pasteurization, however, removes the most problematic organisms and still allows fermentation and coagulation of the milk. Therefore, in many cases, low-temperature pasteurization is the only pretreatment applied to raw milk before cheese production (30). Alternatively, bacteria and bacterial spores may be removed using a centrifugation step, the so-called "bactofugation" (31). Besides safety considerations, raw milk is subjected to substantial variations resulting in varying cheese qualities. Consequently, before cheese production, milk is often standardized to achieve consistently high quality. Such standardization targets the adjustment of fat, protein, and calcium concentrations

by using, for example, filtration technology, the addition of milk protein concentrate, or $CaCl_2$ (31). Pasteurized and standardized milk is often referred to as cheese milk.

Fermentation

Essential for most cheese varieties is the fermentation with selected LAB. This process is initiated by the addition of starter culture (see 1.4) and is usually finalized within 24 h, but often much earlier, for example, after approximately 5 h for Cheddar cheese or 6-12 h for Gouda. Most importantly, during the fermentation, the cheese milk is acidified by LAB, which metabolize lactose and produce mainly L-lactic acid. Simultaneously, casein is partially degraded and utilized as a nitrogen source by the LAB. Consequently, during the fermentation, the pH of the milk drops from around pH 6.5 to pH 5.2. Besides the significant contribution to the flavor of the cheese, the acidification has several other benefits. First, acidification inhibits the growth of bacteria that are potentially pathogenic or that can cause spoilage. Second, the activity and stability of the coagulant are influenced by acid production, and thereby the coherence of the coagulum is influenced. Third, syneresis of the cheese curd, which is discussed in the next section, is affected by acid production (32). Through fermentation, not only acid is produced, but the starter bacteria also grow, which is essential for the ripening phase and flavor compound production (33). During this early stage of cheese production, the activities and functions of the LAB are rather simple and include acidification and proliferation, but become more complex during later parts of the process, especially during the ripening process (34,35).

Coagulation of Milk Proteins and Curd Syneresis

Simultaneous to the fermentation process, the milk is coagulated by the action of a coagulant, which in most cases is an aspartyl proteinase (36). Coagulation can, however, also be achieved by acidification to pH 4.6 or acidification to pH 5.2 combined with heating to 90°C (32). Coagulation happens in two phases, the primary enzymatic phase and the secondary non-enzymatic phase, which generally overlap (27). To understand the molecular phenomena of coagulation, first, the structure of casein micelles is explained. Four different casein fractions exist α_{s1}-, α_{s2}-, β- and κ-casein, in approximately 4:1:3.5:1.5 ratio, which form a micelle that contains wide pores and channels allowing the incorporation and release of other proteins or chemicals (37–39). κ-Caseins form the outer surface of the micelles and stabilize the macrostructure by pointing their hydrophilic C-termini outward (40). Most aspartyl proteinases acting as coagulants cleave κ-casein at a specific position (Phe_{105}-Met_{106}), thereby releasing the hydrophilic C-terminus. This loss results in destabilization of the micelle and in protein aggregation, which is visible as flocculation. In the secondary phase, the aggregated proteins form a firm gel, finally consisting of strands of micelles around 10 micelles long and 14 micelles thick. This gel is referred to as the cheese curd, where bacteria, fat, and other milk solids are incorporated. The remaining liquid phase is called cheese whey or simply whey (27). During curd formation and processing, a

significant fraction of the lactose is lost in the cheese whey so that at the end of the fermentation, hardly any lactose remains (41).

After coagulation, the cheese curd is cut and heated in order to drain the whey from the gel. This process of whey expulsion is called syneresis and is essential for controlling the moisture content and firmness of the cheese. This is where the differentiation of many cheese varieties has its origin since small changes have a tremendous impact on the final product. Generally, whey expulsion is enhanced when the curd is cut into smaller pieces. High temperature and low pH further enhance syneresis. The temperature increase, referred to as cooking or scalding, is achieved by washing the curd particles with hot water, so that the curd is heated up to temperatures between 30-55°C depending on the desired firmness of the cheese and starter cultures used (42).

The cooking step not only influences the moisture content of the cheese curd but also dramatically affects the LAB. In mixed starter cultures, which are the standard for cheese making, it is expected that the cooking temperature influences the microbial composition as the individual bacteria have different temperature tolerance (43). Generally, LAB are separated into mesophilic and thermophilic LAB, characterized by their optimal growth temperatures. Generally, mesophilic LAB are used for cheeses in which the cooking temperature is kept below 40°C, and thermophilic LAB are used above that threshold. Furthermore, the cooking step may influence the behavior of the starter bacteria due to the induction of gene expression (44). As those phenomena are of great importance, the differences between mesophilic and thermophilic LAB are explained in further detail later on (see **Section 1.4**).

Processing the Cheese Curd

Once the initial cheese curd has been cut and cooked, the solid curd particles are separated from the liquid whey by draining and are transferred into molds. Further whey-drainage can be facilitated by milling and pressing the curd particles into the molds, but often they are just filled in and stick together by gravitational force. At this stage, other treatments may be applied, such as “cheddaring” or kneading and stretching for Cheddar cheese or *pasta filata* cheeses (for example, Mozzarella), respectively (26). Common to most varieties is the last step before ripening: the salting. Salt may be applied in different ways, for example, by directly salting the milled cheese curds or by salting the molded curds either with dry salt or by incubation in a salt solution (brine solution). Salting is another way of facilitating syneresis as the salt binds water.

Moreover, salting contributes to cheese ripening by controlling microbial growth and enzyme activity in the cheese. At the same time, the physical properties of the cheese proteins are affected by salting, thus influencing the texture of the cheese. Salting also inhibits the growth of spoilage or pathogenic microorganisms. Flavor formation by LAB is also affected, as the starter bacteria have different tolerance to salt, and thereby salting can facilitate the growth of individual organisms, which is essential to assure their presence during the ripening phase (45,46).

Cheese Ripening

Essential for all rennet-coagulated cheeses is the ripening phase in which the full cheese aroma unfolds. The time for ripening varies from two weeks to several years. Key to ripening is the presence of bacterial cells through which numerous biochemical reactions are facilitated. Those reactions are either catalyzed by intact bacteria or by enzymes, which were released into the cheese matrix from damaged (lysed) cells. Among the numerous reactions that are important for flavor formation, are proteolysis in combination with amino acid catabolism, lipolysis in combination with fatty acid catabolism, as well as residual lactose and citrate fermentation. All those steps are facilitated by a multitude of enzymes and finally result in the formation of volatile flavor compounds (41). Cellular lysis (or autolysis) of the LAB is essential for cheese ripening, as enzymes, such as peptidases, are released into the cheese matrix, which is crucial for protein degradation and avoidance of bitter peptides (47). Similarly, lipolysis is influenced by the autolysis of the starter bacteria (48). The expression of enzymes necessary for ripening, such as peptidases, differs among LAB, and therefore the biochemical reactions that occur during cheese ripening strongly depend on the present microflora (49).

Hence, the microbial community structure determines flavor formation during cheese ripening. Besides the starter bacteria, so-called non-starter LAB (NSLAB) and other microorganisms such as propionic acid bacteria, molds, and yeast become relevant for flavor development. Those organisms are often introduced to the cheese matrix through contact with the environment in the cheese factory or sometimes added purposely as so-called adjunct cultures (adjuncts in short). Among the NSLAB are mainly salt and heat tolerant *Lactobacillus*, *Pediococcus*, *Enterococcus,* and *Leuconostoc* species, which can grow during the ripening, while starter bacteria stopped growing due to lactose depletion or unfavorable conditions (50). The composition and development of the cheese microflora are strongly dependent on the cheese variety (43). Only with the emergence of next-generation-sequencing, detailed insight into the microbial diversity below the species level was obtained, for example, for understanding undefined starter cultures or microbial community development during cheese ripening (51–53).

1.4 Starter Cultures

1.4.1 LAB in Food Fermentations

*(From **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

LAB are generally associated with nutrient-rich habitats, especially with fermented food products such as cheese, yogurt, sourdough, sauerkraut or fermented meat and fish (54,55). Traditionally, fermentation with LAB was applied in order to extend shelf life, thus allowing for safe consumption of foods after long-term storage. This was important in times when fresh food and cold storage were not available. LAB readily consume free sugars, proliferate quickly, and produce lactic acid until a low pH is reached, typically 4 to 4.5. Some LAB species are able to lower the pH even further and are able to sustain growth at pH values as low as 2.8 to 3.0. And by doing so, these

organisms can prevent other, potentially pathogenic bacteria from spoiling the food products (56–58). Fermentation also enhances the flavor and texture of the final product, which has made fermentation with LAB popular and widespread all over the world (21). Traditional fermented food products mainly rely on spontaneous fermentation by microorganisms introduced *via* the environment or by using a process called backslopping (59). Even though the basic principles behind food fermentations have not changed much, nowadays, the production processes in most cases have undergone dramatic changes, especially at industrial scales. This is mainly driven by the desire to gain better control of the fermentation (60), and spontaneous fermentation has been substituted with controlled fermentation using starter cultures with well-defined composition and function. These starter cultures allow for stable, safe, and scalable production of the desired product. Important species in commercial dairy starter cultures are *L. lactis*, *Streptococcus thermophilus,* and *Lactobacillus delbruekii* subsp. *bulgaricus*. Nonetheless, in some cases, undefined starter cultures are still preferred, as they provide appealing features that cannot yet be matched by defined cultures and that originate from their diverse composition consisting of various strains of *L. lactis* or *Leuconostoc* ssp. (53,61,62). However, carefully characterizing such undefined starter cultures is essential for assuring food safety.
*(End of the part taken from **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

The composition of starter cultures varies significantly according to the desired texture and flavor of the products. Generally, for fast growth in milk, dairy LAB utilize milk proteins, the caseins, as a nitrogen source, and therefore extracellular proteases are required. Not all LAB are required to express those proteases because protease-negative strains can metabolize peptides deriving from protein degradation by protease-positive strains. Since excessive proteolysis can result in bitterness, typically, only a fraction of all strains in a starter culture is protease-positive (63).
Today, starter cultures are usually proliferated and provided by specialized companies, which guarantee a consistent performance of the bacteria. The bacteria are provided in high concentrations, often in frozen or lyophilized form. Two main options for starter culture handling and inoculation are practiced today. First, the frozen or lyophilized starter culture is added directly to the cheese milk (direct vat-inoculation) or, second, the bacteria are amplified at the dairy companies to create a bulk starter, which can be used for several batches of cheese and thereby helps reduce costs. At the same time, the additional amplification step increases the risk of contamination, which is why direct vat-inoculation is considered safer compared to inoculation with a bulk starter.

1.4.2 Mesophilic Starter Cultures

As mentioned earlier, mesophilic starter cultures are used at temperatures below 40°C, with optimal growth temperatures being around 20-35°C. Mesophilic starters are suitable for producing soft, semi-hard, and a few harder cheese varieties for which comparably low cooking

temperatures are used. However, producing very hard cheeses with mesophilic starters is challenging due to the high cooking temperatures needed (26). In almost all mesophilic starter cultures, defined and undefined, *L. lactis* is predominant. Dairy lactococci exist in three distinct phenotypes and belong to two subspecies: *lactis* and *cremoris* (64–66). *L. lactis* subsp. *lactis* is believed to originate from plant environments and has gradually adapted to the dairy environment. Since *L. lactis* subsp. *cremoris* is until now exclusively found in dairy environments, it is believed that this strain has developed from *L. lactis* subsp. *lactis* (67). This process occurred through reductive evolution and horizontal gene transfer during which, for example, amino acid biosynthesis genes were lost or inactivated, and genes for protein degradation were gained (9,68).

Phenotypically, the two subspecies are characterized by their different resistance to temperature and salt stress, as well as their ability to produce ammonia from arginine. More specifically, *L. lactis* subsp. *lactis* can grow at 40°C or in the presence of 4% NaCl, while *L. lactis* subsp. *cremoris* is not able to grow under those conditions. Furthermore, *L. lactis* subsp. *lactis* exclusively produces arginine deiminase, thereby enabling arginine metabolism. Further differences between the two subspecies exist, but they are usually of minor relevance. Confusingly, some strains, for example, *L. lactis* subsp. *cremoris* MG1363, belonging to the *cremoris* subspecies by genotype show a *lactis* phenotype, which has caused many discussions in the scientific literature regarding their taxonomic description (64–66).

The third phenotype, *L. lactis* subsp. *lactis* biovar diacetylactis is distinct from *L. lactis* subsp. *lactis* by its ability to metabolize citrate due to the presence of a plasmid-encoded citrate transporter. This diacetylactis biovariant (biovar) is particularly important as it produces CO_2, and the aroma compounds acetoin and diacetyl, which are responsible for a buttery aroma in many dairy products (64).

In typical mesophilic starter cultures, both *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are represented by one or several strains. Both subspecies are responsible for acid production, while *L. lactis* subsp. *lactis* is considered to acidify faster due to its higher robustness (69). For the same reason, *L. lactis* subsp. *cremoris* is considered to contribute stronger to flavor formation during cheese ripening, as they lyse more efficiently after lactose depletion (33,70). A mesophilic starter culture that consists of those two species only is termed an “O-culture.” They are used, for example, for producing Cheddar cheese (69). *L. lactis* subsp. *lactis* biovar diacetylactis is usually added in concentration to avoid texture defects due to excessive CO_2-formation (71). Since the citrate metabolism is only activated at low pH, it is considered to be important during the later stages of cheese production (72). Another important member of mesophilic starter cultures is *Leuconostoc* spp., which contributes to flavor formation by metabolizing citrate similar to *L. lactis* subsp. *lactis* biovar diacetylactis. *Leuconostoc* spp. usually grow much slower compared to *L. lactis* and therefore do not contribute to acidification but become more active during the ripening phase (61,73). Cultures consisting of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis*

subsp. *lactis* biovar diacetylactis and *Leuconostoc* spp. are called “DL-cultures” and are used among others to produce Gouda cheese and most Scandinavian cheese variants (69).

1.4.3 Thermophilic Starter Cultures

Thermophilic starter cultures are used for cheeses, which are produced by using cooking temperatures above 40°C. Most of them contain *Streptococcus thermophilus* or thermophilic lactobacilli, such as *Lactobacillus delbrueckii* subsp. *lactis* or *Lactobacillus helveticus*, resulting in unique tastes. Other varieties, for example, Emmentaler or Jarlsberg cheese, rely on thermophilic propionibacteria, which provide a nutty and sweet aroma (74). Similar to mesophilic starters, usually, several different strains are found in the starter cultures, but the elucidation of their functions is not as advanced as for mesophilic starters (75). Although their primary metabolism is similar, thermophilic LAB (lactobacilli and *S. thermophilus*) are quite different compared to mesophilic LAB. For example, the presence of lactobacilli during ripening is often accompanied by D-lactic acid formation (71). Furthermore, regarding their proteolytic enzymes, there is a great variety, which provides each strain with unique flavor forming capacities during cheese ripening (76,77). Consequently, thermophilic and mesophilic starters are by no means interchangeable as the resulting products have different tastes.

1.4.4 Phage Resistance of Starter Cultures

Resistance against bacteriophages is an essential property of starter cultures. Since raw milk and cheese production plants usually are not fully sterile, the occurrence of bacteriophages is unavoidable, which potentially causes immense monetary loss due to production failure. Phages are troublesome for both mesophilic and thermophilic starter cultures. Considerable research efforts have been invested in understanding phage host and phage resistance mechanisms (78). This has, among others, led to the groundbreaking discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated genes (Cas) system, which is an adaptive immune system in bacteria (79,80). This discovery has led to a revolution of gene editing technologies applicable to both microorganisms and higher organisms (81).

Starter culture companies and cheese manufacturers have developed methodologies for overcoming or avoiding bacteriophage attacks during cheese production. First, sanitation and proper equipment design are essential for avoiding the transfer of phages from one batch to another. Then, since phages are present in raw milk and in rather high concentrations in whey, appropriate handling of both raw and waste material is crucial. Bacteriophages are quite selective and only infect specific bacteria. Therefore, starter culture rotation, using two or more cultures with complementary phage sensitivity in rotation, is a common strategy for avoiding increasing phage titers. Direct vat-inoculation is less prone to phage attacks compared to bulk starters, as during bulk starter production, the risk of accumulating phages is higher (82). Alternatively, starter cultures based on bacteriophage-insensitive strains or undefined starter cultures with a high strain

diversity, which are usually less prone to phage attacks, might be used (51,62,83,84). Nevertheless, such approaches are never entirely safe, and ultimately, phages may mutate and start causing problems (85).

1.4.5 Stress Physiology of LAB during Cheese Production

For the starter bacteria, cheese production is a journey from one stressful situation to the next (**Figure 1.4**), which will be covered individually in the next sections. Consequently, the stress physiology of LAB and their cellular stress response mechanisms have been studied extensively (86–88). Stress response mechanisms often overlap, which has been demonstrated several times. For example, starved *L. lactis* cultures showed increased resistance against environmental stress compared to control cultures, which were not starved, revealing the existence of a multi-stress resistant phenotype (89). A multi-stress resistant phenotype was also observed when exposing *L. lactis* cells to mildly acidic conditions, followed by exposure to typically lethal stress levels concerning acid, heat, salt, oxidative, and solvent stress (90). Starved or acid-stressed cells showed increased survival against the other stress factors, revealing that a universal stress response was activated. Similar studies confirmed this observation and linked it to nucleotide metabolism and the expression of universal stress molecules such as the alarmone (p)ppGpp (91,92).

Sensing Environmental Changes and Cellular Damage

LAB have several mechanisms for sensing environmental changes and translating them into appropriate cellular responses. For sensing extracellular signals, one-component and two-component systems exist in LAB. The latter is comprised of a membrane-bound histidine kinase, which senses extracellular signals, such as the presence of metal ions or a bacteriocin (93,94). The signal is propagated by phosphorylating a specific cytosolic response regulator protein, which then triggers the respective cellular response, for example, by activating or repressing transcription (95). Besides that, one-component systems are widespread in LAB and function similarly as two-component systems with the difference that the sensor and regulator function are united in one molecule (96). For example, the agmatine pathway is regulated by a one-component system in which AguR senses agmatine in the medium and activates transcription *via* a C-terminal DNA-binding domain (97). For sensing intracellular signals such as the concentration of amino acids or nucleotides, similar systems are in place. One famous example is the transcriptional regulator CodY, which senses branched-chain amino acids in LAB and represses the transcription of genes involved in amino acid metabolism accordingly (98). PurR regulates purine biosynthesis by sensing the intracellular phosphoribosyl pyrophosphate (PRPP) concentration, which is required for purine, pyrimidine, histidine, and nicotinamide coenzyme biosynthesis (99).

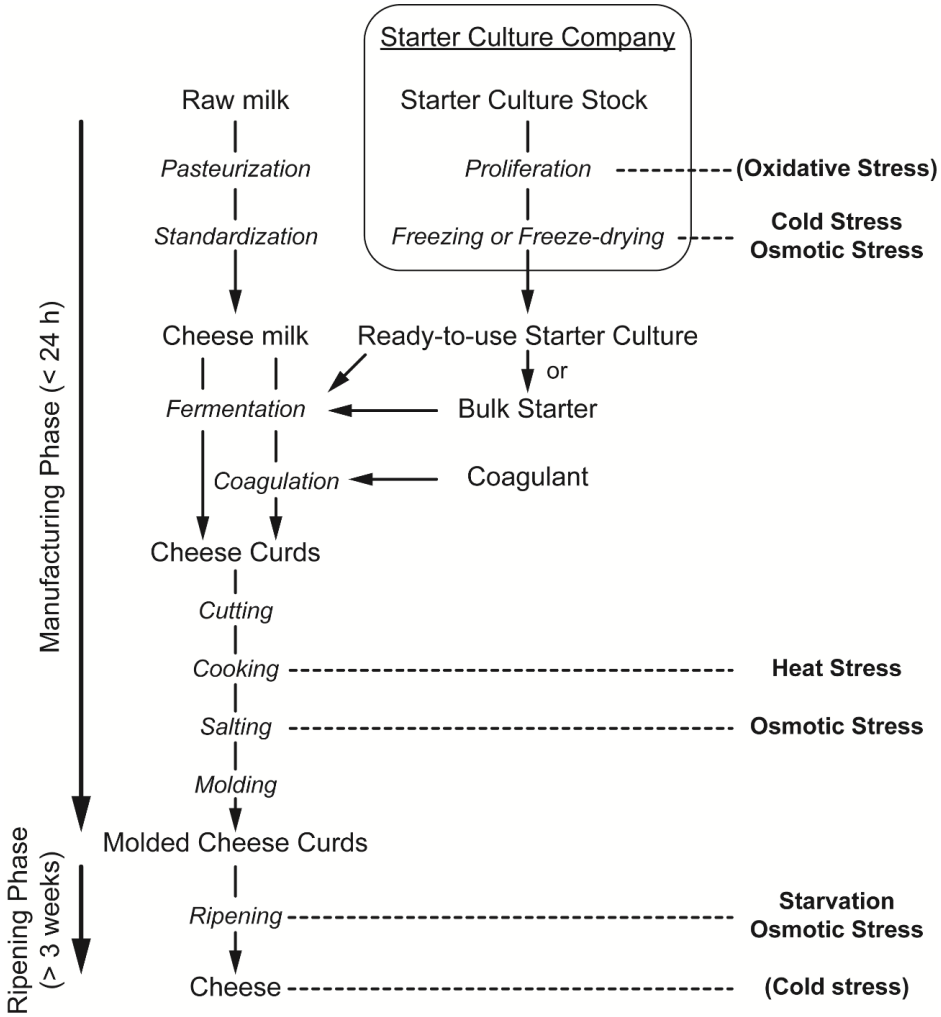

Figure 1.4 Overview of a typical cheese production workflow with *an* emphasis on the environmental stress exposed to the starter bacteria. Starting materials, intermediates, and products are shown in regular font. Processing steps are shown in italic font. Stress imposed on the starter bacteria is indicated in bold font.

Alternatively, other cellular components may act as sensor molecules. The stringent response is a conserved stress response mechanism in bacteria. It is mediated through the accumulation of the alarmone guanosine tetra- or pentaphosphate ((p)ppGpp), which is synthesized by specialized synthetases, called RelA or SpoT. Those synthetases are bound to ribosomes and produce (p)ppGpp when they recognize uncharged tRNAs, which occur during amino acid starvation (100). It has been shown that (p)ppGpp production is also triggered by other factors, for example, during fatty acid starvation (101). The accumulation of (p)ppGpp causes a complete remodeling of bacterial metabolism and physiology, aiming at supporting the pro-survival mechanisms of the cell (102).

On a molecular level, temperature changes are sensed by so-called thermosensors, which can be proteins, or DNA/RNA structures reacting to temperature changes by conformational changes and thereby modulate activities. For instance, in LAB, CtsR is a global transcription repressor,

which binds DNA at low but not at elevated temperatures and thereby regulates the expression of heat-shock proteins (103). Alternatively, temperature changes can be sensed indirectly by recognizing the accumulation of misfolded proteins. Under normal conditions, chaperones mediate protein folding, for example, of heat-shock regulator proteins, which can act as transcriptional modulators. When misfolded proteins accumulate due to stress, the chaperones prioritize the refolding of these proteins, which leads to malfunction of the heat-shock regulator proteins, resulting in activation or repression of transcription (104).

In many bacteria, DNA damage is sensed by LexA and RecA and results in the so-called SOS-response, which is a well-studied stress response mechanism in *Escherichia coli*. In a simplified version, LexA represses transcription by binding to the so-called SOS-box, a DNA motif in promoter regions of affected genes. The second player in the SOS-response is RecA, which senses and binds single-stranded DNA (ssDNA), a sign of DNA damage. The RecA-ssDNA-complex stimulates proteolytic cleavage of LexA and its dissociation from the SOS-box, resulting in the activation of downstream genes (105). The SOS-regulon includes many genes involved in DNA-repair, as summarized previously (106). In *L. lactis,* a protein similar to LexA was identified, HdiR, which functions as a transcriptional repressor and is activated by RecA-mediated self-cleavage. Other than described above, HdiR requires additional processing for full dissociation from the DNA. This processing happens through proteolytic cleavage by ClpP, a protease involved in the degradation of misfolded proteins (107,108).

In the next sections, stress response and alleviation mechanisms are summarized for individual stress situations, and alternative sensing mechanisms are mentioned when appropriate.

Cold Stress Response

LAB are provided by the starter culture companies in either frozen or lyophilized form. For starter cultures, the ability to grow at low temperatures is not essential, as dairy fermentations are usually carried out at temperatures favoring LAB growth. However, the starter bacteria are required to survive the freezing process and then recover quickly and reliably when thawed. Significant optimization of those processes has led to improved survival of LAB after freezing or lyophilization. For example, exposing cultures to mildly stressful conditions (starvation, osmotic, cold, heat or acid) before freezing or freeze-drying is beneficial for increasing the survival rate of LAB, by triggering a general stress response as described above (109).

When exposed to low temperatures, the cellular physiology is affected by two means: membrane fluidity decreases and secondary structures in DNA, RNA, and proteins are stabilized and more rigid. Consequently, transcription and translation efficiencies are reduced, and proteins may malfunction (110). As a counteraction, LAB express so-called cold-shock proteins (CSP) and change the fatty acid composition of the cell membrane (111,112). CSPs are believed to act as transcriptional modulators and RNA-chaperones, thereby facilitating ribosome functionality (113). When drying is involved, the accumulation of compatible solutes, such as betaine or proline, is

beneficial for the survival of the LAB (114,115). These solutes help to avoid dehydration by increasing the intracellular osmolarity avoiding the diffusion of water outside the cell.

Heat Stress Response

As soon as the cheese fermentation is initiated, conditions are favorable for the LAB to achieve fast growth and acid production in milk. The subsequent step, cooking, however, is not to the favor of the LAB. In mesophilic cheese fermentations, the temperature is raised to as high as 40°C, and cell growth is hampered. The higher the cooking temperature, the more stressful the situation is for the LAB. Typically, the temperature is reduced back to 30°C or lower within a few hours, so that the cells recover. In some rare cases, high temperatures are kept for several hours, and the recovery of mesophilic bacteria is dramatically reduced (116).

Other than cold stress, heat stress causes direct damage to macromolecules within the cell. Proteins unfold or even denature, which can have detrimental effects on the survival of the cells. Furthermore, membrane fluidity increases at high temperatures, which may result in altered transport kinetics through the membranes (117). Under heat stress, the expression of chaperones and other heat-shock proteins (HSP) is induced (118–121). Chaperones, such as DnaK, GroEL, or ClpB, mediate protein folding and refolding of denatured proteins (104). Other proteins, such as the membrane-bound metalloprotease FtsH, HtrA, or ClpP mediate removal of misfolded proteins (107,122–124). Changes in the fatty acid composition of the bacterial cell membrane have been observed (117).

Acid Stress Response

After curd formation, cooking, and whey drainage, LAB face a self-imposed new challenge: acid stress due to lactic acid production. LAB are rather acid-tolerant, which confers a growth advantage compared to other competing species. Generally, bacteria keep the intracellular pH (pH_i) more alkaline compared to the medium for guaranteeing proper solute transport and activity of metabolic enzymes (16). *L. lactis* grows optimally at pH 6.3 and can maintain growth at pH 4.5, which contributes to a pH_i of around 5.7 (125,126). Typically, during acidification, a pH gradient between extracellular and intracellular space of 0.6 to 1.4 is maintained in *L. lactis* (127). Below pH 5.0, the cells are damaged transiently (125). When cells are subjected to a rapid extracellular pH drop, the intracellular pH also drops rapidly, showing a flexible and fast acid stress response (128).

Within the acceptable pH range, LAB maintain their pH_i by ATPase mediated proton translocation (129). Generally, increased ATPase activity was detected at lower pH, and accordingly, the survival after a critical acid shock was increased, when the cells had been adjusted to a low pH beforehand. It was also demonstrated that survival depends on the intracellular ATP level, which is maintained through glycolysis (90,130,131). In summary, survival at low pH is rather energy demanding and results in a lower biomass yield (128).

Other mechanisms exist, which are induced at low pH and contribute to maintaining a high intracellular pH either through releasing NH_3 or consuming H^+. First, two three-enzyme deiminase pathways have been described, acting on arginine or agmatine (132,133). In a first step, NH_3 is released from both substrates, thereby increasing the intracellular pH, resulting in citrulline or carbamoyl putrescine, respectively. Next, carbamoyl transferases convert citrulline or carbamoyl putrescine into carbamoyl phosphate and ornithine or putrescine, respectively. In the last step, carbamoyl phosphate is converted by carbamate kinases to NH_3 and CO_2, while ATP is formed from ADP in the same reaction. Ornithine and putrescine are exported in exchange with arginine and agmatine, respectively, making the transport energy independent.

Similarly, decarboxylation reactions contribute to the maintenance of the intracellular pH by consuming intracellular protons for achieving CO_2 release, mediated by several enzymes. Examples for enzymes enabling these reactions are glutamate decarboxylase (GAD) (134), aspartic acid decarboxylase (AspD) (135), and histidine decarboxylase (HDC) (136). The respective substrates, glutamate, aspartate, and histidine, are imported in exchange with the products γ-amino butyrate (GABA), alanine, and histamine, respectively. In *S. thermophilus* and some lactobacilli, extracellular urea can contribute to pH maintenance by producing NH_3 and CO_2 through a rather complex urease system (137,138). As the last example, citrate metabolism is beneficial for the survival of LAB at low pH, because protons are utilized during citrate metabolism. In the first step, divalent citrate is imported in exchange with monovalent lactic acid. Then citrate is converted to acetate and oxaloacetate, which is decarboxylated into pyruvate consuming one proton. The product of this pathway is the non-acidic compound acetoin. This pathway results in the formation of membrane potential due to the electrogenic citrate transport and a pH gradient due to the proton consumption yielding additional energy for survival at low pH (139). Other than the action of ATPase mediated proton export, all those mechanisms depend on the presence of those substrates in the growth medium and are therefore not considered universal acid stress response mechanisms.

Salt Stress Response

Towards the end of the cheese production process, the cheese curds are salted either with crystalline salt or by using a salt solution (brine solution). At this stage, LAB are entrapped in the curd, and fermentation is still ongoing. As the salt is applied at the outside of the curd, most LAB are not in direct contact with the salt. Nevertheless, due to the water-binding capacity, the environment for the LAB changes towards higher osmolarity due to water removal. As mentioned above, also during freeze- or spray-drying, LAB experience water shortage and osmotic stress.

In bacteria, compatible solutes, so-called osmoprotectants, are accumulated in response to osmotic stress. They retain a high intracellular osmolarity so that water does not diffuse outwards (140). In *L. lactis*, mainly glycine-betaine and proline act as osmoprotectants, which were shown to be transported into the cell via a transporter system named OpuA or BusA

(141,142). The transporter system is specific for glycine-betaine or proline-betaine, but also accepts other substrates such as proline with a reduced affinity (143). Not only is OpuA/BusA responsible for solute transport, but also for sensing the osmotic gradient over the membrane by reacting to changes in membrane properties (144). Moreover, the cystathionine-β-synthase domains of OpuA/BusA sense the intracellular ionic strength and surface charge of the membrane *via* an anionic peptide chain (145,146). The expression of the transporter is controlled at the transcription level through the transcriptional repressor BusR, which acts as a sensor for intracellular ionic strength (147,148).

The accumulation of other osmoprotectants, for example, trehalose, was shown to have a positive effect on *L. lactis* regarding their survival of stressful conditions. Trehalose is not naturally synthesized or accumulated by *L. lactis*, and therefore genetic engineering was required to achieve biosynthesis (149,150). It has also been suggested that protein and peptide metabolism may play a role in osmoprotection in LAB by increasing intracellular amino acid concentrations as it has been shown for *B. subtilis* (151,152).

Carbohydrate Starvation Response

At last, during the ripening phase, LAB experience carbohydrate starvation due to the depletion of lactose. Consequently, the fermentation stops and growth of the starter bacteria arrests. Since dairy-associated LAB typically live in a nutrient-rich environment, by nature, carbohydrate starvation does not occur often. As mentioned above, growth would first arrest due to acidification rather than carbohydrate depletion. Since dairy-associated LAB originate from plant sources, where nutrient limitation may occur more frequently, it is not surprising that LAB have many cellular mechanisms for handling carbohydrate starvation (67).

In LAB, carbohydrate metabolism is regulated by the catabolite control protein CcpA, which binds a particular DNA region, the catabolite responsive element (cre), and thereby modulates transcription. The binding is strongly enhanced in the presence of phosphorylated HPr kinase and fructose 1,6-bisphosphate (153). CcpA can either be enhancing or repressing depending on the location of cre in the promoter region. Thereby, many genes involved in the uptake of carbohydrates or amino acids are differentially affected (154). Another important regulatory mechanism is the stringent response, which was already mentioned above. It has been shown that glucose depletion triggers the accumulation of (p)ppGpp depending on the presence of RelA, even if another less-favorable carbohydrate is available. The rapid accumulation of (p)ppGpp results in arrested cell growth, while slow accumulation allows the cells to change to the less-favorable substrate (155).

When starved for carbohydrates for a prolonged time, *L. lactis* become non-culturable but remains metabolically active. The amount of time until this viable but non-culturable state (VNC) is reached, is strain-dependent and varies between one week to three months. Transcription and metabolism in the VNC state are dramatically changed compared to normal growth. Genes

responsible for cell division were repressed, which is the reason for the inability to form colonies. Moreover, genes required for DNA replication, sugar metabolism, autolysis, and cell wall metabolism were repressed. Contrarily, transport systems for oligopeptides remained active, and the transcription factor CodY was induced, which resulted in repression of some peptidases. Notably, some peptidases and enzymes required for amino acid utilization remained active. VNC cells retained their ability to produce ATP from amino acids, such as branched-chain amino acids (BCAA). Similarly, arginine, glutamine, and leucine were utilized for producing ATP (156,157). The mechanisms for arginine and glutamine utilization were described above, and the mechanism for leucine utilization has been described elsewhere (158). Some LAB do not enter a VNC state, but instead, they autolyze when starved for carbohydrates, which is essential for flavor formation during cheese ripening (159–162).

Starter bacteria respond differently to carbohydrate starvation depending on their strain-specific characteristics. Several studies have assessed the fraction of damaged or intact cells during ripening and found that the number of cells with damaged cell walls increased with time (52,163). Interestingly, many starter bacteria survive surprisingly long in the cheese matrix after potentially entering a VNC state (164,165). Another relevant physiological state is the state of near-zero growth in which the LAB are exposed to minimal amounts of carbohydrates in retentiostat cultivations. Here, the LAB remain culturable for a prolonged time, and thereby this state is distinct from the starvation-induced VNC state (166). In *L. lactis* subsp. *lactis* KF147, the transition from regular to near-zero growth was accompanied by a switch from homolactic to “mixed-acid”-fermentation, which is more energy-efficient. Furthermore, the culturability remained high throughout several weeks (167). In this strain, catabolite repression was released during near-zero growth, enabling the consumption of alternative carbon sources. At the same time, amino acid metabolism remained active and only decreased slowly (168). Interestingly, in similar experiments using the dairy strain, *L. lactis* subsp. *lactis* biovar diacetylactis Fm03-V1, a significant fraction of the cells entered the VNC state during retentiostat cultivation and produced cheese-like flavor compounds underlining the importance of this physiological state during cheese ripening (169,170).

Nitrogen Starvation Response

L. lactis and other dairy associated LAB are auxotrophs for several amino acids, which are available in sufficient amounts in milk (11). Nevertheless, LAB might experience amino acid or more general nitrogen starvation in other environments or growth media such as plant materials or waste-derived media with high carbohydrate but low protein concentrations (171).

As mentioned above, the stringent response and the CodY transcriptional regulator are essential for sensing intracellular amino acids, especially branched-chain amino acids. Furthermore, GlnR is involved in sensing and regulating nitrogen availability in LAB, which partly overlaps with CodY regulation (172,173). The global response of *L. lactis* to isoleucine starvation as an example for

amino acid starvation has been described in detail based on transcriptomic and proteomic analysis (174). Upon isoleucine starvation, the growth rate was dramatically reduced, although glucose was still available. The authors found that genes related to cell growth (transcription, translation, and fatty acid metabolism) and the stringent response (carbohydrate and nucleotide metabolism) were strongly reduced during amino acid starvation. Contrarily, the molecular machinery responsible for amino acid supply, modulated by CodY, was induced, including amino acid biosynthesis genes and transporters. The experiment was designed carefully to avoid other secondary stress factors such as acid or temperature stress, and because of that, it is interesting that differential modulation of stress response proteins, such as chaperones and proteases, was observed. More importantly, proteins involved in oxidative stress and aerobic metabolism were enhanced, although no oxygen was present during cultivation (174). Besides their importance for protein biosynthesis, certain amino acids are involved in peptidoglycan structure of the bacterial cell wall (175). Recently, amino acid supply has been linked to the flexibility and stability of the cell wall, and a limited amino acid supply has substantial effects on the cell walls (176,177).

Even though LAB are prototroph for nucleotides, nucleotide starvation may occur due to limited ability for de novo biosynthesis in some strains (99,178). Naturally, milk has low amounts of purine precursors but contains orotate, which is a precursor for pyrimidines (179). Purine starvation has been studied in *L. lactis* MG1363, and several genes involved in de novo biosynthesis were found to be stimulated under purine starvation (180). Interestingly, purine starvation induces a multi-stress resistant phenotype in *L. lactis* (92). Pyrimidine starvation also affects cellular physiology. The genes for UTP biosynthesis are regulated by PyrR, which represses pyrimidine biosynthesis genes when UMP is present, as summarized previously (99). CTP starvation leads to slower growth and the expression of *pyrG*, which catalyzes CTP formation from UTP and is regulated differently compared to the other *pyr* genes (181).

Oxidative Stress in LAB

Commonly, oxidative stress is not encountered frequently during cheese production and is therefore not covered here in detail. It is still an important aspect of LAB physiology as dissolved oxygen and respiratory growth have a substantial impact on industrially relevant parameters such as biomass yield or flavor formation.

*(From **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

As most LAB are anaerobic or facultatively anaerobic, exposure to oxygen or reactive oxygen species can be harmful or inhibitory to bacterial growth. Oxidative stress can be induced by dissolved oxygen present in the growth medium or when fermentation of mixed species is carried out, and one or several of the present organisms produce H_2O_2. Particularly some lactobacilli have been shown to produce H_2O_2 (182). The adverse effects are further made difficult by the lack of self-sustained functional respiration or catalase in many LAB, which quench reactive oxygen species (ROS) or H_2O_2 in other organisms. Despite its fermentative metabolism, it was

shown that in *L. lactis* subsp. *cremoris* MG1363 respiration can be activated when heme is added to the growth medium. Respiration can also provide a growth-stimulating effect under aerobic conditions (183,184). Alternatively, NADH oxidase (Nox) can consume dissolved oxygen and help release oxidative stress (185). By activating and fine-tuning of the respiration of *L. lactis*, Liu *et al*. could show in two cases that aerobic conditions can be extremely beneficial for producing certain chemicals in LAB (186,187). Other LAB besides *L. lactis* are able to respire, but the ability to do so varies with genera, species, and even subspecies as reviewed elsewhere (188).

When the addition of heme is not desired, or the strains lack the required enzymes for respiration, oxidative stress can be a severe problem for LAB, especially when combined with other stress factors such as elevated temperatures (189). Probiotic strains also suffer from oxidative damage during production, storage, and passage through the gastrointestinal tract (GIT). While oxygen tolerance increases the robustness of starter bacteria or probiotic strains, respiring strains are desired as growth rate and yield are usually increased under respiratory growth (190,191).

*(End of the part taken from **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

1.5 Methods for Starter Culture Optimization

1.5.1 Improving Stress Tolerance

*(From **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

During industrial food fermentations, various environmental stress factors can be imposed on the organisms. Amongst others, stress can be imposed by suboptimal changes in temperature, acidity, oxygen, osmotic pressure, or nutrient availability. As the bacterial stress response is complex, and no reliable model exists that can predict beneficial mutations, stress tolerance is usually targeted and improved using random mutagenesis or evolutionary approaches (**Figure 1.5**) (87). Adaptive laboratory evolution (ALE) (**Figure 1.5, A)** has been gaining much attention recently due to the advancements in sequencing technologies and applications have been reviewed extensively (192–194). ALE is one of the most promising and reliable methods to produce desired phenotypes or gain insight into physiological stress responses. The latter is mostly achieved by genome sequencing and in-depth characterization of the obtained mutants.

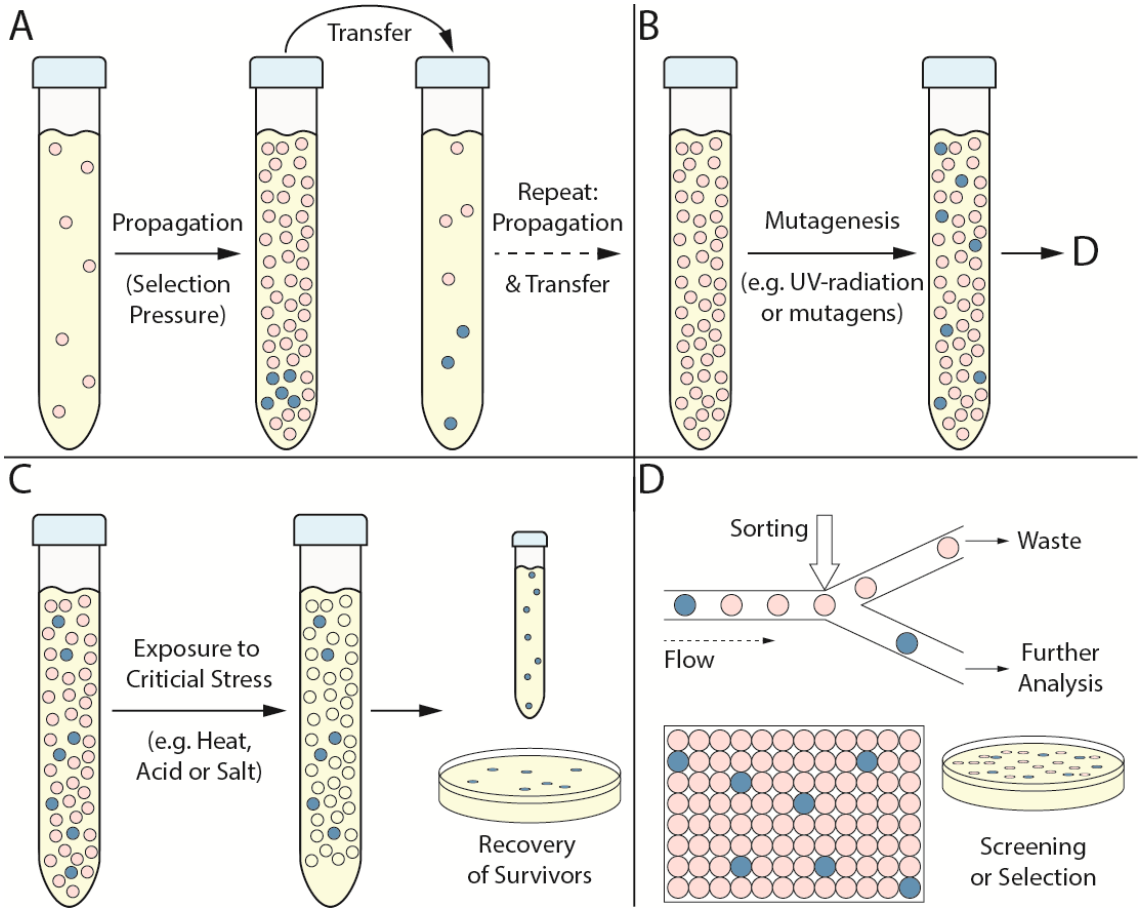

Figure 1.5 Methods for strain development applied to obtain superior mutants, which are not considered to be genetically modified organisms (GMO). A: Principle of adaptive laboratory evolution. During incubation, wild-type bacteria (red) are exposed to stressful conditions such as high or low temperature, high salt, or oxygen. This imposes a selection pressure, which favors the proliferation of spontaneously occurring superior mutant strains (blue). By repeated sequential incubation under increasing selection pressure, desirable phenotypes are obtained. B: Random mutagenesis, for example, induced by UV-radiation, chemical mutagens, or genome shuffling, is powerful but not selective. Nearly all bacteria have mutations after random mutagenesis, and superior mutants (blue) are obtained by screening using various means (D). C: From large or mutagenized populations, superior mutant strains (blue) are obtained but exposing the culture to high stress for a short time, which is adjusted to cause the death of most bacteria (red). Rare survivors are recovered and contain beneficial mutations. D: Screening methods applied for obtaining superior mutants (blue) after random mutagenesis (B) or spontaneous mutation (C). Upper row: Cell or droplet sorting is powerful for screening large populations in a short time. The phenotype must be linked to a fluorescent signal to be suited for sorting. Lower row: Screening methods based on colorimetric assays in microtiter plates or on agar plates.

Furthermore, this approach is particularly suitable for LAB as the resulting strains are considered as non-genetically modified organisms (GMO), which eases their application in food production chains or similar fields. Harvesting the natural diversity of LAB can be an attractive alternative source of microorganisms with high stress-tolerance (195,196). A common phenomenon among

LAB is the occurrence of multi-stress-resistance, which means that resistance to one type of stress often is accompanied by resistance to other stress factors, most likely mediated by expression of chaperone proteins (87,91). Also, when used as probiotics, LAB are exposed to various unfavorable environments during the passage through the gastrointestinal tract. Survival is critical for the probiotic in order to colonize the gut and unfold its beneficial potential.

1.5.2 Heat Tolerance

Heat stress can occur easily in industrial fermentations or during biomass preparation and transport, especially when mesophilic strains are applied. Chen *et al.* used ALE (**Figure 1.5, A**) to increase the thermotolerance of *L. lactis* subsp. *cremoris* MG1363, a well-studied model strain, and obtained a mutant (TM29), which showed increased acidification rates at 40°C, a temperature well above the natural optimum of *L. lactis* subsp. *cremoris*. It was shown that adaptation to an elevated suboptimal growth temperature goes along with an increased expression of chaperones and transporter proteins. Furthermore, it was concluded that the unique thermotolerance of TM29 is caused by many synergistically acting mutations (117).

Other strategies, applying short-term exposure to sublethal high temperatures for up to a few days, can also yield strains with improved survival at high temperatures (**Figure 1.5, C**), which was shown for *L. lactis* subsp. *cremoris* MG1363 and *Lactobacillus helveticus* DSM 20075 (197,198). In both cases, resulting strains were more thermotolerant but, at the same time, more sensitive to salt exposure. The above-mentioned cross-resistance between different sorts of stress can be exploited to isolate strains with desired phenotypes. Dijkstra *et al.* have isolated several mutant strains of a subpopulation of *L. lactis* subsp. *cremoris* SK11 after repeated exposure to heat stress. This subpopulation was shown to be more resistant to heat stress and, at the same time, also to spray-drying, which is a technology used for preparing bacteria for long-term storage (199). Similarly, the adaptation of *Lactobacillus acidophilus* NCFM, a probiotic strain, was shown to be more thermotolerant and tolerant to acid stress (pH 2) after gradual adaptation to surviving a critical temperature for a prolonged time (200).

In most cases, when short term adaptation of LAB was applied (197–200), the survival rates after heat-stress combined with subsequent recovery at the optimal growth temperature were evaluated as a criterion for increased thermotolerance and until now, Chen *et al.* provided the only example for increasing the maximal growth temperature of a LAB using ALE (117). Short term adaptation or the isolation of a subpopulation can result in mutant strains with only one or a few mutations, whereas the long term adaptation of *L. lactis* subsp. *cremoris* MG1363 resulted in a robust thermotolerant strain with many mutations, less readily reverted (117).

Besides the non-targeted approaches to increase the thermotolerance of LAB, rational ones have been carried out successfully mainly by overexpressing heat-shock related proteins. Expression of the small heat shock protein Lo18 from *Oenococcus oeni* ATCC BAA-1163 in *L. lactis* subsp. *cremoris* MG1363 improved survival after short exposure to heat and acid stress (201). Similarly,

homologous overexpression of *groESL* from *Lactobacillus paracasei* subsp. *paracasei* NFBC 338 increased the strains' survival after heat shock and solvent exposure, but not after salt exposure. Heterologous expression of the same protein complex in *L. lactis* subsp. *cremoris* NZ9800 had a similar effect on the strains robustness (202). Heterologous expression of DnaK from *E. coli* JM109, a chaperone protein, in *L. lactis* subsp. *cremoris* NZ9000 allowed growth at 40°C and conferred a multiple-stress tolerance phenotype towards salt, acid, and solvent stress (203). Lastly, as shown by Wu *et al.,* the heterologous expression of RecO, a protein involved in DNA-repair, from *Lactobacillus casei* Zhang in *L. lactis* subsp. *cremoris* NZ9000 also confers multiple-stress tolerance but interestingly not towards heat stress (204). Even though expressing chaperones or heat-shock proteins seems generally beneficial for stress tolerance, the effects can also be specific for certain types of stresses.

When produced as starter cultures, LAB are often exposed to repeated freeze-thaw cycles, which cause the viable cell count to decrease. As a first example, *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 had been exposed to 30 cycles of freezing, thawing, and growing in milk, which resulted in subpopulations with increased survival rates after freezing (205). Similarly, *Lactobacillus rhamnosus* GG has been optimized using ALE by exposing the strain 150 times to freeze-thaw cycles and subsequent growth. This procedure resulted in mutant strains with improved reactivation properties after reactivation from a frozen culture (206).

1.5.3 Acid Tolerance

Particularly for lactic acid fermentation, low pH preferentially below 3.8 is desired, since, under these conditions, lactic acid is in its free form and easily removable, for instance using electrodialysis or ultrafiltration (207,208). Neutralization of the lactic acid is undesired due to the increasing osmotic stress during the process and complicated recovery procedures for the resulting salts (208,209). Lactobacilli are naturally somewhat tolerant to low pH, but optimization is still required for most industrial strains (210). In a landmark study, Patnaik *et al.* have successfully applied genome shuffling using two mutagenized populations (**Figure 1.5, B**) to improve the fermentative capacity of a high-performing *Lactobacillus* strain at low pH without prior knowledge about the genomics or metabolism of this strain. The authors demonstrate an approximately fivefold increased tolerance to protonated lactic acid at pH 3.8 and a twofold increase in lactic acid production at pH 4.0 (211). Other technologies such as ALE have also been applied successfully to increase the tolerance of LAB to low pH or high acid concentrations. *Lactobacillus delbrueckii* Uc-3 (NCIM 5219) has been subject to a series of optimizations including UV mutagenesis (**Figure 1.5, C**), protoplast fusion with *Acetobacter pasteurianus* (NCIM 2314) and ALE, resulting in an acid-tolerant strain, which was shown to produce up to 50 g/L lactic acid without the need to adjust the pH, which greatly simplifies down-stream processing (209). *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3718 has been adapted for one year to tolerate high concentrations (70 g/L) of lactic acid at pH 6.5 and was shown to produce up to

76.8 g/L in a batch fermentation using pH control (212). In this example, the strain has been adapted to tolerate high salt concentrations rather than low pH, which is typically referred to as acid stress. Nevertheless, this study addresses the importance of considering that in prolonged fermentation, the strains eventually suffer from both acid stress and osmotic stress caused by high product concentrations.

An exciting approach combining rational strain engineering and ALE was presented by Overbeck *et al*. (213). By harvesting the knowledge of previous ALE experiments (214), two deletion strains were created based on *Lactobacillus casei* 12A and ATCC 334 that each had a deletion in the *mutS* gene conferring a hyper-mutator phenotype. These hyper-mutator strains were used in a comparative ALE experiment towards increasing lactic acid resistance at low pH. Simultaneously, the hyper-mutator and wild-type parent strains were carried through the ALE experiment. Subsequently, the *mutS* mutations were reversed, and the resulting strains were compared. In both cases, pH-tolerant strains were obtained, but as anticipated, the hyper-mutator strains resulted in superior tolerance compared to the mutants deriving from the wild-type starting strains (213). This study demonstrates that ALE can efficiently be accelerated using hyper-mutator strains as starting points.

1.5.4 Oxygen Tolerance

(A section was removed and inserted in ***Section 1.4.5*** **- Oxidative Stress in LAB**)

Similar approaches, as described above, have been applied to increase the tolerance towards oxidative stress in LAB. Spontaneous mutants of *L. lactis* subsp. *cremoris* MG1363 were obtained through selection after exposure to H_2O_2 (**Figure 1.5, C**). These mutants were able to survive longer in aerated conditions and in co-culture with *Lactobacillus delbrueckii* subsp. *delbrueckii*, an H_2O_2-producing strain (215). In a similar experiment, DTT-resistant mutants of *L. lactis* subsp. *cremoris* MG1363 were shown to exhibit resistance to oxygen and the reducing agent DTT as well as resistance to copper and zinc. The conferring mutations were shown to be linked to the inactivation of a phosphate transporter (*pstFEDCBA*). Another indirect way of obtaining oxygen resistant LAB was demonstrated using the dairy strains *L. lactis* subsp. *lactis* IPLA947. Adaptation applying increasing concentrations of the bacteriocin Lcn972, which causes cell envelope stress, resulted in a stable mutant with increased resistance to H_2O_2. Chen *et al.* adapted and isolated a mutant strain of *L. lactis* subsp. *cremoris* MG1363, in which *trxB2* was deleted coding for a ferredoxin reductase essential to aerobic growth. Consequently, the mutant strain grew very poorly under aerobic conditions. Subsequently, via ALE, this phenotype could be reversed, thus reestablishing a mechanism for releasing oxidative stress (216).

By taking advantage of the natural diversity of LAB, it has been possible to find strains with high oxygen tolerance and the ability to respire. Firstly, a selection of 76 strains comprising *Lactobacillus*, *Leuconostoc,* and *Weissella* strains was screened for aerobic growth and respiration, when heme, menaquinone, or both were added. Only for a few strains (*Lactobacillus*

brevis E06 and E31, *Weissella minor* E14), heme supplementation improved the growth capacity, and generally, a great diversity was observed between the different strains, also indicating that various mechanisms for releasing oxidative stress exist in LAB (190). In one study where the focus was on two probiotic species of LAB *(Lactobacillus johnsonii* and *Lactobacillus gasseri*), 34 strains were screened for their oxygen tolerance and respiratory growth. From this collection, three candidates were obtained that displayed engaging probiotic activities and were more tolerant to oxidative stress, partly because they produced catalase. The authors claim that such strains could be produced more efficiently on a large scale due to their respiratory capacity (191).
*(End of the part taken from **Manuscript 5**: "Metabolic Engineering of Lactic Acid Bacteria")*

1.6 References

1. Kindstedt PS. The history of cheese. In: Global Cheesemaking Technology: Cheese Quality and Characteristics. Editors: Papademas P, Bintsis T. John Wiley & Sons Ltd. **2017**, 3–19.
2. Lahtinen S, Ouwehand AC, Salminen S, Von Wright A. Lactic Acid Bacteria - Microbiological and functional aspects. Editors: Lahtinen S, Ouwehand AC, Salminen S, Von Wright A. CRC Press, Taylor & Francis Group **2012**, 779 p.
3. Food and Agriculture Organization on the United Nations (FAO). Dairy Market Review - Overview of global dairy market developments in 2018. **2019**.
4. Food and Agriculture Organization on the United Nations (FAO). Fermented fruits and vegetables. A global perspective. **1998**.
5. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* **2006**, 7(7):688–93.
6. George F, Daniel C, Thomas M, Singer E, Guilbaud A, Tessier FJ, Revol-Junelles AM, Borges F, Foligné B. Occurrence and dynamism of lactic acid bacteria in distinct ecological niches: A multifaceted functional health perspective. *Front Microbiol* **2018**, 9:1–15.
7. Heeney DD, Gareau MG, Marco ML. Intestinal *Lactobacillus* in health and disease, a driver or just along for the ride? *Curr Opin Biotechnol* **2018**, 49:140–7.
8. Del Rio B, Redruello B, Fernandez M, Cruz Martin M, Ladero V, Alvarez MA. Lactic acid bacteria as a live delivery system for the in situ production of nanobodies in the human gastrointestinal tract. *Front Microbiol* **2019**, 9:1–16.
9. Bachmann H, Starrenburg MJC, Molenaar D, Kleerebezem M, Vlieg JETVH. Microbial domestication signatures of *Lactococcus lactis* can be reproduced by experimental evolution. *Genome Res* **2012**, 22:115–24.
10. Teusink B, Molenaar D. Systems biology of lactic acid bacteria: For food and thought. *Curr Opin Syst Biol* **2017**, 6:7–13.
11. Chopin A. Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiol Rev* **1993**, 12:21–37.
12. Boekhorst J, Siezen RJ, Zwahlen MC, Vilanova D, Pridmore RD, Mercenier A, Kleerebezem M, de Vos WM, Brüssow H, Desiere F. The complete genomes of *Lactobacillus plantarum* and *Lactobacillus johnsonii* reveal extensive differences in chromosome organization and gene content. *Microbiology* **2004**, 150(11):3601–11.
13. Koebmann BJ, Andersen HW, Solem C, Jensen PR. Experimental determination of control of glycolysis in *Lactococcus lactis*. *Antonie Van Leeuwenhoek* **2002**, 82(1–4):237–48.

14. Sun Z, Harris HMB, McCann A, Guo C, Argimón S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF, Liu W, Song Y, Salvetti E, Wrobel A, Rasinkangas P, Parkhill J, Rea MC, O'Sullivan O, Ritari J, Douillard FP, Paul Ross R, Yang R, Briner AE, Felis GE, De Vos WM, Barrangou R, Klaenhammer TR, Caufield PW, Cui Y, Zhang H, O'Toole PW. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nat Commun* **2015**, 6:1–13.
15. Douillard FP, de Vos WM. Functional genomics of lactic acid bacteria: From food to health. *Microb Cell Fact* **2014**, 13(S8):1–21.
16. Endo A, Dicks LMT. Physiology of the LAB. In: Lactic Acid Bacteria - Biodiversity and Taxonomy. Editors: Holzapfel WH, Wood BJB. John Wiley & Sons, Ltd **2017**, 13–30.
17. Petersen KV, Liu J, Chen J, Martinussen J, Jensen PR, Solem C. Metabolic characterization and transformation of the non-dairy *Lactococcus lactis* strain KF147, for production of ethanol from xylose. *Biotechnol J* **2017**, 12:1–10.
18. Tanaka K, Komiyama A, Sonomoto K, Ishizaki A, Hall S, Stanbury P. Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. *Appl Microbiol Biotechnol* **2002**, 60:160–7.
19. Shinkawa S, Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H, Kondo A. Improved homo L-lactic acid fermentation from xylose by abolishment of the phosphoketolase pathway and enhancement of the pentose phosphate pathway in genetically modified xylose-assimilating Lactococcus lactis. *Appl Microbiol Biotechnol* **2011**, 91(6):1537–44.
20. Goel A, Eckhardt TH, Puri P, de Jong A, Branco dos Santos F, Giera M, Fusetti F, de Vos WM, Kok J, Poolman B, Molenaar D, Kuipers OP, Teusink B. Protein costs do not explain evolution of metabolic strategies and regulation of ribosomal content: Does protein investment explain an anaerobic bacterial Crabtree effect? *Mol Microbiol* **2015**, 97(1):77–92.
21. Smid EJ, Kleerebezem M. Production of Aroma Compounds in Lactic Fermentations. *Annu Rev Food Sci Technol* **2014**, 5(1):313–26.
22. Neves AR, Pool WA, Kok J, Kuipers OP, Santos H. Overview on sugar metabolism and its control in *Lactococcus lactis* - The input from in vivo NMR. *FEMS Microbiol Rev* **2005**, 29:531–54.
23. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **1983**, 154(1):1–9.
24. Górecki RK, Koryszewska-Bagińska A, Gołebiewski M, Zylińska J, Grynberg M, Bardowski JK. Adaptative potential of the *Lactococcus lactis* IL594 strain encoded in its 7 plasmids. *PLoS One* **2011**, 6(7):1–12.
25. Aleksandrzak-Piekarczyk T, Kok J, Renault P, Bardowski J. Alternative lactose catabolic pathway in *Lactococcus lactis* IL1403. *Appl Environ Microbiol* **2005**, 71(10):6060–9.
26. Fox PF, Ottogalli G, McSweeney PLH. Diversity and Variety of Cheese: An Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 781–808.
27. Horne DS, Lucey JA. Rennet-Induced Coagulation of Milk. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 115–43.
28. Cunliffe HR, Blackwell JH, Dors R, Walker JS. Inactivation of Milkborne Foot-and-Mouth Disease Virus at Ultra-High Temperatures. *J Food Prot* **1979**, 42(2):135–7.
29. Stulova I, Kabanova N, Kriščiunaite T, Laht TM, Vilu R. The effect of milk heat treatment on the growth characteristics of lactic acid bacteria. *Agron Res* **2011**, 9:473–8.
30. Kelly AL, Huppertz T, Sheehan JJ. Pre-treatment of cheese milk: Principles and developments. *Dairy Sci Technol* **2008**, 88:549–72.
31. Panthi RR, Jordan KN, Kelly AL, Sheehan JJD. Selection and Treatment of Milk for Cheesemaking. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 23–50.

32. Fox PF, Mcsweeney PLH. Cheese: An Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 5–21.
33. Crow VL, Coolbear T, Holland R, Pritchard GG, Martley FG. Starters as finishers: Starter properties relevant to cheese ripening. *Int Dairy J* **1993**, 3:423–60.
34. Smit G, Smit BA, Engels WJM. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol Rev* **2005**, 29:591–610.
35. Olson NF. The impact of lactic acid bacteria on cheese flavor. *FEMS Microbiol Rev* **1990**, 87:131–47.
36. Uniacke-Lowe T, Fox PF. Chymosin, Pepsins and Other Aspartyl Proteinases: Structures, Functions, Catalytic Mechanism and Milk-Clotting Properties. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 69–113.
37. Swaisgood HE. Chemistry of the caseins. In: Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects,. Editors: Fox PF, McSweeney PLH. **2003**, 135–60.
38. Trejo R, Dokland T, Jurat-Fuentes J, Harte F. Cryo-transmission electron tomography of native casein micelles from bovine milk. *J Dairy Sci* **2011**, 94(12):5770–5.
39. Sahu A, Kasoju N, Bora U. Fluorescence study of the curcumin-casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules* **2008**, 9(10):2905–12.
40. Dalgleish DG. On the structural models of bovine casein micelles - Review and possible improvements. *Soft Matter* **2011**, 7(6):2265–72.
41. McSweeney PLH. Biochemistry of Cheese Ripening: Introduction and Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 379–87.
42. Fagan CC, O'Callaghan DJ, Mateo MJ, Dejmek P. The Syneresis of Rennet-Coagulated Curd. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 145–77.
43. Cotter PD, Beresford TP. Microbiome Changes During Ripening. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 389–409.
44. Feirtag JM, McKay LL. Thermoinducible Lysis of Temperature-Sensitive *Streptococcus cremoris* Strains. *J Dairy Sci* **1987**, 70(9):1779–84.
45. Guinee TP, Fox PF. Salt in Cheese: Physical, Chemical and Biological Aspects. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: Mcsweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 207–59.
46. Guinee TP, Sutherland BJ. Salting of Cheese. In: Encyclopedia of Dairy Sciences. Editors: Fuquay JW. **2011**, 595–606.
47. Crow VL, Coolbear T, Gopal PK, Martley FG, McKay LL, Riepe H. The role of autolysis of lactic acid bacteria in the ripening of cheese. *Int Dairy J* **1995**, 5(8):855–75.
48. Collins YF, McSweeney PLH, Wilkinson MG. Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *J Dairy Res* **2003**, 70:105–13.
49. Christensen JE, Dudley EG, Pederson JA, Steele JL. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* **1999**, 76(1–4):217–46.
50. Irlinger F, Helinck S, Jany JL. Secondary and Adjunct Cultures. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 273–300.
51. Erkus O, de Jager VC, Spus M, van Alen-Boerrigter IJ, van Rijswijck IM, Hazelwood L, Janssen PW, van Hijum SA, Kleerebezem M, Smid EJ. Multifactorial diversity sustains microbial community stability. *ISME J* **2013**, 7(11):2126–36.

52. Erkus O, de Jager VCL, Geene RTCM, van Alen-Boerrigter I, Hazelwood L, van Hijum SAFT, Kleerebezem M, Smid EJ. Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. *Int J Food Microbiol* **2016**, 228:1–9.
53. Frantzen CA, Kleppen HP, Holo H. *Lactococcus lactis* Diversity in Undefined Mixed Dairy Starter Cultures as Revealed by Comparative Genome Analyses and Targeted Amplicon Sequencing of *epsD*. *Appl Environ Microbiol* **2018**, 84(3):1–15.
54. Helmut K, Fröhlich J. Lactic Acid Bacteria. In: Biology of Microorganisms on Grapes, in Must and in Wine. Editors: König H, Unden G, Fröhlich J. Springer-Verlag Berlin Heidelberg **2009**, 3–30.
55. Gänzle MG. Lactic metabolism revisited: Metabolism of lactic acid bacteria in food fermentations and food spoilage. *Curr Opin Food Sci* **2015**, 2:106–17.
56. Kleynmans U, Heinzl H, Hammes WP. *Lactobacillus suebicus* sp. nov., an Obligately Heterofermentative *Lactobacillus* Species Isolated from Fruit Mashes. *Syst Appl Microbiol* **1989**, 11(3):267–71.
57. Leroy F, De Vuyst L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **2004**, 15(2):67–78.
58. Nisiotou AA, Dourou D, Filippousi ME, Diamantea E, Fragkoulis P, Tassou C, Banilas G. Genetic and technological characterisation of vineyard- and winery-associated lactic acid bacteria. *Biomed Res Int* **2015**, 2015:1–8.
59. Parente E, Cogan TM, Powell IB. Starter Cultures: General Aspects. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 201–26.
60. Venema G, Kok J. Improving dairy starter cultures. *Trends Biotechnol* **1987**, 5:144–9.
61. Frantzen CA, Kot W, Pedersen TB, Ardö YM, Broadbent JR, Neve H, Hansen LH, Bello FD, Østlie HM, Kleppen HP, Vogensen FK, Holo H. Genomic characterization of dairy associated *Leuconostoc* species and diversity of Leuconostocs in undefined mixed mesophilic starter cultures. *Front Microbiol* **2017**, 8:1–14.
62. Smid EJ, Erkus O, Spus M, Wolkers-rooijackers JCM, Alexeeva S. Functional implications of the microbial community structure of undefined mesophilic starter cultures. *Microb Cell Fact* **2014**, 13(S2):1–9.
63. Bachmann H, Molenaar D, Kleerebezem M, Van Hylckama Vlieg JE. High local substrate availability stabilizes a cooperative trait. *ISME J* **2011**, 5(5):929–32.
64. Kim W. The genus Lactococcus. In: Lactic Acid Bacteria: Biodiversity and Taxonomy. **2014**, 429–43.
65. Torres Manno M, Zuljan F, Alarcón S, Esteban L, Blancato V, Espariz M, Magni C. Genetic and phenotypic features defining industrial relevant *Lactococcus lactis*, *L. cremoris* and *L. lactis* biovar. diacetylactis strains. *J Biotechnol* **2018**, 282(June):25–31.
66. Rademaker JLW, Starrenburg MJC, Naser SM, Gevers D, Kelly WJ, Hugenholtz J, Swings J, Vlieg JETVH. Diversity Analysis of Dairy and Nondairy *Lactococcus lactis* Isolates, Using a Novel Multilocus Sequence Analysis Scheme and. *Appl Environ Microbiol* **2007**, 73(22):7128–37.
67. Cavanagh D, Fitzgerald GF, McAuliffe O. From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiol* **2015**, 47:45–61.
68. Godon J, Delorme C, Bardowski J, Chopin M, Ehrlich SD, Renault P. Gene Inactivation in *Lactococcus lactis*: Branched-Chain Amino Acid Biosynthesis. *J Bacteriol* **1993**, 175(14):4383–90.
69. Ardö Y, Varming C. Bacterial influence on characteristic flavour of cheeses made with mesophilic DL-starter. *Aust J Dairy Technol* **2010**, 65(3):153–8.
70. Fox PF, Wallace JM, Morgan S, Lynch CM, Niland EJ, Tobin J. Acceleration of cheese ripening. *Antonie Van Leeuwenhoek* **1996**, 70:271–97.

71. McSweeney PLH, Fox PF, Ciocia F. Metabolism of Residual Lactose and of Lactate and Citrate. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 411–21.
72. van Mastrigt O, Mager EE, Jamin C, Abee T, Smid EJ. Citrate, low pH and amino acid limitation induce citrate utilization in *Lactococcus lactis* biovar diacetylactis. *Microb Biotechnol* **2018**, 11(2):369–80.
73. Pedersen TB, Vogensen FK, Ardö Y. Effect of heterofermentative lactic acid bacteria of DL-starters in initial ripening of semi-hard cheese. *Int Dairy J* **2016**, 57:72–9.
74. Fröhlich-Wyder MT, Bisig W, Guggisberg D, Jakob E, Turgay M, Wechsler D. Cheeses With Propionic Acid Fermentation. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 889–910.
75. Oberg CJ, Broadbent JR. Thermophilic Starter Cultures: Another Set of Problems. Vol. 76. **1993**, 2392–406.
76. Van Kranenburg R, Kleerebezem M, Van Hylckama Vlieg J, Ursing BM, Boekhorst J, Smit BA, Ayad EHE, Smit G, Siezen RJ. Flavour formation from amino acids by lactic acid bacteria: Predictions from genome sequence analysis. *Int Dairy J* **2002**, 12:111–21.
77. Kunji ERS, Mierau I, Hagfing A, Poolman B, Konings WN. The Proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek* **1996**, 70:187–221.
78. Mahony J, McDonnell B, Casey E, van Sinderen D. Phage-Host Interactions of Cheese-Making Lactic Acid Bacteria. *Annu Rev Food Sci Technol* **2016**, 7(1):267–85.
79. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **2005**, 60(2):174–82.
80. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero D a, Horvath P. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science (80-)* **2007**, 315:1709–12.
81. Donohoue PD, Barrangou R, May AP. Advances in Industrial Biotechnology Using CRISPR-Cas Systems. *Trends Biotechnol* **2018**, 36(2):134–46.
82. Marcó MB, Moineau S, Quiberoni A. Bacteriophages and dairy fermentations. *Bacteriophage* **2012**, 2(3):149–58.
83. O'Sullivan D, Coffey A, Fitzgerald GF, Hill C, Ross RP. Design of a Phage-Insensitive Lactococcal Dairy Starter via Sequential Transfer of Naturally Occurring Conjugative Plasmids. *Appl Environ Microbiol* **1998**, 64(11):4618–22.
84. McDonnell B, Mahony J, Hanemaaijer L, Kouwen TRHM, van Sinderen D. Generation of bacteriophage-insensitive mutants of *Streptococcus thermophilus* via an antisense RNA CRISPR-Cas silencing approach. *Appl Environ Microbiol* **2018**, 84(4):1–14.
85. Josephsen J, Petersen A, Neve H, Nielsen EW. Development of lytic *Lactococcus lactis* bacteriophages in a Cheddar cheese plant. *Int J Food Microbiol* **1999**, 50(3):163–71.
86. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **2002**, 82(1–4):187–216.
87. Papadimitriou K, Alegría Á, Bron PA, Angelis M De, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, Sinderen D Van, Varmanen P, Ventura M, Zúñiga M, Tsakalidou E. Stress Physiology of Lactic Acid Bacteria. *Microbiol Mol Biol Rev* **2016**, 80(3):837–90.
88. Tsakalidou E, Papadimitriou K. Stress Responses of Lactic Acid Bacteria. Editors: Tsakalidou E, Papadimitriou K. Springer **2011**,.
89. Hartke A, Bouche S, Gansel X, Boutibonnes P, Auffray Y. Starvation-Induced Stress Resistance in *Lactococcus lactis* subsp. *lactis* IL1403. *Appl Environ Microbiol* **1994**, 60(9):3474–8.
90. O'Sullivan E, Condon S. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Appl Environ Microbiol* **1997**, 63(11):4210–5.

91. Rallu F, Gruss A, Ehrlich SD, Maguin E. Acid- and multistress-resistant mutants of *Lactococcus lactis*: Identification of intracellular stress signals. *Mol Microbiol* **2000**, 35(3):517–28.
92. Ryssel M, Meisner Hviid AM, Dawish MS, Haaber J, Hammer K, Martinussen J, Kilstrup M. Multi-stress resistance in *Lactococcus lactis* is actually escape from purine-induced stress sensitivity. *Microbiology* **2014**, 160(2014):2551–9.
93. Khunajakr N, Liu CQ, Charoenchai P, Dunn NW. A plasmid-encoded two-component regulatory system involved in copper-inducible transcription in *Lactococcus lactis*. *Gene* **1999**, 229(1–2):229–35.
94. Kuipers OP, Beerthuyzen MM, De Ruyter PGGA, Luesink EJ, De Vos WM. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J Biol Chem* **1995**, 270(45):27299–304.
95. Barakat M, Ortet P, Whitworth DE. P2CS: A database of prokaryotic two-component systems. *Nucleic Acids Res* **2011**, 39:771–6.
96. Cuthbertson L, Nodwell JR. The TetR Family of Regulators. *Microbiol Mol Biol Rev* **2013**, 77(3):440–75.
97. Linares DM, del Rio B, Redruello B, Ladero V, Cruz Martin M, de Jong MCM, Kuipers OP, Fernandez M, Alvarez MA. AguR, a transmembrane transcription activator of the putrescine biosynthesis operon in *Lactococcus lactis*, acts in response to the agmatine concentration. *Appl Environ Microbiol* **2015**, 81(18):6145–57.
98. Den Hengst CD, Van Hijum SAFT, Geurts JMW, Nauta A, Kok J, Kuipers OP. The *Lactococcus lactis* CodY regulon: Identification of a conserved *cis*-regulatory element. *J Biol Chem* **2005**, 280(40):34332–42.
99. Kilstrup M, Hammer K, Jensen PR, Martinussen J. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* **2005**, 29:555–90.
100. Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL, Berninghausen O, Hauryliuk V, Beckmann R, Wilson DN. The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Res* **2016**, 44(13):6471–81.
101. Sinha AK, Winther KS, Roghanian M, Gerdes K. Fatty acid starvation activates RelA by depleting lysine precursor pyruvate. *Mol Microbiol* **2019**, 112(4):1339–49.
102. Potrykus K, Cashel M. (p)ppGpp: Still Magical? *Annu Rev Microbiol* **2008**, 62(1):35–51.
103. Elsholz AKW, Michalik S, Zühlke D, Hecker M, Gerth U. CtsR, the Gram-positive master regulator of protein quality control, feels the heat. *EMBO J* **2010**, 29(21):3621–9.
104. Schramm FD, Schroeder K, Jonas K. Protein aggregation in bacteria. *FEMS Microbiol Rev* **2019**, 44(1):54–72.
105. Sutton MD, Smith BT, Godoy VG, Walker GC. The Sos Response: Recent Insights into *umuDC*-Dependent Mutagenesis and DNA Damage Tolerance. *Annu Rev Genet* **2000**, 34:479–97.
106. Maslowska KH, Makiela-Dzbenska K, Fijalkowska IJ. The SOS system: A complex and tightly regulated response to DNA damage. *Environ Mol Mutagen* **2019**, 60(4):368–84.
107. Frees D, Ingmer H. ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Mol Microbiol* **1999**, 31(1):79–87.
108. Savijoki K, Ingmer H, Frees D, Vogensen FK, Palva A, Varmanen P. Heat and DNA damage induction of the LexA-like regulator HdiR from *Lactococcus lactis* is mediated by RecA and ClpP. *Mol Microbiol* **2003**, 50(2):609–21.
109. Santivarangkna C, Kulozik U, Foerst P. Storing Lactic Acid Bacteria: Current Methodologies and Physiological Implications. In: Stress Responses of Lactic Acid Bacteria. Editors: Tsakalidou E, Papadimitriou K. Springer **2011**, 479–504.
110. Phadtare S. Recent developments in bacterial cold-shock response. *Curr Issues Mol Biol* **2004**, 6(2):125–36.
111. Capozzi V, Fiocco D, Spano G. Responses of Lactic Acid Bacteria to Cold Stress. In: Stress Responses of Lactic Acid Bacteria. Editors: Tsakalidou E, Papadimitriou K. Springer **2011**, 91–110.

112. Broadbent JR, Lin C. Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology* **1999**, 39:88–102.
113. Wouters JA, Rombouts FM, Kuipers OP, De Vos WM, Abee T. The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. *Syst Appl Microbiol* **2000**, 23(2):165–73.
114. Kets EPW, Teunissen PJM, De Bont JAM. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Appl Environ Microbiol* **1996**, 62(1):259–61.
115. Molenaar D, Hagting A, Alkema H, Driessen AJM, Konings WN. Characteristics and osmoregulatory roles of uptake systems for proline and glycine betaine in *Lactococcus lactis*. *J Bacteriol* **1993**, 175(17):5438–44.
116. Rehn U, Petersen MA, Saedén KH, Ardö Y. Ripening of extra-hard cheese made with mesophilic DL-starter. *Int Dairy J* **2010**, 20(12):844–51.
117. Chen J, Shen J, Ingvar Hellgren L, Ruhdal Jensen P, Solem C. Adaptation of *Lactococcus lactis* to high growth temperature leads to a dramatic increase in acidification rate. *Sci Rep* **2015**, 5:1–15.
118. Kilstrup M, Jacobsen S, Hammer K, Vogensen FK, Kilstrup M, Jacobsen S, Hammer K. Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. *Appl Environ Microbiol* **1997**, 63(5):1826–37.
119. Dijkstra AR, Alkema W, Starrenburg MJC, Hugenholtz J, Hijum SAFT Van, Bron PA. Fermentation-induced variation in heat and oxidative stress phenotypes of *Lactococcus lactis* MG1363 reveals transcriptome signatures for robustness. *Microb Cell Fact* **2014**, 13(148):1–11.
120. Meulen SB Van Der, Jong A De, Kok J, Kok J. Early Transcriptome Response of *Lactococcus lactis* to Environmental Stresses Reveals Differentially Expressed Small Regulatory RNAs and tRNAs. *Front Microbiol* **2017**, 8:1–15.
121. Xie Y, Chou L, Cutler A, Weimer B. DNA Macroarray Profiling of *Lactococcus lactis* subsp. *lactis* IL1403 Gene Expression during Environmental Stresses. *Appl Environ Microbiol* **2004**, 70(11):6738–47.
122. Nilsson D, Lauridsen AA, Tomoyasu T, Ogura T. A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli ftsH* gene product. *Microbiology* **1994**, 140(10):2601–10.
123. Arnau J, Sorensen KI, Appel KF, Vogensen FK, Hammer K. Analysis of heat shock gene expression in *Lactococcus lactis* MG1363. *Microbiology* **1996**, 142(7):1685–91.
124. Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A. HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. *Mol Microbiol* **2000**, 35(5):1042–51.
125. Harvey RJ. Damage to *Streptococcus lactis* resulting from growth at low pH. *J Bacteriol* **1965**, 90(5):1330–6.
126. Kashket ER. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol Lett* **1987**, 46(3):233–44.
127. Nannen NL, Hutkins RW. Intracellular pH Effects in Lactic Acid Bacteria. *J Dairy Sci* **1991**, 74(3):741–6.
128. Siegumfeldt H, Rechinger KB, Jakobsen M. Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Appl Environ Microbiol* **2000**, 66(6):2330–5.
129. Kobayashi H, Suzuki T, Unemoto T. Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. *J Biol Chem* **1986**, 261(2):627–30.
130. O'Sullivan E, Condon S. Relationship between acid tolerance, cytoplasmic pH, and ATP and H^+- ATPase levels in chemostat cultures of *Lactococcus lactis*. *Appl Environ Microbiol* **1999**, 65(6):2287–93.

131. Zhu Z, Yang P, Wu Z, Zhang J, Du G. Systemic understanding of *Lactococcus lactis* response to acid stress using transcriptomics approaches. *J Ind Microbiol Biotechnol* **2019**, 46(11):1621–9.
132. Marquis RE, Bender GR, Murray DR, Wong A. Arginine deiminase system and bacterial adaptation to acid environments. *Appl Environ Microbiol* **1987**, 53(1):198–200.
133. Ladero V, Rattray FP, Mayo B, Martín MC, Fernández M, Alvarez MA. Sequencing and transcriptional analysis of the biosynthesis gene cluster of putrescine-producing *Lactococcus lactis*. *Appl Environ Microbiol* **2011**, 77(18):6409–18.
134. Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J. A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. *Mol Microbiol* **1998**, 27(2):299–310.
135. Abe K, Hayashi H, Maloney PC. Exchange of Aspartate and Alanine. *J Biol Chem* **1996**, 271(6):3079–84.
136. Trip H, Mulder NL, Lolkema JS. Improved acid stress survival of *Lactococcus lactis* expressing the histidine decarboxylation pathway of *Streptococcus thermophilus* CHCC1524. *J Biol Chem* **2012**, 287(14):11195–204.
137. Mobley HLT, Island MD, Hausinger RP. Molecular biology of microbial ureases. *Microbiol Rev* **1995**, 59(3):451–80.
138. Mora D, Monnet C, Parini C, Guglielmetti S, Mariani A, Pintus P, Molinari F, Daffonchio D, Manachini PL. Urease biogenesis in *Streptococcus thermophilus*. *Res Microbiol* **2005**, 156(9):897–903.
139. Magni C, De Mendoza D, Konings WN, Lolkema JS. Mechanism of citrate metabolism in *Lactococcus lactis*: Resistance against lactate toxicity at low pH. *J Bacteriol* **1999**, 181(5):1451–7.
140. Csonka LN. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev* **1989**, 53(1):121–47.
141. Bouvier J, Bordes P, Romeo Y, Fourçans A, Bouvier I, Gutierrez C. Characterization of OpuA, a glycine-betaine uptake system of *Lactococcus lactis*. *J Mol Microbiol Biotechnol* **2000**, 2(2):199–205.
142. Obis D, Guillot A, Gripon JC, Renault P, Bolotin A, Mistou MY. Genetic and biochemical characterization of a high-affinity betaine uptake system (BusA) in *Lactococcus lactis* reveals a new functional organization within bacterial ABC transporters. *J Bacteriol* **1999**, 181(20):6238–46.
143. Wolters JC, Ronnie PAB, Gul N, Karasawa A, Andy-Mark WHT, Slotboom DJ, Poolman B. Ligand binding and crystal structures of the substrate- binding domain of the ABC transporter OpuA. *PLoS One* **2010**, 5(4).
144. Van Der Heide T, Poolman B. Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane. *Proc Natl Acad Sci* **2000**, 97(13):7102–6.
145. Biemans-Oldehinkel E, Mahmood NABN, Poolman B. A sensor for intracellular ionic strength. *Proc Natl Acad Sci* **2006**, 103(28):10624–9.
146. Mahmood NABN, Biemans-Oldehinkel E, Patzlaff JS, Schuurman-Wolters GK, Poolman B. Ion specificity and ionic strength dependence of the osmoregulatory ABC transporter OpuA. *J Biol Chem* **2006**, 281(40):29830–9.
147. Romeo Y, Obis D, Bouvier J, Guillot A, Fourçans A, Bouvier I, Gutierrez C, Mistou MY. Osmoregulation in *Lactococcus lactis*: BusR, a transcriptional repressor of the glycine betaine uptake system BusA. *Mol Microbiol* **2003**, 47(4):1135–47.
148. Romeo Y, Bouvier J, Gutierrez C. Osmotic regulation of transcription in *Lactococcus lactis*: Ionic strength-dependent binding of the BusR repressor to the busA promoter. *FEBS Lett* **2007**, 581(18):3387–90.

149. Termont S, Vandenbroucke K, Iserentant D, Neirynck S, Steidler L, Remaut E, Rottiers P. Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance. *Appl Environ Microbiol* **2006**, 72(12):7694–700.
150. Carvalho AL, Cardoso FS, Bohn A, Neves AR, Santos H. Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance. *Appl Environ Microbiol* **2011**, 77(12):4189–99.
151. Piuri M, Sanchez-Rivas C, Ruzal SM. Adaptation to high salt in *Lactobacillus*: Role of peptides and proteolytic enzymes. *J Appl Microbiol* **2003**, 95(2):372–9.
152. Zaprasis A, Brill J, Thüring M, Wünsche G, Heun M, Barzantny H, Hoffmann T, Bremer E. Osmoprotection of *Bacillus subtilis* through import and proteolysis of proline-containing peptides. *Appl Environ Microbiol* **2013**, 79(2):576–87.
153. Warner JB, Lolkema JS. CcpA-Dependent Carbon Catabolite Repression in Bacteria. *Microbiol Mol Biol Rev* **2003**, 67(4):475–90.
154. Zomer AL, Buist G, Larsen R, Kok J, Kuipers OP. Time-resolved determination of the CcpA regulon of *Lactococcus lactis* subsp. *cremoris* MG1363. *J Bacteriol* **2007**, 189(4):1366–81.
155. Solopova A, Gestel J Van, Weissing FJ, Bachmann H, Teusink B, Kok J. Bet-hedging during bacterial diauxic shift. *Proc Natl Acad Sci* **2014**, 111(20):7427–32.
156. Ganesan B, Stuart MR, Weimer BC. Carbohydrate starvation causes a metabolically active but nonculturable state in *Lactococcus lactis*. *Appl Environ Microbiol* **2007**, 73(8):2498–512.
157. Stuart MR, Chou LS, Weimer BC. Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. lactis. *Appl Environ Microbiol* **1999**, 65(2):665–73.
158. Ganesan B, Dobrowolski P, Weimer BC. Identification of the leucine-to-2-methylbutyric acid catabolic pathway of *Lactococcus lactis*. *Appl Environ Microbiol* **2006**, 72(6):4264–73.
159. Boutrou R, Sepulchre A, Gripon JC, Monnet V. Simple Tests for Predicting the Lytic Behavior and Proteolytic Activity of Lactococcal Strains in Cheese. *J Dairy Sci* **1998**, 81:2321–8.
160. Riepe HR, Pillidge CJ, Gopal PK, Mckay LL. Characterization of the highly autolytic *Lactococcus lactis* subsp. *cremoris* strains CO and 2250. *Appl Environ Microbiol* **1997**, 63(10):3757–63.
161. Hannon JA, Wilkinson MG, Delahunty CM, Wallace JM, Morrissey PA, Beresford TP. Use of autolytic starter systems to accelerate the ripening of Cheddar cheese. *Int Dairy J* **2003**, 13(4):313–23.
162. O'Donovan CM, Wilkinson MG, Guinee TP, Fox PF. An investigation of the autolytic properties of three lactococcal strains during cheese ripening. *Int Dairy J* **1996**, 6:1149–65.
163. Porcellato D, Skeie SB. Bacterial dynamics and functional analysis of microbial metagenomes during ripening of Dutch-type cheese. *Int Dairy J* **2016**, 61:182–8.
164. Ruggirello M, Dolci P, Giordano M, Doyle C, Osullivan O, Cotter P, Cocolin L. Study of *Lactococcus lactis* during advanced ripening stages of model cheeses characterized by GC-MS. *Food Microbiol* **2016**, 74:132–42.
165. Ruggirello M, Cocolin L, Dolci P. Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models. *Food Microbiol* **2016**, 59:112–8.
166. Ercan O, Bisschops MMM, Overkamp W, Jørgensen TR, Ram AF, Smid EJ, Pronk JT, Kuipers OP, Daran-Lapujade P, Kleerebezem M. Physiological and transcriptional responses of different industrial microbes at near-zero specific growth rates. *Appl Environ Microbiol* **2015**, 81(17):5662–70.
167. Ercan O, Smid EJ, Kleerebezem M. Quantitative physiology of *Lactococcus lactis* at extreme low-growth rates. *Environ Microbiol* **2013**, 15(8):2319–32.

168. Ercan O, Wels M, Smid EJ, Kleerebezem M. Molecular and metabolic adaptations of *Lactococcus lactis* at near-zero growth rates. *Appl Environ Microbiol* **2015**, 81(1):320–31.
169. van Mastrigt O, Abee T, Lillevang SK, Smid EJ. Quantitative physiology and aroma formation of a dairy *Lactococcus lactis* at near-zero growth rates. *Food Microbiol* **2018**, 73:216–26.
170. van Mastrigt O, Gallegos Tejeda D, Kristensen MN, Abee T, Smid EJ. Aroma formation during cheese ripening is best resembled by *Lactococcus lactis* retentostat cultures. *Microb Cell Fact* **2018**, 17:1–8.
171. Liu J, Chen L, Dorau R, Lillevang SK, Jensen PR, Solem C. From waste to taste – Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) *Lactococcus lactis* dairy isolate. *J Agric Food Chem* **2020**, 68(21):5891–9.
172. Larsen R, Kloosterman TG, Kok J, Kuipers OP. GlnR-mediated regulation of nitrogen metabolism in *Lactococcus lactis*. *J Bacteriol* **2006**, 188(13):4978–82.
173. Groot Kormelink T, Koenders E, Hagemeijer Y, Overmars L, Siezen RJ, de Vos WM, Francke C. Comparative genome analysis of central nitrogen metabolism and its control by GlnR in the class Bacilli. *BMC Genomics* **2012**, 13:191.
174. Dressaire C, Redon E, Gitton C, Loubière P, Monnet V, Cocaign-Bousquet M. Investigation of the adaptation of *Lactococcus lactis* to isoleucine starvation integrating dynamic transcriptome and proteome information. *Microb Cell Fact* **2011**, 10:1–11.
175. Chapot-Chartier M-P, Kulakauskas S. Cell wall structure and function in lactic acid bacteria. *Microb Cell Fact* **2014**, 13:1–23.
176. Huang C, Hernandez-Valdes JA, Kuipers OP, Kok J. Lysis of a *Lactococcus lactis* Dipeptidase Mutant and Rescue by Mutation in the Pleiotropic Regulator CodY. *Appl Environ Microbiol* **2020**, 86(8):1–15.
177. Solopova A, Formosa-Dague C, Courtin P, Furlan S, Veiga P, Péchoux C, Armalyte J, Sadauskas M, Kok J, Hols P, Dufrêne YF, Kuipers OP, Chapot-Chartier MP, Kulakauskas S. Regulation of cell wall plasticity by nucleotide metabolism in *Lactococcus lactis*. *J Biol Chem* **2016**, 291(21):11323–36.
178. Martinussen J, Martinussen J, Wadskov-hansen SLL, Wadskov-hansen SLL, Hammer K, Hammer K. Two Nucleoside Uptake Systems in *Lactococcus lactis*: Competition between Purine Nucleosides and Cytidine Allows for Modulation of Intracellular Nucleotide Pools. *J Bacteriol* **2003**, 185(5):1503–8.
179. Sundekilde UK, Gustavsson F, Poulsen NA, Glantz M, Paulsson M, Larsen LB, Bertram HC. Association between the bovine milk metabolome and rennet-induced coagulation properties of milk. *J Dairy Sci* **2014**, 97(10):6076–84.
180. Beyer NH, Roepstorff P, Hammer K, Kilstrup M. Proteome analysis of the purine stimulon from *Lactococcus lactis*. *Proteomics* **2003**, 3(5):786–97.
181. Jørgensen CM, Hammer K, Martinussen J. CTP Limitation Increases Expression of CTP Synthase in *Lactococcus lactis*. *J Bacteriol* **2003**, 185(22):6562–74.
182. Marty-Teysset C, De La Torre F, Garel JR. Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: Involvement of an NADH oxidase in oxidative stress. *Appl Environ Microbiol* **2000**, 66(1):262–7.
183. Duwat P, Sourice S, Cesselin B, Lamberet G, Vido K, Gaudu P, Le Loir Y, Violet F, Loubière P, Gruss A. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *J Bacteriol* **2001**, 183(15):4509–16.
184. Blank LM, Koebmann BJ, Michelsen O, Lars K, Jensen PR, Michelsen OLE, Nielsen LK. Hemin Reconstitutes Proton Extrusion in an H^+-ATPase-Negative Mutant of *Lactococcus lactis*. *J Bacteriol* **2001**, 183(22):6707–9.
185. De Felipe FL, Starrenburg M, Hugenholtz J. The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis* subsp. *lactis* MG1363. *FEMS Microbiol Lett* **1997**, 156:15–9.

186. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. *Metab Eng* **2016**, 36:57–67.
187. Liu J, Wang Z, Kandasamy V, Lee SY, Solem C, Jensen PR. Harnessing the respiration machinery for high-yield production of chemicals in metabolically engineered *Lactococcus lactis*. *Metab Eng* **2017**, 44:22–9.
188. Pedersen MB, Gaudu P, Lechardeur D, Petit M-A, Gruss A. Aerobic Respiration Metabolism in Lactic Acid Bacteria and Uses in Biotechnology. *Annu Rev Food Sci Technol* **2012**, 3:37–58.
189. Chen J, Shen J, Solem C, Jensen PR. Oxidative Stress at High Temperatures in *Lactococcus lactis* Due to an Insufficient Supply of Riboflavin. *Appl Environ Microbiol* **2013**, 79(19):6140–7.
190. Zotta T, Ricciardi A, Ianniello RG, Storti L V., Glibota NA, Parente E. Aerobic and respirative growth of heterofermentative lactic acid bacteria: A screening study. *Food Microbiol* **2018**, 76:117–27.
191. Maresca D, Zotta T, Mauriello G. Adaptation to aerobic environment of *Lactobacillus johnsonii*/*gasseri* strains. *Front Microbiol* **2018**, 9:1–11.
192. Sandberg TE, Salazar MJ, Weng LL, Palsson BO, Feist AM. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metab Eng* **2019**, 56:1–16.
193. Johansen E. Use of Natural Selection and Evolution to Develop New Starter Cultures for Fermented Foods. *Annu Rev Food Sci Technol* **2018**, 9:411–28.
194. Bachmann H, Molenaar D, Branco F, Teusink B. Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria. *FEMS Microbiol Rev* **2017**, 24:1–19.
195. Dijkstra AR, Setyawati MC, Bayjanov JR, Alkema W, Van Hijum SAFT, Bron PA, Hugenholtz J. Diversity in robustness of *Lactococcus lactis* strains during heat stress, oxidative stress, and spray drying stress. *Appl Environ Microbiol* **2014**, 80(2):603–11.
196. Boguta AM, Bringel F, Martinussen J, Jensen PR. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. *Microb Cell Fact* **2014**, 13(97):1–16.
197. Smith WM, Pham TH, Lei L, Dou J, Soomro AH, Beatson SA, Dykes GA, Turner MS. Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of llmg_1816 (*gdpP*) induced by high-temperature growth. *Appl Environ Microbiol* **2012**, 78(21):7753–9.
198. Spus M, Liu H, Wels M, Abee T, Smid EJ. Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **2017**, 241:173–80.
199. Dijkstra AR, Starrenburg MJC, Todt T, Van Hijum SAFT, Hugenholtz J, Bron PA. Transcriptome analysis of a spray drying-resistant subpopulation reveals a zinc-dependent mechanism for robustness in *L. lactis* SK11. *Front Microbiol* **2018**, 9:1–10.
200. Kulkarni S, Haq SF, Samant S, Sukumaran S. Adaptation of *Lactobacillus acidophilus* to thermal stress yields a thermotolerant variant which also exhibits improved survival at pH 2. *Probiotics Antimicrob Proteins* **2018**, 10(4):717–27.
201. Weidmann S, Maitre M, Laurent J, Coucheney F, Rieu A, Guzzo J. Production of the small heat shock protein Lo18 from *Oenococcus oeni* in *Lactococcus lactis* improves its stress tolerance. *Int J Food Microbiol* **2017**, 247:18–23.
202. Desmond C, Fitzgerald GF, Stanton C, Ross RP, Al DET, Icrobiol APPLENM. Improved Stress Tolerance of GroESL-Overproducing *Lactococcus lactis* and Probiotic *Lactobacillus paracasei* NFBC 338. *Appl Environ Microbiol* **2004**, 70(10):5929–36.
203. Abdullah-Al-Mahin, Sugimoto S, Higashi C, Matsumoto S, Sonomoto K. Improvement of multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli dnaK*. *Appl Environ Microbiol* **2010**, 76(13):4277–85.

204. Wu C, Zhang J, Du G, Chen J. Heterologous expression of *Lactobacillus casei* RecO improved the multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 during salt stress. *Bioresour Technol* **2013**, 143:238–41.
205. Monnet C, Béal C, Corrieu G. Improvement of the resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* to freezing by natural selection. *J Dairy Sci* **2003**, 86(10):3048–53.
206. Kwon YW, Bae JH, Kim SA, Han NS. Development of freeze-thaw tolerant *Lactobacillus rhamnosus* gg by adaptive laboratory evolution. *Front Microbiol* **2018**, 9:1–10.
207. Hongo M, Nomura Y, Iwahara M. Novel method of lactic acid production by electrodialysis fermentation. *Appl Environ Microbiol* **1986**, 52(2):314–9.
208. Othman M, Ariff AB, Rios-Solis L, Halim M. Extractive fermentation of lactic acid in lactic acid bacteria cultivation: A review. *Front Microbiol* **2017**, 8:1–7.
209. Singhvi M, Zendo T, Gokhale D, Sonomoto K. Greener L-lactic acid production through in situ extractive fermentation by an acid-tolerant *Lactobacillus strain*. *Appl Microbiol Biotechnol* **2018**, 102(15):6425–35.
210. Bosma EF, Forster J, Nielsen AT. Lactobacilli and pediococci as versatile cell factories – Evaluation of strain properties and genetic tools. *Biotechnol Adv* **2017**, 35(4):419–42.
211. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, Cardayré S. Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nat Biotechnol* **2002**, 20:707–12.
212. Ju SY, Kim JH, Lee PC. Long-term adaptive evolution of *Leuconostoc mesenteroides* for enhancement of lactic acid tolerance and production. *Biotechnol Biofuels* **2016**, 9:1–12.
213. Overbeck TJ, Welker DL, Hughes JE, Steele JL, Broadbent JR. Transient MutS-based hypermutation system for adaptive evolution of *Lactobacillus casei* to low pH. *Appl Environ Microbiol* **2017**, 83(20):1–15.
214. Lenski RE, Sniegowski P, Gerrish P. Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* **1997**, 387:703–5.
215. Rochat T, Gratadoux JJ, Corthier G, Coqueran B, Nader-Macias ME, Gruss A, Langella P. *Lactococcus lactis* SpOx spontaneous mutants: A family of oxidative-stress-resistant dairy strains. *Appl Environ Microbiol* **2005**, 71(5):2782–8.
216. Chen J, Shen J, Solem C, Jensen PR. A new type of *yumC*-like ferredoxin (flavodoxin) reductase is involved in ribonucleotide reduction. *MBio* **2015**, 6(6):1–8.

2. Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis SD96

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2.1 Abstract

The genome of L*actococcus lactis* subsp. *lactis* biovar diacetylactis SD96, a strain used for cheese production, is presented. SD96 is refractory to phage attack, which is a desired property for starter bacteria. Its ten plasmids provide industrially important traits, such as lactose and citrate metabolism, proteolytic activity, and phage resistance.

2.2 Main Text

Lactococcus lactis (*L. lactis*), considered to be the model organism of lactic acid bacteria (LAB), has a long history of use in the dairy industry. Many mesophilic starter cultures applied for cheese production contain several LAB, which are usually *Lactococcus* and *Leuconostoc* species (1). *L. lactis* subsp. *lactis* biovar diacetylactis is present in many mesophilic starter cultures and is responsible for a buttery aroma and gas formation from citrate. It has been shown that a large genetic variety exists among lactococci, even between members of the same subspecies (2,3).

Here, we present the whole genome of the *L. lactis* subsp. *lactis* biovar diacetylactis SD96, a bacteriophage insensitive mutant of strain SD16, which was originally isolated from an Italian cheese by starter culture provider Sacco S.r.l. To date, none of the phages contained in the comprehensive phage collection at Sacco S.r.l. has been found to be able to infect SD96, and dairies using SD96 have not reported any phage attack for this strain. Because of the contributions of plasmid-encoded genes to industrially relevant phenotypes, we were particularly interested in the plasmid content of this strain.

DNA for sequencing was isolated from an early-stationary-phase culture in M17 broth supplemented with 0.5% lactose, which was inoculated from a single colony. Total DNA was isolated using the Genomic DNA Purification Kit (product number K0512) from Thermo Scientific. Sequencing was carried out on both PacBio Sequel and Illumina HiSeq4000 platforms, using two distinct DNA preparations. Illumina sequencing using a PE150 PCR-free library provided 8.78 million raw paired-end 150 bp reads (SRA accession number SRX6686433). The sequences were processed and assembled using various software programs with default settings unless

otherwise stated. Raw Illumina reads were processed using Geneious Prime (version 2019.1.3) (4). Briefly, adapter sequences and low-quality sequences were trimmed from both ends; finally, all reads above 50 bp were kept. This process resulted in 7.25 million sequences, with an average length of 138 bp. From the PacBio Sequel project using a 20K library, we received 331,855 raw reads, with an average length of 7,575 bp and a maximal read length of 44,814 bp (SRA accession number SRX6711783). For de novo assembly, the PacBio reads alone were processed and assembled using the Canu assembler (version 1.8) (5), which provided 13 linear scaffolds. The Circulator-tool (version 1.5.5) (6) provided a closed chromosome and four plasmids. Another six plasmids were closed by identifying repeating regions on the scaffolds using the Repeat Finder plugin (version 1.0) in Geneious, followed by manual evaluation and trimming. Furthermore, the presence of replication-associated genes was confirmed for all ten plasmids by sequence comparison with previously characterized *L. lactis* plasmids. Two scaffolds (4,944 and 5,967 bp) could not be closed or associated with the genome and did not contain replication-associated genes. Finally, the circularized sequences were polished using Pilon (version 1.23), for which the processed Illumina paired-end reads had been mapped to the circular sequences using Bowtie2 (version 2.3.0) in Geneious. The genome and plasmids were annotated using the RAST-annotation server (Version 2.0) (7).

The 2.42-Mbp chromosome of SD96 has a GC content of 35.3%. The ten plasmids have an average GC content of 34.4% (minimum, 30.3%; maximum, 36.5%) and sizes of 64.9, 49.6, 37.4, 25.4, 11.7, 8.3, 7.9, 7.3, 4.6, 2.7 kbp (GenBank accession numbers CP043518 – CP043528). Plasmids associated with protein degradation, lactose and citrate metabolism, and phage defense, as well as two cryptic plasmids with unknown purpose, were identified.

2.3 Data Availability

The genome sequences of *L. lactis* subsp. *lactis* biovar diacetylactis SD96, including its ten plasmids, are available in GenBank under the accession numbers CP043523 (chromosome), CP043525 (pSD96_01), CP043526 (pSD96_02), CP043527 (pSD96_03), CP043528 (pSD96_04), CP043524 (pSD96_05), CP043518 (pSD96_06), CP043519 (pSD96_07), CP043520 (pSD96_08), CP043521 (pSD96_09), CP043522 (pSD96_10). Raw data has been deposited at the Sequence Read Archive (SRA) under accession numbers SRX6686433 (Illumina data) and SRX6711783 (PacBio Sequel data).

2.4 Acknowledgment

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2.5 References

1. Leroy F, De Vuyst L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **2004**, 15(2):67–78.

2. Erkus O, de Jager VC, Spus M, van Alen-Boerrigter IJ, van Rijswijck IM, Hazelwood L, Janssen PW, van Hijum SA, Kleerebezem M, Smid EJ. Multifactorial diversity sustains microbial community stability *ISME J* **2013**, 7(11):2126–36.
3. Frantzen CA, Kleppen HP, Holo H. *Lactococcus lactis* Diversity in Undefined Mixed Dairy Starter Cultures as Revealed by Comparative Genome Analyses and Targeted Amplicon Sequencing of *epsD*. *Appl Environ Microbiol* **2018**, 84(3):1–15.
4. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, 28(12):1647–9.
5. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive *k*-mer weighting and repeat separation. *Genome Res* **2017**, 722–36.
6. Hunt M, Silva N De, Otto TD, Parkhill J, Keane JA, Harris SR. Circlator: Automated circularization of genome assemblies using long sequencing reads. *Genome Biol* **2015**, 16(1):1–10.
7. Aziz RK, Bartels D, Best A, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics* **2008**, 9:1–15.

3. Systems Biology Approach for Understanding Thermotolerant *Lactococcus lactis* Mutants

This chapter serves as a manuscript for publication in an international scientific journal.

3.1 Abstract

Lactococcus lactis (*L. lactis*) is a model lactic acid bacterium and one of the main components in cheese starter cultures used for producing soft or semi-hard cheeses. Since most dairy *L. lactis* strains grow optimally at around 30°C, they are not particularly well-adapted to the elevated temperatures (37-39°C) that are often used during cheese manufacturing, and this hampers industrial performance. To increase the thermal robustness of the industrial strain *L. lactis* subsp. *lactis* biovar diacetylactis SD96, we applied long-term adaptive laboratory evolution (ALE) for one and a half years. Three mutants with improved growth and acidification rates at both high and low temperatures were obtained at different time points during the ALE (JA24: 160 generations, RD01: 150 generations, and RD07: 400 generations). The most thermotolerant strain isolated, RD07, could acidify milk at 41°C, which is a unique property of dairy lactococci. Genomics and transcriptomics analysis revealed the potential underlying causes responsible for enhanced growth. These included the downregulation of the pleiotropic transcription factor CodY and overexpression of genes involved in guanidine nucleotide drainage. The obtained results are invaluable for guiding future studies investigating the adaptation of *L. lactis* to elevated growth temperatures.

In conclusion, we demonstrate that long-term ALE at high temperatures is a powerful method for obtaining unique and valuable strains useful for the starter culture industry. We show that the thermotolerant mutants are suitable for producing hard cheese with high cooking temperatures at 40°C or above, where normal mesophilic starter bacteria are not applicable. Cheese trials at Arla Foods, Denmark, using JA24 and RD01 in combination with a commercial starter culture, resulted in cheese with superior taste compared to control cheese judged by an expert panel.

3.2 Background

Lactic acid bacteria (LAB) are widely applied in food fermentations, and they are particularly well-known for their essential role within the dairy industry (1). In 2019, approximately 852 million tons of milk were produced globally, from which up to 30% was fermented using LAB, resulting in products like cheese, yogurt, and fermented butter (2,3). Ever since the first cheeses were produced more than 9000 years ago, the urge to produce new products has driven the diversification and optimization of production processes and the responsible LAB (1,4,5). Nowadays, more than 75% of all cheeses are produced following the same basic steps (6). First, raw milk is pasteurized for inactivating pathogens and spoilage microorganisms, and

standardized regarding fat and protein content (7,8). Next, the treated and standardized milk is coagulated by the action of proteolytic enzymes (for example, rennet) and fermented by LAB (6). Moisture content (hardness) and flavor of the cheese are controlled by heating the curd during the fermentation, which is called cooking or scalding (9). The temperature and duration of the cooking step determine which LAB can be used. For cooking temperatures below 40°C, mesophilic LAB, such as *Lactococcus lactis* (*L. lactis*), are used, whereas for higher temperatures, thermophilic LAB, such as *Streptococcus thermophilus* (*S. thermophilus*), are used (10). *L. lactis* has outstanding flavor forming capacities and therefore constitutes the main component of mesophilic cheese starter cultures.

Nevertheless, the stress resistance of *L. lactis* limits the range of cheese variants producible with this bacterium (11). Cheddar cheese is produced using *L. lactis* in combination with high cooking temperatures up to 39°C (12). For ensuring robust acidification at even slightly higher cooking temperatures, *S. thermophilus* is often supplemented to the starter cultures (13,14). In some rare cheeses, such as the Swedish Västerbottensost, mesophilic starter bacteria are pushed to their boundaries by cooking at 40°C for several hours, followed by a long ripening time of several months (15). This process is costly as long-term storage in special ripening facilities is required. Using thermophilic LAB for the same process would potentially reduce the ripening time, but also would result in a differently flavored cheese. Consequently, thermotolerant *L. lactis* strains would allow the production of high-cooked cheddar cheese or similar hard cheeses with reduced ripening time and similar flavor as the original softer variants.

The tremendous industrial importance inspired much research to target the understanding and improvement of the stress resistance of LAB (16,17). Environmental stress in LAB is relieved through numerous stress response mechanisms (16,18,19). One example of a highly conserved stress response mechanism is the stringent response mediated by the so-called alarmone guanosine tetra- or pentaphosphate ((p)ppGpp) (16). The stringent response is activated by various factors, including amino acid starvation or environmental stress (20). In the example of amino acid starvation, RelA, a (p)ppGpp synthetase, is activated due to the presence of uncharged tRNAs, and then starts to produce (p)ppGpp from GTP (21). As a consequence, (p)ppGpp levels increase, and GTP levels drop, which initiates several downstream cascades, ultimately leading to an arrested growth by the inhibition of rRNA synthesis, DNA replication, and transcription (20,22).

Due to the complexity of the underlying mechanisms, stress tolerance is usually targeted by applying random mutagenesis or by evolutionary engineering (4,5). In this context, adaptive laboratory evolution (ALE) has recently gained much attention as advancements in laboratory automation, sequencing technologies and genome editing have emerged (23,24). Sequencing technologies, especially second- and third-generation-sequencing technologies, such as Illumina PacBio, and NanoPore sequencing, are vital for ALE experiments as the beneficial mutations can be identified easily and studied subsequently by editing the genome of the parent strain (25).

Taken together, ALE has become one of the most reliable methods for producing desired phenotypes and gaining insight into the underlying mechanisms that control physiological stress responses. Lastly, this approach is particularly suitable for optimizing LAB, which are intended to be applied in the food industry, as the resulting strains are considered not genetically modified (natural).

Previous studies have investigated the adaptation of LAB to heat stress by several means. One of the model strains of the LAB, *L. lactis* subsp. *cremoris* MG1363 has previously been adapted to grow at high temperatures using a long-term ALE. The resulting mutant (TM29) showed an increased acidification rate at elevated temperatures and could grow at 40°C, which is well above the average temperature optimum of *L. lactis* subsp. *cremoris*. Increased expression of chaperones and transporter proteins was observed in TM29, but finally, many synergistically acting mutations were found to cause the thermotolerance of TM29 (26). So far, this is the only example where the maximal growth temperature of a LAB has been increased by using ALE. Long term propagation of LAB can be troublesome, as, for example, undesired mutations or plasmid loss may occur (27).

As an alternative to ALE, short-term exposure to sublethal high temperatures for several hours was used to obtain strains with improved survival at high temperatures based on spontaneous mutations. This method was first demonstrated by Smith *et al.*, who grew *L. lactis* subsp. *cremoris* MG1363 at 37°C and frequently obtained spontaneous thermotolerant mutants. The mutants had mutations in *gdpP*, which is involved in the stringent response (28). Similarly, thermotolerant mutants of *Lactobacillus helveticus* DSM 20075 were obtained by Spus *et al.*, which had different mutations (29). Furthermore, it has been shown that spontaneous thermotolerant mutants of *L. lactis* subsp. *cremoris* SK11 have cross-resistance to other environmental stresses (30). Such cross-protection is based on the assumption that different stresses cause similar damage to the cells on a molecular level, and thereby protection against one stress causes resistance against another (31,32). Besides tremendous work and promising results using laboratory strains of *L. lactis*, transferring those findings to industrial strains is still not trivial and might be difficult or even impossible sometimes (33,34).

Here, we demonstrate how long-term ALE of *L. lactis* subsp. *lactis* biovar *diacetylactis* SD96 (SD96 in short), an industrial dairy strain, was applied to obtain thermotolerant mutants with superior acidification properties. During the long-term propagation, a particular focus was laid on maintaining plasmid-encoded growth and flavor forming capacities by using milk as the growth medium for the ALE. SD96 was sequenced, allowing the in-depth analysis of the mutations acquired during the ALE (see **Chapter 2**, (35)). Furthermore, RNA-sequencing analysis revealed how the transcriptome of the mutant strains was altered during the ALE.

3.3 Results

3.3.1 Isolation of Mutant Strains with Increased Thermotolerance

As a starting point for the ALE, we chose *L. lactis* SD96, a dairy isolate, which grows well in milk, and is generally insensitive to phage attack (see **Chapter 2**, (35)). Although the optimum growth temperature for this particular strain is between 30 and 37°C, growth was possible at 39°C in both LM17 broth and reconstituted skim milk (RSM) (data not shown).

SD96 was subjected to two different adaptation procedures: [1] growth during recurring heat shock (42°C for 1 h) and [2] growth at constant high temperature, which finally resulted in the three mutant strains JA24 [1], RD01 and RD07 [2]. JA24 and RD01 were both isolated after approx. 150 generations, and RD07 after approx. 400 generations in ultra-high-temperature (UHT) treated milk. RD01 was isolated at 40°C, and RD07 was isolated at 41°C. Noteworthy, growth under adaptation procedure [2] was much slower compared to procedure [1], which had the effect that RD01, even though similar generations had passed, was adapted several months longer than JA24. Likewise, RD07 was adapted nearly one and a half years longer than JA24.

3.3.2 ALE Results in Strains that Acidify Milk Faster at Both Low and High Temperature

SD96 and the mutant strains JA24, RD01, and RD07 were tested in various milk fermentations. The time required for the strain to acidify to pH 5.2 was used to compare the mutant strains in UHT milk (**Figure 3.1**) and pasteurized milk (PM) (**Figure 3.2**). In UHT milk, the effect of adaptation was most apparent as SD96 acidified this growth medium rather poorly even at 30°C (13.1 ± 0.2 h). All three mutant strains grew faster than SD96 at 30°C (JA24: 7.4 ± 0.2 h, RD01: 6.4 ± 0.1 h, RD07: 6.0 ± 0.1 h). At 37°C, SD96 acidified the UHT milk slowly as well (12.9 ± 0.2 h), but the mutant strains were comparably fast (JA24: 6.6 ± 0.2 h, RD01: 6.3 ± 0.1 h, RD07: 5.7 ± 0.2 h). Throughout all temperatures, RD07 displayed the fastest acidification, whereas the difference was most striking at 40°C (7.8 ± 0.4 h) and 41°C (11.1 ± 0.4 h), where the other mutant strains were either slowed down dramatically (40°C) or did not grow at all (41°C). When the 42°C heat shock treatment scheme used for isolating JA24, was applied to the mutant strains, they also displayed improved performance (SD96: 16.0 ± 0.3 h, JA24: 9.1 ± 0.8 h, RD01: 7.3 ± 0.3 h, RD07: 7.3 ± 0.1 h).

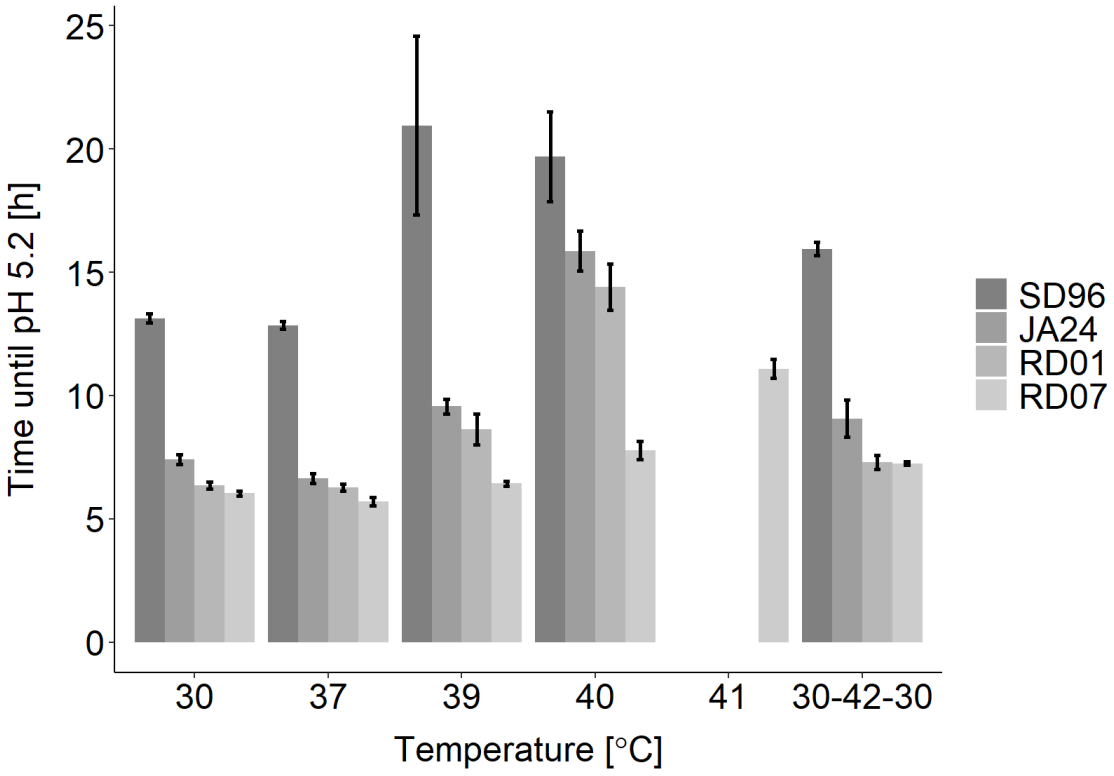

Figure 3.1 Time until reaching pH 5.2 in ultra-high-temperature (UHT) treated milk. SD96 was compared to the mutant strains JA24, RD01 and RD07 at 30°C, 37°C, 39°C, 40°C and 41°C. Additionally, the strains were tested by applying the temperature profile "30-42-30," meaning that the strains were grown for 1 h at 30°C, followed by incubation for 1 h at 42°C and then returning to 30°C for 24 h. Experiments were carried out in duplicates.

Because of the weak performance of SD96 in UHT milk, the effect of the high-temperature adaptation was more revealing, when PM was used, in which SD96 grew fast at 30°C (6.3 ± 0.2 h). Again, RD07 displayed the fastest acidification throughout all temperatures and interestingly, even at 22°C and 30°C the mutant strains outperformed SD96 (30°C: SD96: 6.3 ± 0.2 h, JA24: 5.5 ± 0.1 h, RD01: 5.6 ± 0.2 h, RD07: 5.3 ± 0.2 h). At 37°C SD96 already showed slower acidification compared to 30°C (7.2 ± 0.1 h), while JA24 and RD01 were less effected (6.1 ± 0.2 h and 6.1 ± 0.1 h) and RD07 acidified even slightly faster (5.1 ± 0.1 h). At 41°C RD01 and RD07 were the only strains, which were able to acidify PM. While RD07 acidified reasonably fast (10.9 ± 0.3 h), RD01 was severely hampered (19.8 ± 0.4 h). As we used RSM for the transcriptomic analysis, we also tested acidification in this medium and found that it was similar to acidification in PM. At 30°C, RD07 was fastest with 5.5 ± 0.8 h, followed by JA24 (6.5 ± 0.1 h), RD01 (7.0 ± 0.2 h) and SD96 (7.3 ± 0.1 h). At 39°C the same order was observed: RD07 (6.7 ± 0.5 h), followed by JA24 (8.7 ± 0.6 h), RD01 (9.0 ± 0.4 h) and SD96 (12.2 ± 1.6 h).

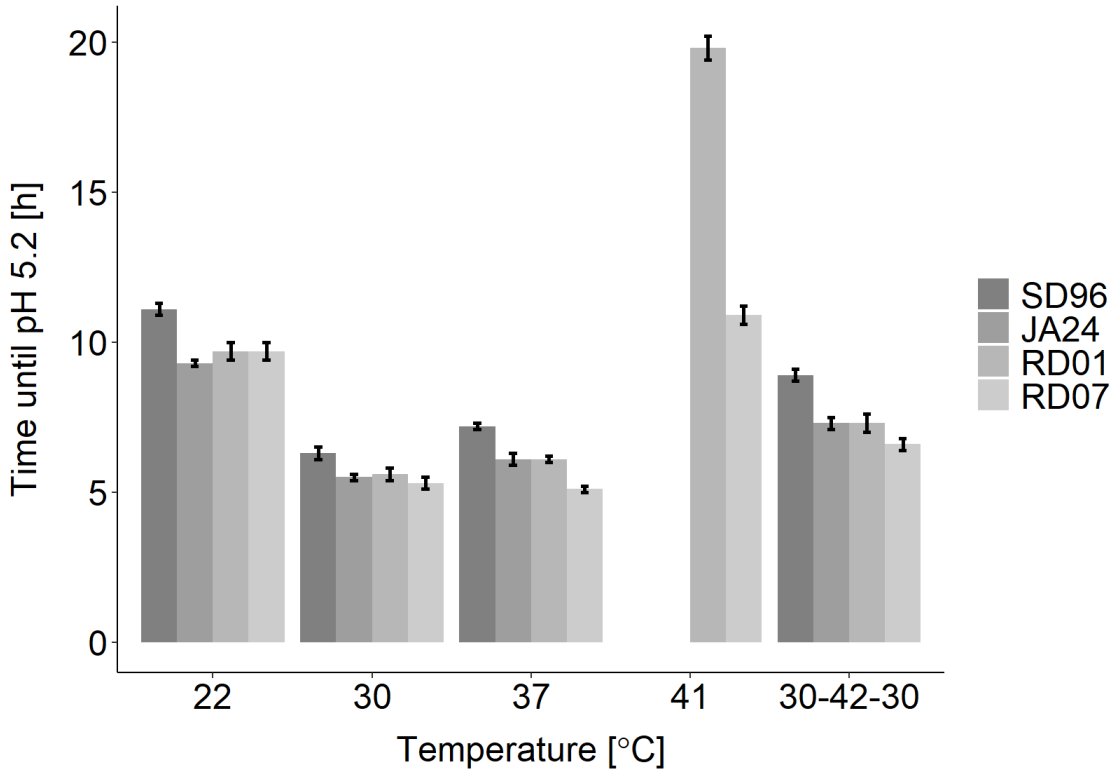

Figure 3.2 Time until reaching pH 5.2 in pasteurized milk (PM). SD96 was compared to the mutant strains JA24, RD01 and RD07 at 22°C, 30°C, 37°C and 41°C. Additionally, the strains were tested applying the temperature profile "30-42-30" meaning that the strains were grown for 1 h at 30°C, followed by incubation for 1 h at 42°C and then returning to 30°C for 24 h. Experiments were carried out in duplicates.

3.3.3 JA24 and RD01 Provide Appealing Flavor in High-Cooked Havarti-Style Cheese

As an initial test for their applicability in a starter culture, the mutant strains JA24 and RD01 have been tested in cheese trials at Arla Foods, Denmark. Two batches of cheese were made, one including the mutant strains and the other without them. Before inoculation, JA24 and RD01 were mixed 1:5 (vol/vol) with a commercially available undefined mesophilic starter culture (Flora-Danica, Chr. Hansen, Hørsholm, Denmark) produced as a bulk starter. Havarti-style cheeses were made according to Arla's internal protocols using an exceptionally high cooking temperature of 39.5°C. The resulting Havarti-style cheeses were evaluated by a panel of six experts at Arla Foods. Cheeses made with starter cultures including the mutants were found to have a more appealing taste compared to the control cheeses according to the expert's judgment. More cheese trials, also including RD07, are required to explore the benefits of mutant strains for flavor development fully. Furthermore, detailed flavor profiling, for example, by GC-MS, would provide a quantifiable measurement of the superior taste of harder cheeses made by using the mutant strains.

3.3.4 RD01 and RD07 are More Susceptible to Attack by Selected Phages

It is well known that ALE, especially at high temperatures, can result in plasmid loss or gene deletions (27). Such genome reduction may provide a growth advantage due to the reduced metabolic burden for maintaining the plasmids, which are often not necessarily required for survival under the provided conditions. SD96 is a fully phage-resistant strain, which carries ten plasmids. Some plasmids, including pSD96_01, pSD96_06, pSD96_07, and pSD96_10 encode phage-defense systems (see **Section 3.3.5**). As phages were absent during the ALE, we speculated that the plasmids responsible for phage-defense might have been lost. To evaluate whether the mutants had indeed obtained a higher susceptibility to phage attack, they were tested against the comprehensive phage collection at the Italy-based company Sacco S.r.l., the company that initially provided SD96 (see **Chapter 2**, (35)).

As expected, SD96 was resistant to all of the tested phages (> 100) (**Figure 3.3**). Similarly, JA24 was also entirely resistant to all phages, which was in alignment with its comparably short adaptation time. RD01 and RD07, however, which had been adapted at constant high temperature for a much longer time (several months, see **Section 3.6.1**), were susceptible to ten phages. Both strains were attacked by the same phages, and in particular by ϕSa1, ϕSa3, ϕSa4, ϕSa5, ϕSa6, ϕSa7 from Sacco's private collection (phage identifiers were changed due to confidentiality) and ϕBil67, ϕC6A and ϕP001 from Félix d'Hérelle Reference Center for Bacterial Viruses (Université Laval, Canada). These last three phages belong to the c2 group of the Siphoviridae family, and phages belonging to this family are rarely involved in failed dairy fermentations (36,37). Additionally, RD01 was attacked by ϕSa8, but RD07 was resistant to this phage. In contrast to that, RD07 was sensitive to ϕSa2, but RD01 was not. Otherwise, the two strains differed slightly regarding the passage in which the attack was observed. The increased susceptibility of RD01 and RD07 suggested that phage-defense systems had been lost during the ALE, which was analyzed further using genome sequencing (see **Section 3.3.6**).

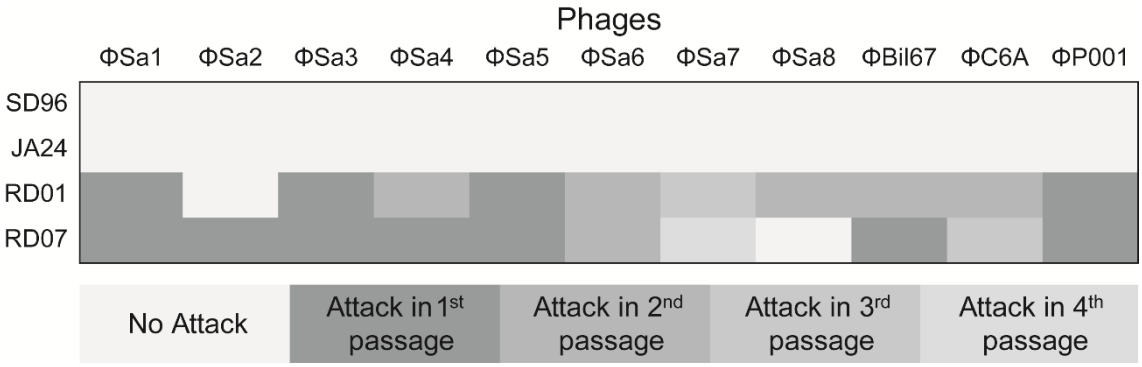

Figure 3.3 Phage profiling of SD96 and the mutant strains JA24, RD01, and RD07. Over 100 phages were tested, of which 26 were from Félix d'Hérelle Reference Center for Bacterial Viruses (Université Laval, Canada). Only phages, which attacked at least one of the mutants, are shown. The phages were enriched in four sequential passages in the presence of the respective strain.

3.3.5 Genome Analysis of the Parent Strain SD96

In order to reveal the genetic changes, which had occurred during the ALE experiment, we first de novo sequenced the whole genome of SD96, and this is described elsewhere (see **Chapter 2**, (35)). The chromosome of SD96 (NCBI reference sequence NZ_CP043523) (2.4 mb) contains 2368 coding sequences (CDS), 19 ribosomal RNAs, and 64 tRNAs. According to NCBI, its closest relative reference genome is IL1403 (92.6% symmetric identity), and the overall closest sequenced strain is Fm03 (92.9% symmetric identity) (38).

Several genes of industrial relevance are located on the ten plasmids of SD96 (**Table 3.1**). The largest plasmid pSD96_02 (64.9 kb, NZ_CP043526) contains genes essential for growth in milk, and most importantly, the cell wall-bound protease PrtP (FTN78_RS13140). Genes for lactose utilization (FTN78_RS12660- FTN78_RS12700), which are also essential for growth in milk, were found on plasmid pSD96_05 (49.6 kb, NZ_CP043524). This plasmid also encodes an *opp* operon responsible for oligopeptide transport (FTN78_RS12805 to FTN78_RS12815 & FTN78_RS12525 to FTN78_RS12535). A second *opp* operon is located on the chromosome (FTN78_RS07325 to FTN78_RS07345). As characteristic for *L. lactis* subsp. *lactis* biovar diacetylactis strains, SD96 contains a plasmid, pSD96_04 (NZ_CP043528), that carries the citrate permease (CitP, FTN78_RS13490), which enables citrate utilization and diacetyl formation. Besides these crucial and well-described genetic elements, SD96 was found to harbor seven other plasmids, of which three were comparably large: pSD96_01 (11.7 kb, NZ_CP043525), pSD96_03 (37.4 kb, NZ_CP043527) and pSD96_06 (27.4 kb, NZ_CP043518). The remaining four smaller plasmids ranged in size from 2.7 kb to 7.9 kb. pSD96_01 encodes amongst other genes the *cadA* gene (FTN78_RS12845), which is involved in cadmium translocation, and one specificity subunit of a type I restriction-modification system (RMS) (FTN78_RS12835). Among other genes, pSD96_06 encodes a complete type I RMS, including specificity, modification, and restriction subunits (FTN78_RS00005 to FTN78_RS00015). The function of pSD96_03 was elusive, but many genes were assigned to the Clusters-of-Orthologous-Groups (COG) category “Intracellular Trafficking and Secretion,” implying a potential role in mediating transport across the cell envelope. Similarly, the four small plasmids pSD96_07 to pSD96_10 could not be assigned to a specific function, but both pSD96_07 (NZ_CP043519) and pSD96_10 (NZ_CP043522) encode specificity subunits of type I RMSs (FTN78_RS00140 and FTN78_RS00240, respectively). pSD96_08 (NZ_CP043520) encodes genes for bacteriocin production (lactoccocin family), which was confirmed using BAGEL4 (39). Many of the plasmid-encoded genes required for mobilizing the plasmid (*mobA* or *mobC*).

Table 3.1 Plasmids of SD96. Putative functions were derived from gene annotations provided through the NCBI Prokaryotic Genome Annotation Pipeline. Accession numbers for the NCBI reference sequences are as follows: pSD96_01: NZ_CP043525, pSD96_02: NZ_CP043526, pSD96_03: NZ_CP043527, pSD96_04: NZ_CP043528, pSD96_05: NZ_CP043524, pSD96_06: NZ_CP043518, pSD96_07: NZ_CP043519, pSD96_08: NZ_CP043520, pSD96_09: NZ_CP043521, pSD96_10: NZ_CP043522.

Name	Size [kb]	Function	Relevant Annotated Genes or Operons
pSD96_01	11.7	Phage defense	*cadA* (Cd transport), specificity subunit of type I RMS, *mobC*
pSD96_02	64.9	Protein degradation	PrtP protease, *mobC*, *amaP*, Cu and Cd transport
pSD96_03	37.4	Unknown function	Conjugation protein TraE, thioredoxin, *mobC*
pSD96_04	8.3	Citrate transport	CitP – citrate transporter, specificity subunit of type I RMS
pSD96_05	49.9	Lactose metabolism	*opp* operon, *lac* operon, Mn^{2+}-transport, universal stress protein, D-LDH, *mobA*
pSD96_06	25.4	Phage defense	Complete type I RMS, *mobC*
pSD96_07	7.3	Phage defense	specificity subunit of type I RMS
pSD96_08	4.6	Unknown function	lactococcin family bacteriocin, *mobC*
pSD96_09	2.7	Unknown function	-
pSD96_10	7.9	Phage defense	specificity subunit of type I RMS, *mobC*

3.3.6 Genome Resequencing Reveals Plasmid Loss and Diverse Mutations

The three mutant strains JA24, RD01, and RD07 were sequenced, and mutations in both the chromosome and the plasmids were evaluated. The mutations are summarized in **Table S 3.1** and shown in **Figure 3.4**.

JA24 still contained all ten plasmids, and only two specific mutations in the genome were identified. The first mutation (JA24-1) was synonymous, and the second mutation (JA24-2) was located in *codY*, a pleiotropic transcriptional regulator (FTN78_RS09305), where arginine at position 237 was changed to serine (R237S).

RD01 and RD07 were derived from the same lineage, and therefore it was not surprising that they shared some mutations. Different from JA24, both strains had lost genetic material during the ALE through plasmid loss and a large chromosomal deletion (approx. 80 kb) (RD01-Gap/RD07-Gap). Within the deleted region, several CDS were located, and below we discuss this further (**Table S 3.2**). Additionally, the following plasmids had been lost in both strains: pSD96_01, pSD96_03, pSD96_06, and pSD96_10, in total approx. 82 kb. RD01 and RD07 had two mutations in common: one synonymous mutation (RD01-1/RD07-3) and another in UDP-N-acetylmuramate-L-alanine ligase (RD01-4/RD07-8, FTN78_RS11940). This mutation caused a change from

phenylalanine to leucine at position 54 (F54L). The affected enzyme is involved in the biosynthesis of peptidoglycan and is also denoted as “MurC.” Furthermore, RD01 bore two further mutations: RD01-2 was located 64 bp upstream of *codY* (FTN78_RS09305) and thereby potentially affected transcription by altering the promoter region. A CodY-box, a sequence mediating CodY-binding and thereby auto-regulation of *codY*-transcription, was previously identified to be located 86 bp upstream of *codY*, which is close to the RD01-2 mutation (40). RD01-3 caused V469L mutation in bifunctional (p)ppGpp synthetase/guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase (FTN78_RS10010), which is also called RelA.

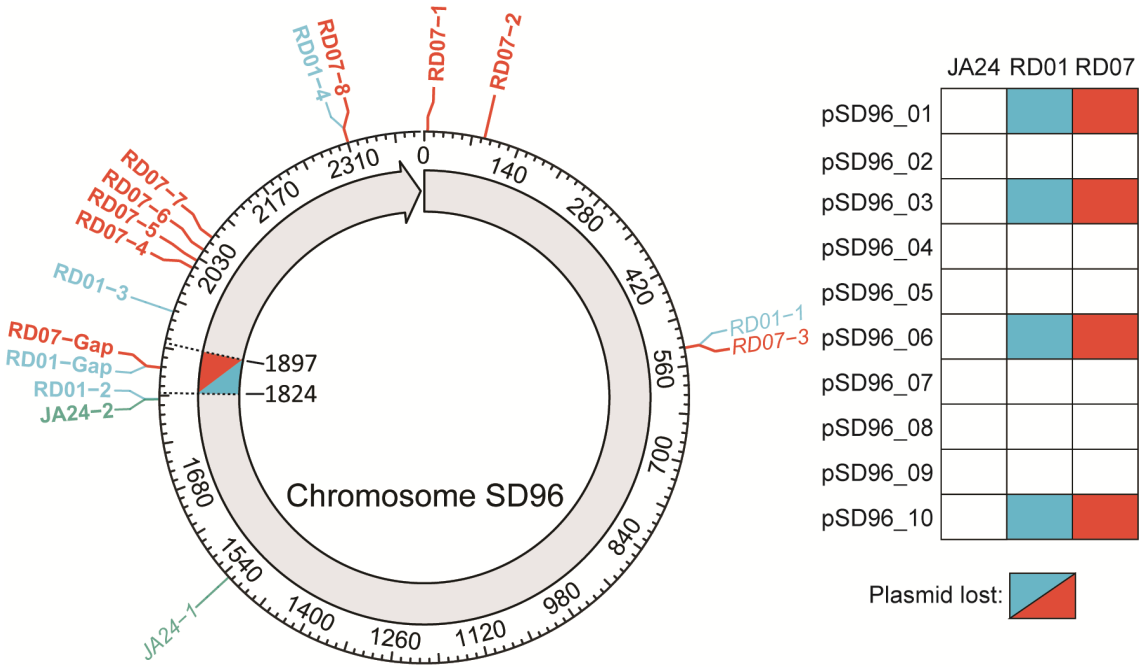

Figure 3.4 Results from genome resequencing of the mutant strain JA24, RD01, and RD07. Left: Mutations on the chromosome are indicated in different colors (JA24: green; RD01; blue, RD07, red). RD01 and RD07 share some mutations: RD01-4 and RD07-8, RD01-1, and RD07-3. Furthermore, an approx. 80 kb-deletion was identical (RD01-Gap and RD07-Gap), which is indicated on the track. Silent mutations are written in italic font (JA24-1, RD01-1, RD07-3). Right: Plasmid profile of the mutant strains. Plasmid loss is indicated by a colored field.

In RD07, in total, eight mutations were identified, of which two have been described above (RD07-3 and RD07-8). RD07-1 causing R546S was found in the phenylalanine-tRNA ligase β-subunit (FTN78_RS00265). RD07-2 was responsible for an amino acid change V957E in a hypothetical protein (FTN78_RS00795), which shares similarity with a phage protein from *L. lactis* subsp. *lactis* KF147 (96.26% sequence identity) and belongs to a prophage in SD96, which were identified using the PHASTER tool (41). RD07-4 was located in a DNA primase gene (FTN78_RS10440), which also belongs to a prophage, and caused A410S mutation. RD07-5 was found upstream of *hflB*/*ftsH*, which encoded the ATP-dependent zinc metalloprotease FtsH (FTN78_RS10530). RD07-6 was responsible for a mutation (D311Y) in RexA, the ATP-

dependent exonuclease subunit A (FTN78_RS10615). Finally, RD07-7 was located in *purR* encoding the *pur* operon regulator (FTN78_RS10700), which is a phosphoribosyl pyrophosphate (PRPP) sensing transcriptional activator in *L. lactis*, and caused a G206S mutation.

3.3.7 RNA-Sequencing Revealed Changes in the Global Transcription Patterns Caused by Mutations in Transcriptional Regulators

To further characterize the changes that had taken place in the mutant strains JA24, RD01, and RD07, we decided to use RNA-Sequencing to conduct a global transcription analysis for each of the three strains. This type of analysis is useful for studying pleiotropic effects; in our case, the effects of having mutations in CodY and PurR, which are transcription factors and control the expression of many genes. The strains were compared on three levels:

1) JA24, RD01, and RD07 each at 30°C *versus* SD96 at 30°C
    - DEGs responsible for the faster acidification of the mutants at 30°C compared to SD96 would be identified.
    - The differential expression would reveal how the mutants adapted to the UHT milk environment.
2) SD96/JA24/RD01/RD07 at 39°C *versus* SD96/JA24/RD01/RD07 at 30°C
    - Genes induced or repressed at high temperatures are identified in this comparison.
    - By analyzing SD96, differentially expressed genes could be identified, and their temperature-induced expression or repression in the mutants could be studied, possibly revealing genes or operons essential for thermotolerance.
3) JA24, RD01, and RD07 each at 39°C *versus* SD96 at 39°C
    - Genes that are differentially expressed at high temperatures are identified using this comparison. This comparison complements comparison 2.
    - The differential expression would reveal how thermotolerance was achieved in the mutants.

We chose 39°C because this temperature allowed for reasonable growth and acidification in RSM for all strains. Sample distance and principal component analysis (PCA) confirmed the clustering of the biological replicates, and thus the eligibility of the data for the above-described analysis (**Figure S 3.2** and **Figure S 3.1**). As another quality check, the mutations within CDS regions were confirmed for all mutant strains using the RNA sequencing data. Next, before analyzing the effects of the mutations on transcription, Clusters-of-Orthologous-Groups (COG) analysis was conducted to evaluate the global expression patterns of the mutant strains.

As an overview, for the three mutants, volcano plots were derived, showing significantly enhanced and reduced genes (absolute log2-fold change > 1 and adjusted p-value (p_{adj}) < 0.05) in

comparison to SD96 at 30°C and at 39°C (**Figure 3.5**). Only genes, which were present in both SD96 and the mutant strains, were considered, while genes located on lost plasmids or the deleted region of RD01 and RD07 were removed from the figures. These deleted genes were removed because they appear as strongly downregulated and thereby distort the analysis. Gene-deletions and plasmid loss are analyzed and discussed separately. Generally, with increased adaptation time, the number of DEGs increased. This trend was observed for both the differential gene expression at low temperature (30°C) and high temperature (39°C). At 39°C, the numbers of DEGs were higher for all strains, and differential expression occurred with greater log2-fold changes.

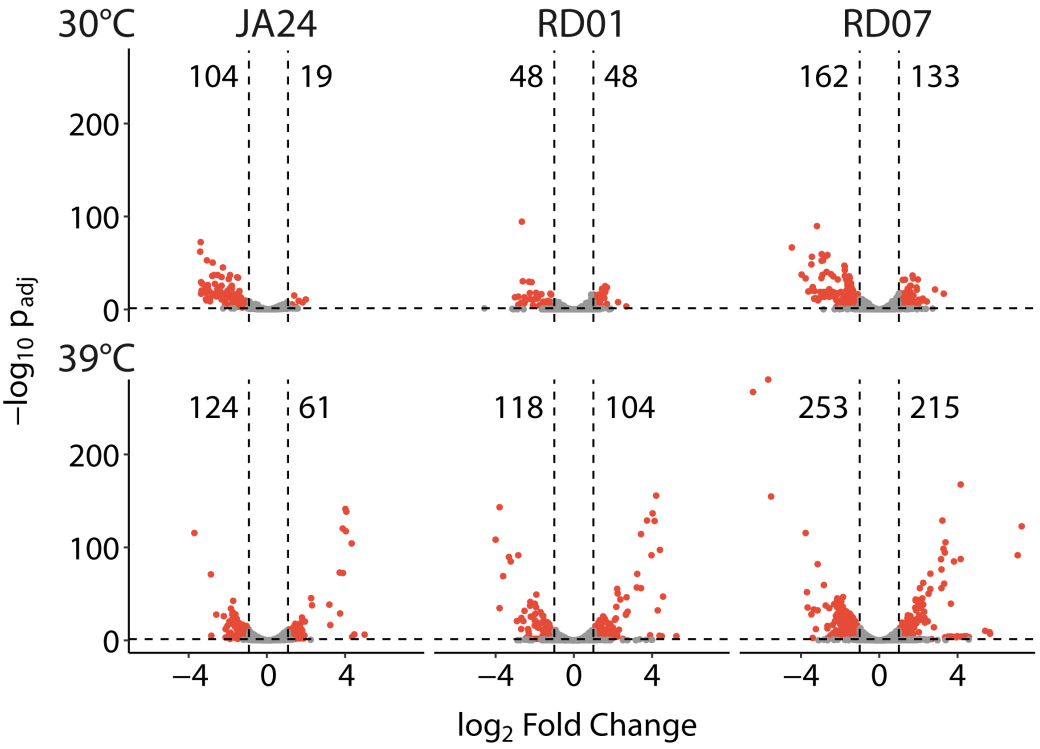

Figure 3.5 Volcano plots showing DEGs for the three mutant strains JA24, RD01, and RD07 compared to SD96 at 30°C (upper panel) and 39°C (lower panel). The adjusted p-value (-$\log_{10}$) calculated by DESeq2 was plotted against log2-fold change. Numbers were derived from two biological replicates. Significant DEGs are shown in red with absolute log2-fold change > 1 and $p_{adj} < 0.05$ and non-significant DEGs in grey. Deleted genes in RD01 and RD07 were not counted here. Total numbers of significantly DEGs are shown in the figures.

COG analysis for comparison 1 (JA24, RD01, and RD07 each at 30°C *versus* SD96 at 30°C) (**Figure 3.5**, upper panel) revealed that most DEGs were within the category “Amino Acid Metabolism and Transport” (“Amino Acids”) (**Figure 3.6**). In this category, RD07 showed the most considerable changes with 45 DEGs, of which 37 were reduced, and 8 were enhanced. JA24 also showed many DEG in this category (Up: 0, Down: 30), while RD01 showed the weakest changes

in the category (Up: 7, Down: 9). The latter, however, provided a nearly balanced differential gene expression in this category and was thereby distinct from JA24 or RD07. Noteworthy, a large amount of DEGs fell into the category “Function Unknown” (JA24: 13, RD01: 18, RD07: 32), which was the second category. The third category was “Inorganic Ion Transport and Metabolism” (“Inorganic Ions” in short), and here, RD07 showed the most noticeable changes compared to JA24 and RD01. Here, RD07 showed a nearly balanced pattern of DEGs (RD07: Up: 12, Down: 18), while RD01 and JA24 showed more reduced DEGs (RD01: Up: 2, Down: 9, JA24: Up: 0, Down: 13). Furthermore, it was remarkable that for RD07, many genes in the categories “Carbohydrate Metabolism and Transport” (“Carbohydrates” in short) and “Nucleotide Metabolism and Transport” (“Nucleotides” in short) were enhanced, while this was not the case for JA24 and RD01. Moreover, the overall numbers of DEGs for JA24 and RD01 were less enhanced compared to RD07 (**Figure 3.5)**. The genes located on the lost plasmids and the deleted 80 kb fragment in RD01 and RD07, mostly belonged in the categories “Replication/Repair,” “Function Unknown,” “Transcription” and “Defense Mechanisms” with descending representation.

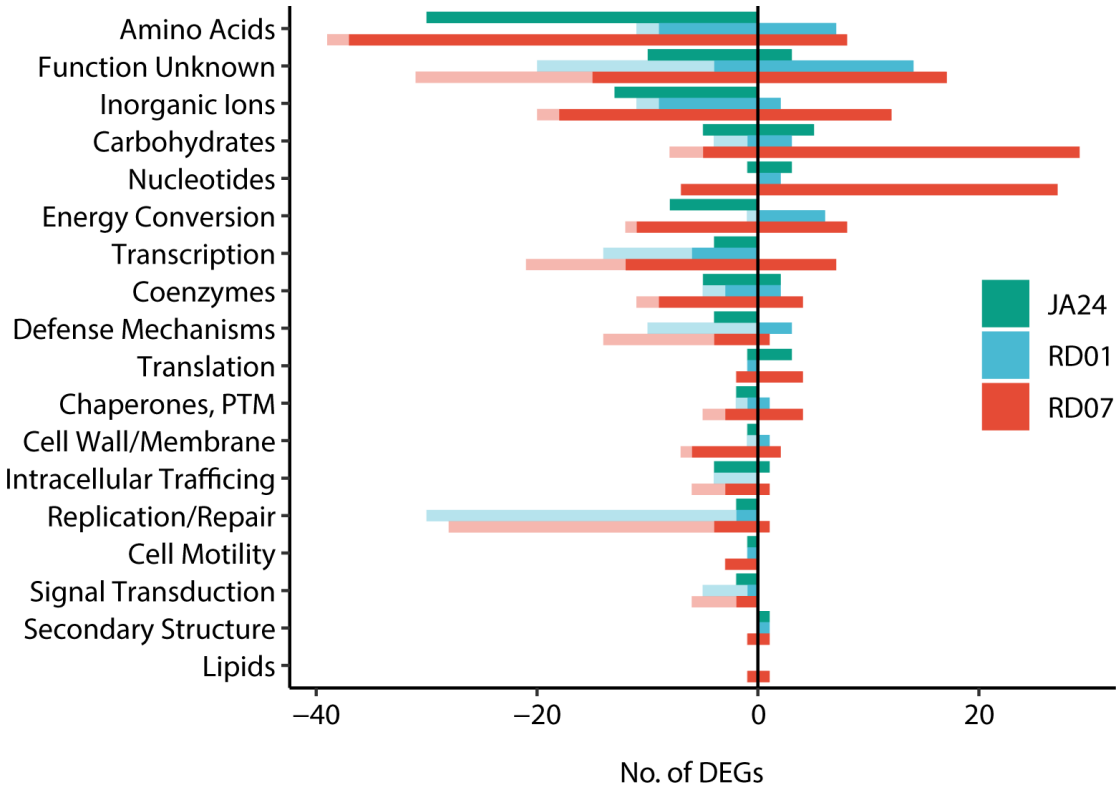

Figure 3.6 Clusters-of-Orthologous-Groups analysis of JA24 (green), RD01 (light blue), and RD07 (red) at 30°C compared to SD96 as reference. The number of DEGs with significant enhancement or reduction are shown (absolute log2-fold change > 1 and padj < 0.05). Categories were sorted from top to bottom with descending numbers of DEGs. Category names were abbreviated for better visualization. Genes located on lost genetic elements in RD01 and RD07 are shown in light colors and were not included in the ranking.

3.3.8 The Number of DEGs Induced by Growth at High Temperatures Decreased with Adaptation Time

DEGs induced by high temperatures were determined for each strain (comparison 2), revealing a disproportional effect. For example, with increasing adaptation time, the total number of DEGs induced by growth at high temperatures decreased. While SD96 showed the most DEGs with 348 enhanced and 370 reduced genes, RD07 only showed 101 enhanced genes, and 191 reduced genes at high temperatures (**Figure 3.7**). Thus, the physiological state of the mutant strains was less affected at high temperatures compared to the parent strain. Many strongly enhanced genes (log2-fold changes > 3.0) in SD96 and JA24 were located on the chromosomal region, which had been lost in RD01 and RD07 (RD01-Gap/RD07-Gap) (**Figure 3.7**).

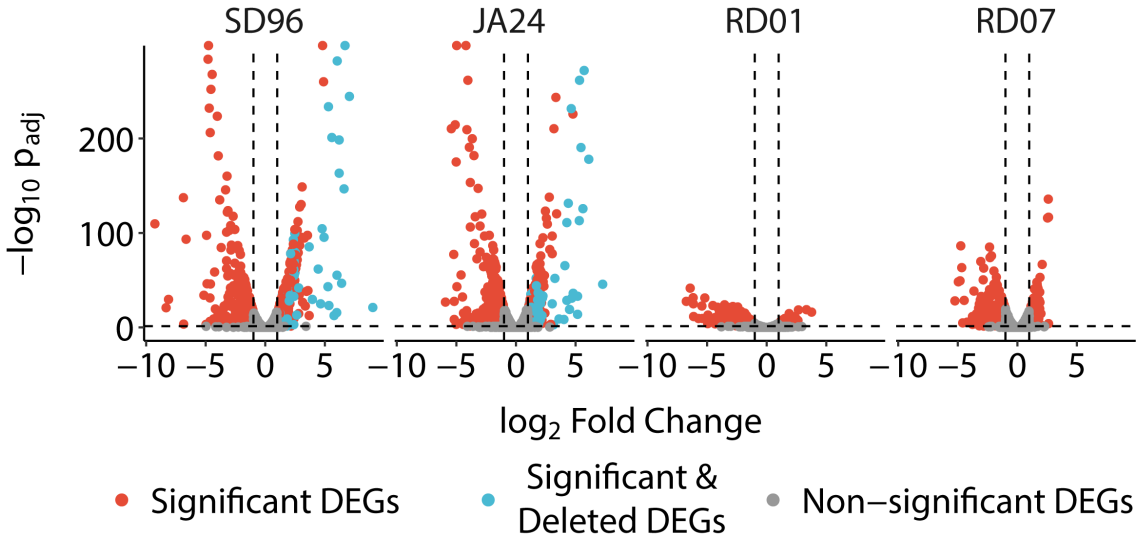

Figure 3.7 Volcano plots showing DEGs for SD96 and the three mutant strains JA24, RD01, and RD07 comparing expression at 30°C with expression at 39°C. The adjusted p-value (-$\log_{10}$) calculated by DESeq2 was plotted against log2-fold change. Numbers were derived from two biological replicates. Significant DEGs are shown in red with absolute log2-fold change > 1 and p_{adj} < 0.05 and non-significant DEGs in grey. Numbers of enhanced ("Up") and repressed ("Down") DEGs are as follows: SD96: Up: 348, Down: 370; JA24: Up: 256, Down: 298; RD01: Up: 139, Down: 245; RD07: Up: 101, Down: 191. Significant DEGs in SD96 and JA24 located on regions, which were deleted in RD01 and RD07, are shown in blue.

For comparison 2 (SD96/JA24/RD01/RD07 at 39°C *versus* SD96/JA24/RD01/RD07 at 30°C), many DEGs were assigned to the category "Function Unknown" (SD96: 115, JA24: 91, RD01: 63, RD07: 53) (**Figure 3.8**). Large fractions of these DEGs are shared with SD96, 86% for JA24, 76% for RD01, and 87% for RD07. Next, the category "Translation" was most effected at high temperature, and many genes in all strains were reduced, which suggested that at high temperature, overall protein synthesis was reduced due to reduced growth rates, as the effect was decreasing with adaptation time. In SD96 and JA24, 66 DEGs fell into this category, while in

RD01, only 45, and in RD07, only 31 DEGs were assigned to “Translation.” Of those DEGs, 85%, 78%, and 68% were shared with SD96, respectively. The decreasing number of DEGs in this category stressed that in RD07, the protein synthesis machinery was less affected by the high temperature compared to SD96, JA24, or RD01. The next category was “Amino Acid Metabolism and Transport,” where all strains showed a somewhat balanced differentially gene expression, but again with SD96 having the most DEGs (Total: 63) and RD07 the least DEGs (Total: 27). This trend was observed nearly throughout all categories, except for “Carbohydrate Metabolism and Transport,” in which RD07 showed the most reduced differential expression with 35 DEGs (83% shared with SD96), followed by JA24 (33 reduced DEGs (67%)), RD01 (31 reduced DEGs (65%)) and finally SD96 with only 29 reduced DEGs. Remarkably, the category “PTM, protein turnover, chaperone function” (“Chaperones, PTM” in short) shows many enhanced DEGs for all four strains, which was expected due to the stress exposed by the high temperature. Nevertheless, again RD07 had comparably few DEGs in this category (Total: 8, 75% shared with SD96), while SD96 had the most (Total: 20).

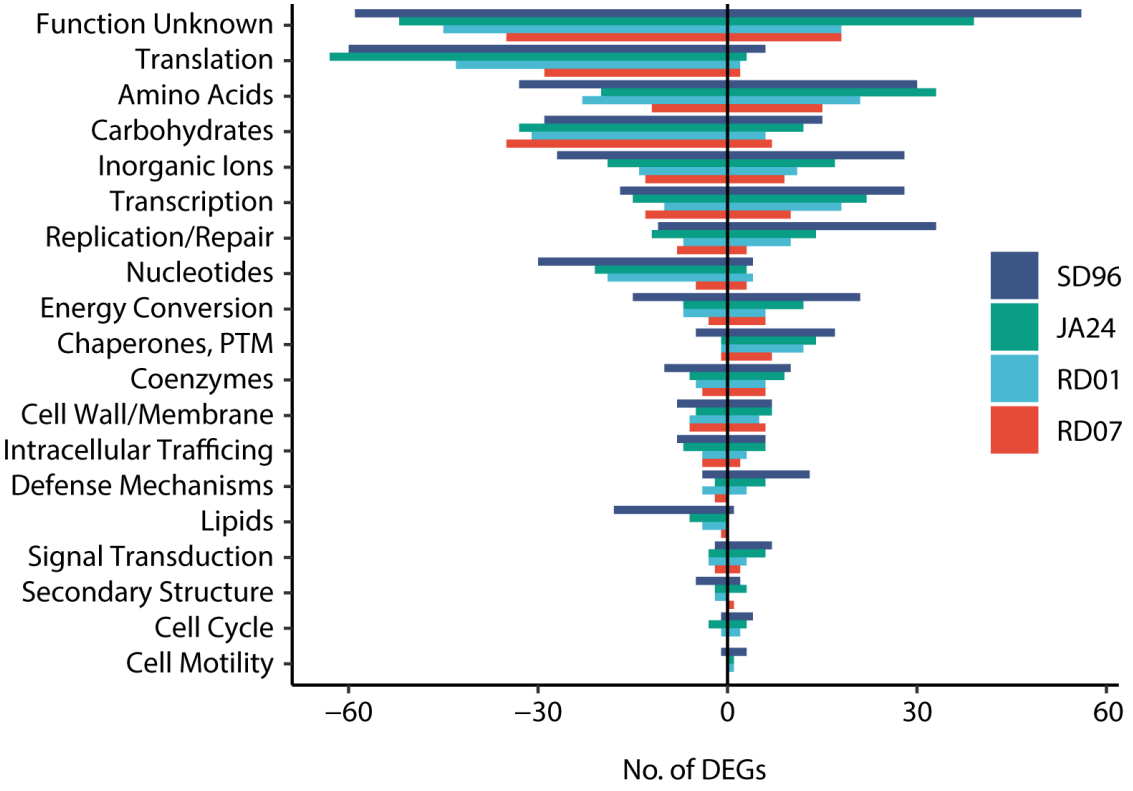

Figure 3.8 Clusters-of-Orthologous-Groups analysis of SD96 (dark blue), JA24 (green), RD01 (light blue), and RD07 (red) comparing gene expression at 39°C and 30°C. DEGs with significant enhancement or reduction (absolute log2-fold change > 1 and padj < 0.05) were counted. The categories were arranged according to the absolute number of DEGs with descending order. Category names were abbreviated for better visualization. The lost genetic elements in RD01 and RD07 were not removed in this analysis as the comparison was not across strains.

The COG analysis shown in **Figure 3.8**, corresponding to comparison 2, revealed which categories had been induced or repressed in the individual strains when grown at high temperatures. Next, the changes across strains during growth at high temperature were determined, corresponding to comparison 3 (JA24, RD01, and RD07 each at 39°C *versus* SD96 at 39°C) (**Figure 3.5**, lower panel, and **Figure 3.9**). Again, as shown for the other two COG analyses, the category "Function Unknown" contained most DEGs. Then, second-most DEGs were assigned to the category "Inorganic Ion Transport and Metabolism," where RD07 showed most DEGs (Up: 21, Down: 32), followed by RD01 (Up: 9, Down: 9) and JA24 (Up: 8, Down: 8). Interestingly, in the next category, "Amino Acid Metabolism and Transport," RD01 showed the most enhanced DEGs (Up: 17), whereas the other two strains only showed 8 or 11 enhanced DEGs (JA24 or RD07, respectively). Conversely, RD07 had more than twice as many reduced DEGs in this category (RD07: 31, JA24: 15, RD01: 5). The only categories, which showed high numbers of enhanced DEGs for some strains were "Nucleotide Metabolism and Transport" and "Translation," where RD07 dominated, but also RD01 showed some enhancement. In the former category, RD07 had 34 enhanced DEGs, and RD01 had 14 DEGs. In RD07, 46 enhanced genes were assigned to "Translation," whereas the other two strains only contained few genes with differential expression in this category (RD01: Up: 5, Down: 4, JA24: Up: 4, Down: 2). These two observations of enhanced differential expression, suggest that RD07 might overexpress genes in the categories "Nucleotide Metabolism and Transport" and "Translation" at high temperature. However, **Figure 3.8** showed that those genes were reduced at high temperatures. Therefore, both comparisons (**Figure 3.8** and **Figure 3.9**) should be considered for analyzing changes to the mutant strains at high temperatures. Again for RD01 and RD07, the categories "Replication/Repair," "Function Unknown," "Transcription," and "Defense Mechanisms" contained most genes, which were located on the lost plasmids or the chromosomal deletion.

Summarizing, the presented data allows the conclusion that compared to the parent strain SD96, all mutant strains showed fewer DEGs when grown at high temperatures and therefore seem to be less affected by the increased temperature (**Figure 3.7**). Nevertheless, the COG analysis revealed that at both 30°C (**Figure 3.6**) and 39°C (**Figure 3.9**), the differences across the strains were more pronounced the longer the mutants were adapted (JA24 < RD01 < RD07). The most significant changes across strains were similar at both temperatures (**Figure 3.6** and **Figure 3.9**) and were found in the categories "Amino Acid Metabolism and Transport," "Inorganic Ion Transport and Metabolism," and "Nucleotide Metabolism and Transport."

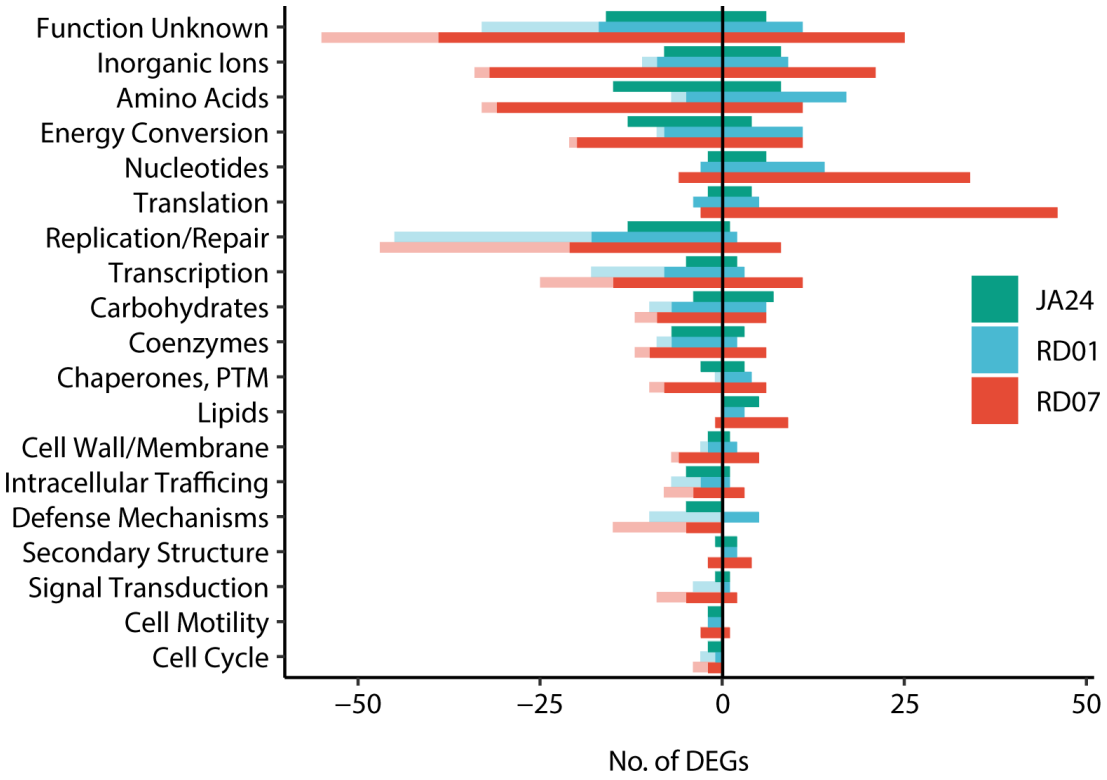

Figure 3.9 Clusters-of-Orthologous-Groups analysis of JA24 (green), RD01 (light blue), and RD07 (red) at 30°C compared to SD96 as reference. The number of DEGs with significant enhancement or reduction are shown (absolute log2-fold change > 1 and padj < 0.05). Categories were sorted from top to bottom with descending numbers of DEGs. Category names were abbreviated for better visualization. Genes located on the lost genetic elements in RD01 and RD07 are shown in light colors and were not included in the ranking.

3.3.9 Influence of the Mutations on Differential Gene Expression

As some of the mutations in the mutant strains were located in promoter regions or transcription factors, we hypothesized that the genes under their regulation might be differentially expressed in the mutant strains and therefore evaluated the expression of all mutated or immediately affected genes (**Table 3.3**). Of the mutated genes, only a few showed differential expression (absolute log2-fold change > 0.5 and $p_{adj} < 0.05$).

In JA24, none of the mutated genes were significantly differentially expressed. In RD01, mutation RD01-2 caused a dramatic reduction of the expression of *codY*, which did not seem to be influenced by growth temperature, as at both 30°C and 39°C *codY* expression was reduced similarly (log2-fold change: -2.7 ± 0.1 and -2.9 ± 0.1, respectively). Accordingly, the downregulation of *codY*-expression leads to the de-repression of *codY*-regulated genes. For example, *dppA*, *ilvD*, *gltD*, *icD*, *citB* and *prtP*, which were all reported to be repressed by CodY (40), were significantly enhanced in RD01 at 30°C (**Table 3.2**). Furthermore, in RD01, the mutated EamA family transporter was differentially expressed at 39°C (log2-fold change = -0.8 ± 0.2) but not at 30°C (**Table 3.3**).

Table 3.2 Differential expression (DE) of selected CodY-regulated genes in JA24, RD01, and RD07 compared to SD96 at both 30°C and 39°C. Log2-fold changes are shown. DE with high p_{adj} (>0.05) and thus low confidence are written in italic font.

CodY-regulon	JA24		RD01		RD07	
	30°C	39°C	30°C	39°C	30°C	39°C
codY	*0.2 ± 0.1*	*0.3 ± 0.1*	-2.7 ± 0.1	-2.9 ± 0.1	0.6 ± 0.1	*0.2 ± 0.1*
dppA	-2.3 ± 0.2	*-0.5 ± 0.3*	1.3 ± 0.2	1.3 ± 0.3	-2.7 ± 0.2	-2.1 ± 0.3
ilvD	-2.0 ± 0.2	-0.7 ± 0.2	1.4 ± 0.1	1.9 ± 0.2	-3.2 ± 0.2	-1.3 ± 0.2
gltD	-0.4 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	2.2 ± 0.1	-1.8 ± 0.1	*-0.3 ± 0.1*
icD	-0.9 ± 0.2	*-0.1 ± 0.3*	1.4 ± 0.2	0.8 ± 0.2	-0.9 ± 0.2	*-0.2 ± 0.3*
citB	-1.5 ± 0.2	*-0.04 ± 0.23*	1.6 ± 0.2	1.1 ± 0.2	-1.4 ± 0.2	-0.7 ± 0.2
prtP	*0.2 ± 0.2*	2.2 ± 0.2	1.6 ± 0.2	3.4 ± 0.2	2.0 ± 0.2	4.2 ± 0.2

Table 3.3 Differential expression (DE) of mutated genes. DE with high p_{adj} (>0.05) and thus low confidence are written in italic font. RD01-2 and RD07-5 were located upstream of *codY* and FtsH, respectively.

Mutation	Affected gene(s)	DE at 30°C	DE at 39°C
JA24-1	LCP family protein	*-0,1 ± 0.1*	0,3 ± 0.1
JA24-2	Pleiotropic transcriptional regulator *codY*	*0,2 ± 0.1*	*0,3 ± 0.1*
RD01-1	EamA family transporter	*-0,7 ± 0.3*	-0,8 ± 0.2
RD01-2	Upstream of pleiotropic transcriptional regulator *codY*	-2,7 ± 0.1	-2,9 ± 0.1
RD01-3	Bifunctional (p)ppGpp synthetase	*-0,3 ± 0.1*	*-0,2 ± 0.2*
RD01-4	UDP-N-acetylmuramate-L-alanine ligase	*0,1 ± 0.2*	0,4 ± 0.1
RD07-1	Phenylalanine-tRNA ligase	0,8 ± 0.2	-1,2 ± 0.1
RD07-2	Hypothetical protein	-0,5 ± 0.2	*-0,1 ± 0.2*
RD07-3	EamA family transporter	*-0,4 ± 0.3*	*0,1 ± 0.2*
RD07-4	DNA primase	*0,7 ± 0.4*	*0,04 ± 0.38*
RD07-5	Upstream of *hflB*/*ftsH* encoding FtsH	-0,5 ± 0.1	-0,8 ± 0.2
RD07-6	RexA, ATP-dependent exonuclease subunit A	*0,2 ± 0.1*	-0,6 ± 0.1
RD07-7	Pur operon regulator, *purR*	1,0 ± 0.2	0,9 ± 0.2
RD07-8	UDP-N-acetylmuramate- L-alanine ligase	*0,2 ± 0.2*	0,5 ± 0.1

In comparison to JA24 and RD01, RD07 provided a more diverse picture. Phenylalanine-tRNA ligase, affected by mutation RD07-1, was enhanced at 30°C (log2-fold change = 0.8 ± 0.2) but downregulated at 39°C (log2-fold change = -1.2 ± 0.1). Mutation RD07-5 in the promoter region of *hflB*/*ftsH* reduced the expression of FtsH at 39° C (log2-fold change = -0.8 ± 0.2), but only moderately at 30°C (log2-fold change = -0.5 ± 1). RexA, ATP-dependent exonuclease subunit A,

which was affected by mutation RD07-6, was also reduced only at 39°C (log2-fold change = -0.6 ± 0.1).

The last mutation (RD07-7) was located in *purR*, the pur operon regulator, whose expression was enhanced at both 30°C and 39°C (log2-fold change: 1.0 ± 0.2 and 0.9 ± 0.2, respectively). Potentially as a consequence of the mutation in *purR*, the purine biosynthesis genes were significantly enhanced in RD07. The purine biosynthesis genes are organized in several operons (42). More specifically at 30°C average log2-fold changes were as follows: *purA*: 1.02, *purB*: 0.88, *purDEK* 1.31 ± 0.11, *purCSQLF* 1.01 ± 0.10, *purMN* 1.341 ± 0.002 and *purH-hprt* 1.05 ± 0.01. At 39°C the differences between RD07 and SD96 were even more pronounced: *purA*: 1.87, *purB*: 1.59, *purDEK* 1.99 ± 0.10, *purCSQLF* 2.09 ± 0.42, *purMN* 2.72 ± 0.46 and *purH-hprt* 2.16 ± 0.15. The strong enhancement of the purine biosynthesis genes in combination with the identified mutation in *purR* indicate that purine or generally nucleotide metabolism is involved in the thermotolerance of RD07.

Regarding the deleted region in RD01 and RD07, transcriptomics analysis revealed that many genes (FTN78_RS09360 – FTN78_RS09480) in the deleted 80-kb area were strongly induced in SD96 at high temperature with an average log2-fold change of 5.2 ± 1.7. Interestingly, of all significantly DEGs in SD96 with log2-fold changes above 3.0 (33 in total), 63 % (21 genes) were located in the deleted area, revealing a hot spot for temperature-induced genes, which can be seen in **Figure 3.7**. The gene with the overall highest enhancement in SD96 (log2-fold change 9.0) was also located in the deleted area and encoded a hypothetical protein (FTN78_RS09425) with similarity to the antirestriction protein (ArdA).

3.4 Discussion

3.4.1 JA24, RD01, and RD07 are Unique Thermotolerant Strains with Industrially Relevant Phenotypes

Here, we reported that it is possible to improve the thermotolerance of *L. lactis* subsp. *lactis* biovar diacetylactis SD96 by using high-temperature ALE. Three mutants JA24, RD01, and RD07 displayed improved acidification at both low and high temperatures. RD07, the most thermotolerant strain, was able to acidify milk at 41°C, which has not been reported for an industrial *L. lactis* until now. Previously, ALE has been used for increasing the thermotolerance of *L. lactis* subsp. *cremoris* MG1363, resulting in the thermotolerant mutant TM29. In this study, a maximal growth temperature of 40°C in GM17 was realized, but pre-incubation at high temperature was required for allowing slow growth at 40°C (26). Here we found that RD07 was able to grow reasonably at 41°C in milk without a pre-incubation step. We also tested RD07 in LM17, and growth at 41°C was possible in this medium as well without a pre-incubation step (data not shown). Generally, strains belonging to the *cremoris* subspecies are less thermotolerant compared to the *lactis* subspecies (43,44), and therefore it is not surprising that RD07 was able

to grow at slightly higher temperatures than TM29. The direct comparison with other thermotolerant *L. lactis* strains is more complicated as different experimental setups have been used. For instance, Dijkstra *et al.* isolated heat-stress-tolerant mutants of *L. lactis* subsp. *cremoris* SK11 and found survival after heat shock at 50°C for 30 min to be improved 1000-fold (30). Similar assays were used by other researchers studying the thermotolerance of *L. lactis* (43,45,46). Such mutants might be resistant to a heat shock, but whether they can grow at elevated temperatures is unknown. Other groups have measured thermotolerance by evaluating growth on solid medium, which does also not correspond to growth in liquid medium (28,31,47).

For understanding the genetic changes leading to thermotolerance in SD96, the genomes of the mutants were sequenced. RD01 and RD07 were isolated from the same lineage, which had been exposed to constant high temperature, and therefore it was not surprising to find that they share some mutations. JA24 was isolated using a different setting, by exposing the culture to reoccurring heat shock (42°C for 1 h) followed by recovery at 30°C. By integrating the genomics analysis with RNA-sequencing-based transcriptomics analysis at both low and high temperatures, it was possible to identify the most likely underlying reason for the thermotolerance, as discussed below. Unambiguous evidence for the mutation's influence is typically presented by re-establishing the isolated mutations in the parent strain. Unfortunately, the genetic manipulation of SD96 was hindered by a low transformation efficiency. Hence further exploration of the effects of the mutations was considered out of scope for this study.

3.4.2 UHT Milk is a Challenging Substrate for SD96

When comparing acidification rates using UHT milk, PM, or RSM, clear differences were apparent. Most strikingly, SD96 acidified UHT milk slowly, which was surprising given that the strain harbors a complete proteolysis machinery. In comparison, its performance in PM or autoclaved RSM (121°C for 5 min) was typical. Similar observations have been described previously (48). We still chose UHT milk as the growth medium for ALE as it is sterile, and proteolysis, as well as caramelization of the lactose, is less pronounced as in fully sterile autoclaved RSM (121°C for 20 min). PM would be the ideal milk for ALE as such milk is usually used for cheese production, but PM was not considered here, because the sterility of the growth medium is crucial for ALE. As the growth of SD96 was slow at high temperatures, contaminating microorganisms would likely take over the culture quickly.

The reason for the different performance of SD96 in UHT milk, PM, and RSM is likely due to different structures of the milk protein caused by the different heat treatments used for preparing the three types of milk. For example, it was shown previously that caseins and whey proteins were more denatured after UHT-treatment compared to pasteurization (49). Furthermore, temperature-induced interaction between casein and whey proteins might also play a role (50). Those structural differences are believed to be the main differences between the milk preparations and might affect the ability of *L. lactis* to metabolize the milk proteins. Consequently,

SD96 potentially suffered from amino acid starvation when grown in UHT milk because of inefficient protein degradation. Therefore, some mutations were likely due to adaptation to UHT milk rather than a high growth temperature. In more detail, adaptation to the UHT-milk environment would be expected to cause mutations in genes responsible for amino acid metabolism and transport.

3.4.3 Adaptation to Growth in UHT Milk Resulted in Mutations Affecting Amino Acid Metabolism

In JA24, only two mutations were identified. JA24-1 is a silent mutation in a LytR-Cps2A-Psr (LCP) family protein, which is homologous to *lcpA* of *L. lactis* subsp. *cremoris* MG1363. *lcpA* deletion strains are not viable, but a conditional deletion showed that LcpA is involved in attaching rhamnan to peptidoglycan and in the organization of the cell wall (51). The mutation found in JA24 results in a codon change from ACC to ACA, which did not affect the expression of the gene in JA24 significantly (**Table 3.3**). Hypothetically, this mutation might influence translation efficiency due to the altered codon, which would result in different LcpA concentrations in JA24 compared to SD96. The codon ACC is less frequently used compared to the ACA codon (13% versus 39% (52)), indicating that the translation efficiency might be increased in the mutant. The second mutation in JA24 was found in the *codY* gene causing R237S mutation in the respective protein. CodY is a branched-chain amino acid (BCAA) sensing pleiotropic transcription regulator, which controls several genes involved in amino acid metabolism (53). CodY was not differentially expressed in JA24, but we still expected mutation JA24-2 to influence the expression of CodY-regulated genes. Indeed several genes, which have been reported to be affected by CodY, were significantly downregulated in JA24 at 30°C compared to SD96, for example, *dppA*, *ilvD*, *ctrA*, *asnB*, *gltA*, *serC, citB* or *dppP* (log2-fold changes < -1.5) (40). Contrarily, several other genes were not affected, including *oppD* or *codY* itself. As the mutated amino acid in CodY is located in the DNA-binding domain (54), it could be speculated that the mutation changed the DNA-binding specificity of CodY. Potentially, the positively charged R237 is involved in binding the negatively charged DNA, and the mutation to a neutral serine disrupted this binding. Consequently, some genes might have been released of the CodY-regulation. This hypothesis is underlined by the finding that genes belonging to the CodY-regulon are regulated to different extents depending on the individual CodY recognition sequences (40).

In RD01, *codY* was also affected by a mutation (RD01-2), located in the promotor region 64 bp upstream of the *codY* CDS. We expected this mutation to change the expression of *codY* in RD01, and indeed we found a strong downregulation of that gene (log2-fold change -2.7 ± 0.1 at 30°C and -2.9 ± 0.1 at 39°C). As CodY represses several genes, it was not surprising that we found many *codY*-regulated genes with significantly enhanced expression compared to SD96. Furthermore, the other mutants mostly showed reduced expression of these genes. For example, *dppA*, which was reported to be strongly affected by CodY (40), was significantly enhanced in

RD01 at 30°C (log2-fold change 1.3 ± 0.2), while it was downregulated in JA24 (log2-fold change -2.3 ± 0.2) and RD07 (log2-fold change -2.7 ± 0.2). Other genes, such as *ilvD*, gltD, *icD,* and *citB*, followed a similar pattern, which was also observed at 39°C. Expression of the cell-wall-bound proteinase PrtP was also suggested to be repressed by CodY (40,55). In agreement with the reduced repression of CodY regulated genes, we found enhanced *prtP* expression in RD01 at 30°C with log2-fold change 1.6 ± 0.2. Interestingly, this was similar in RD07 (log2-fold change 2.0 ± 0.2), but not in JA24 (log2-fold change 0.1 ± 0.12). At 39°C, *prtP*-expression was strongly enhanced for all three mutants (JA24: 2.2 ± 0.2, RD01: 3.4 ± 0.2, RD07: 4.2 ± 0.2).

These findings support the hypothesis that in SD96 amino acid supply was limited during ALE at high temperatures, which was compensated in RD01 by downregulating *codY* and thus upregulating amino acid metabolism. The other two mutants did not have mutations with similar effects, but rather the opposite was observed, as genes in the amino acid metabolism were downregulated compared to SD96. Potentially, JA24 did not suffer from amino acid limitation during ALE due to the different selection pressure it was exposed to, while RD07 presumably found an alternative strategy to deal with the amino acid limitation at high temperature. This hypothesis could be supported by the fact that supplementing the UHT milk with peptone was beneficial for acidification using SD96 at high temperatures and similarly at 30°C after heat shock (data not shown).

3.4.4 Mobile Genetic Elements in RD01 and RD07 were lost during ALE

Plasmid loss frequently occurs during serial propagation, especially when using growth temperatures above the optimal temperature. Growing a plasmid containing strain above its optimal temperature is a standard method for plasmid curing (56). Even at moderate temperatures, loss of the large protease plasmid readily occurs in *L. lactis* when grown in milk, although this plasmid is essential for fast growth (27,57). During ALE, we observed that a significant fraction of the isolated strains was protease-negative after several propagation steps at high temperatures. Nevertheless, the cultures were never completely dominated by protease-negative strains. When slow growth was observed, the cultures were enriched for protease-positive strains, and the ALE was continued.

Furthermore, genome resequencing of the mutant strains revealed that four plasmids (pSD96_01, pSD96_03, pSD96_06, and pSD96_10) had been lost in RD01 and RD07. Three of the plasmids were comparably large (pSD96_01: 12 kb, pSD96_03: 37 kb, pSD96_06: 25 kb), and their loss might confer a growth advantage due to the metabolic burden of replicating the plasmids. Plasmids pSD96_01, pSD96_06, and pSD96_10 encoded genes for phage resistance, including a complete type I RMS on pSD96_06 and two additional specificity subunits on pSD96_01 and pSD96_10. Such a stacking pattern of specificity subunits belonging to type I RMSs is vital for phage defense and has been described previously for *L. lactis* (58).

Additionally, the large chromosomal deletion (80 kb) in RD01 and RD07 also contained a complete RMS assigned to a putative prophage (FTN78_RS09530 - FTN78_RS09570), which was induced at high temperature with an average log2-fold change of 2.4 ± 0.1 in SD96. The loss of phage resistance genes, encoded on the lost plasmids and in the deleted area, explains the increased sensitivity of RD01 and RD07 towards a few of the tested phages. Hypothetically, the phage robustness of RD01 and RD07 could be restored partially by reintroducing the lost plasmids, for instance, by conjugative transfer. The remaining three plasmids (pSD96_07, pSD96_08, and pSD96_09) apparently, they do not burden the cells strongly so that they have been kept during the serial propagation. As mentioned above, generally, many genes in the deleted area were highly induced by growth at high temperature, which most likely imposed an unnecessary burden to the cell, as RD01 and RD07 grew well in milk even without those genes. It is unknown why so many genes located in the deleted area were induced at high temperatures, even though they did not contribute to the thermotolerant phenotype.

3.4.5 In RD01, the Stringent Response and Peptidoglycan Structure are Affected by Mutations

The other mutations in RD01, RD01-3, and RD01-4, did not affect transcription. Bifunctional (p)ppGpp synthetase (RelA), which is affected by RD01-3, plays a crucial role in modulating the stringent response by mediating the synthesis of the second messenger (p)ppGpp (22). The stringent response and CodY-based regulation of transcription are both modulated by the availability of amino acids. Other than CodY, which is activated by BCAA, the stringent response and thereby (p)ppGpp synthesis is activated by uncharged tRNAs (59,60). In *L. lactis,* both mechanisms are not linked to another as it is the case in other firmicutes such as *B. subtilis*, in which CodY is also activated by increased GTP levels caused by low RelA activity (22,55). The mutation in RelA (V469L) is located in a TGS domain, which is named after proteins where it occurs (ThrRS, GTPase, and SpoT/RelA), and is potentially involved in sensing aminoacyl-tRNA (59,60). Hypothetically, the mutation changed the sensitivity of RelA towards amino acid starvation and thereby the regulation of the stringent response. Nevertheless, without further insight into potential changes in (p)ppGpp levels, it is impossible to reveal the impact of this mutation.

RD01-4 causes F54L mutation in UDP-N-acetylmuramate- L-alanine ligase (MurC). MurC catalyzes the addition of L-Ala to UDP-N-acetylmuramic acid, which is followed by the addition of other amino acids resulting in the formation of the UDP-N-acetylmuramoyl-pentapeptide, typically consisting of L-Ala, D-Glu, L-Lys, D-Ala and D-Ala (61,62). Previous studies on the substrate specificity of MurC revealed that, despite a high preference for L-Ala, MurC could also accept other substrates such as L-Ser or L-Ala paralogues (63). Potentially, the mutation in MurC resulted in differential incorporation of other amino acids than L-Ala, which could, in turn, result in an altered peptidoglycan structure. This hypothesis could be underlined by the observation that

RD01 was more resistant to lysozyme treatment, which was applied during DNA isolation. The mutation, however, did not cause increased cell wall thickness, as revealed by transmission electron microscopy (data not shown). Similar observations have been reported previously that the amino acid composition of the pentapeptide affects stress resistance and simultaneously modulates the resistance to peptidoglycan hydrolases in *L. lactis* (64,65). Hypothetically, the mutation in MurC could be a result of the amino acid limitation experienced by SD96 in UHT milk at high temperatures. The mutation implies that specifically L-Ala or the other amino acids forming the pentapeptide might be limiting for SD96 under those conditions.

The last mutation, RD01-1, is found in an EamA family transporter, which in IL1403 is referred to as *yjcD*. Unfortunately, this protein has not been characterized, and consequently, no conclusions can be drawn regarding the effect of this mutation.

3.4.6 Changes in Nucleotide Metabolism Possibly Result in a Multi-Stress Resistant Phenotype in RD07

In RD07, eight mutations were found, and a combined effect causing the unique thermotolerant phenotype of RD07 is expected. Therefore, here we attempt to explain the phenotype using the transcriptomics analysis. First, RD01 and RD07 share two mutations (RD01-1/RD07-3 and RD01-4/RD07-8) and a large deletion (RD01-Gap and RD07-Gap), which have already been discussed. As the thermotolerance of RD07 was improved compared to RD01, we searched for differences between the strains using COG analysis of DEGs (data not shown). At 39°C, the COG category with the most DEGs was “Amino acid metabolism and transport,” where RD07 showed mostly reduced expression compared to RD01, which can be traced back to the downregulation of CodY in RD01. Next, RD07 is standing out with the most enhanced genes in the category “Nucleotide metabolism and transport,” which is likely due to the mutation in *purR*. The mutation in PurR was identified to be located within the PRPP binding pocket, which allows speculating that the modulation of PurR activity by PRPP could have been changed in RD07 (66). Purine metabolism has been linked to a multi-stress-resistant phenotype in *L. lactis* (31). More specifically, the excess of guanine nucleotides was shown to induce stress sensitivity, as exemplified on acid and heat stress. Generally, low purine availability results in low guanine levels and a stress-resistant phenotype, which is the case when milk is used as a substrate (31).

Consequently, it could be speculated that the high thermotolerance of RD07 is due to lower guanine nucleotide levels compared to SD96 or RD01. To strengthen this hypothesis, we examined the genes involved in GMP biosynthesis or scavenging (**Figure 3.10**). Among *adk*, *apt*, *gmk, guaA*, *guaB*, *guaC*, *hpr, hpt, pyk*, *xpt,* and the *pur* genes, only *guaC*, *xpt,* and the *pur* genes were differentially expressed in RD07. At 30°C, in comparison to SD96, *guaC* was enhanced 7.2-fold (log2-fold change 2.8 ± 0.3) in RD07 and 3.7-fold (log2-fold change 1.9 ± 0.3) in comparison to RD01. *xpt* was only moderately enhanced (log2-fold change 1.5 ± 0.2) for both comparisons. As mentioned above, the *pur* genes were also enhanced in RD07. Overall, this

expression pattern could result in a reduced guanidine nucleotide pool, as overexpression of *guaC, purA,* and *purB* would favor adenine nucleotide synthesis (**Figure 3.10**). Consequently, it is likely that in RD07, the guanine nucleotide pool was reduced compared to SD96 or RD01, leading to a more robust phenotype of RD07. A similar observation has been described previously, where the inactivation of *guaA* led to multi-stress resistance (32,67).

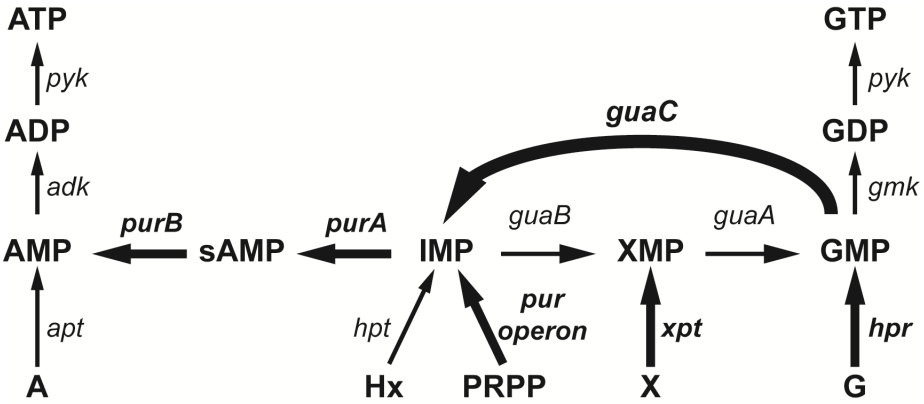

Figure 3.10 Scheme of purine metabolism with emphasis on the differential expression in RD07. Compared to SD96 and RD01, in RD07, *guaC* is strongly enhanced (thick arrow), while the *pur* genes, *xpt,* and *hpr* were only moderately enhanced (medium arrows) and the remaining genes were unaltered (thin arrows). This differential expression pattern suggests a reduced GMP pool, which is beneficial for stress tolerance. Abbreviations are as follows: *adk*: adenylate kinase, *apt*: adenine phosphoribosyltransferase, *gmk*: guanylate kinase, *guaA*: GMP synthase, *guaB*: IMP dehydrogenase, *guaC*: GMP reductase, *hpt*: hypoxanthine/guanine phosphoribosyltransferase, *hpr*: hypoxanthine/guanine phosphoribosyltransferase, *purA*: adenylosuccinate synthase, *purB*: adenylosuccinate lyase, *pur operon*: *purCSQLF, purMN,* and *purDEK*, *pyk*: pyruvate kinase, *xpt*: xanthine phosphoribosyltransferase. A: adenine, Hx: hypoxanthine, PRPP: phosphoribosyl diphosphate, X: xanthine, G: guanine, I: inosine, sAMP: adenylosuccinate. xMP: monophosphate, xDP: diphosphate, xTP: triphosphate (with x: A, G, I, or X). Modified from (31).

3.4.7 Other Mutations in RD07 Contribute to Its Unique Thermotolerant Phenotype

The remaining mutations in RD07 are expected to contribute with various degree to its thermotolerance. Mutation RD07-1 caused R546S mutation in phenylalanine-tRNA ligase (β-subunit), also referred to as PheT (FTN78_RS00265) and is located in the anticodon binding domain. No unambiguous evidence exists about the effect of this mutation, but the literature suggests that aminoacyl-tRNA synthetases are involved in translational quality control (68,69). A potential role in translational quality control or tRNA recognition could be assumed, which might reduce translational errors caused by high temperature. RD07-2 and RD07-4 are both located in prophages. The former mutation caused V957E in a hypothetical protein (FTN78_RS00795), and the latter mutation caused A410S in a DNA primase of another prophage (FTN78_RS10440).

Both genes are transcribed with low abundance in SD96 at 30°C (11.5 ± 0.1 TPM and 1.0 ± 0.2 TPM, respectively) and 39°C (3.0 ± 0.1 TPM and 1.6 ± 0.1 TPM, respectively). Furthermore, the expression levels of both genes were not altered significantly in RD07, and therefore the mutations likely do not exert a significant effect on the phenotype. Mutation RD07-5 lies in the promotor region of *hflB*/*ftsH* coding for ATP-dependent zinc metalloprotease FtsH (FTN78_RS10530), which is involved in the cellular stress regulation (70). This enzyme is of importance for stress resistance and thermotolerance as it contributes to quality control of cytosolic and membrane proteins (71,72). Its expression is triggered among others through cell-envelope stress (73). Transcriptomic analysis revealed that FtsH was slightly downregulated in RD07 at 30°C (log2-fold change -0.4 ± 0.1) and even more at 39°C (log2-fold change -0.8 ± 0.2). This observation is surprising, as the opposite would be expected. The deletion of FtsH in *Lactobacillus plantarum* resulted in a heat-sensitive phenotype, while overexpression produced a robust strain (74). Potentially, FtsH has other functions in *L. lactis* and is, for instance, involved in regulating cell envelope composition, as it has been suggested previously (75). Mutation RD07-6 caused a point mutation (D311Y) in RexA, an ATP-dependent exonuclease (subunit A), which is involved in the repair of double-strand breaks (76). The effect of the point mutation is unclear, but a potential advantage for repairing DNA damage is likely. This enzyme is slightly reduced at 39°C (log2-fold change -0.6 ± 0.1), which was unexpected, as DNA-damage occurs more frequently when the cells are stressed. Generally, we expect all mutations in RD07 to contribute to its unique thermotolerance.

3.5 Conclusion

We presented three natural mutant strains with increased thermotolerance derived from *L. lactis* subsp. *lactis* biovar diacetylactis SD96 by ALE, namely JA24, RD01, and RD07. All mutant strains showed improved acidification in milk at both high and low temperatures, with RD07 being the most thermotolerant and efficient strain. This strain can acidify milk at 41°C, which is unique for a dairy *L. lactis* subsp. *lactis* strain. Besides a slight increase in susceptibility to phage attack, the mutants have kept their ability to grow independently in milk and produce flavor compounds from citrate. Genome and RNA-sequencing revealed genomic and transcriptomic changes, which established during ALE. All three mutants seem to have found different strategies for improved growth in UHT milk and increased survival at high temperatures. RD07, the most thermotolerant strain, also showed the most complex set of mutations, and their potential effects on the phenotype were discussed extensively. We concluded that the drainage of the guanidine nucleotide pool is a likely cause of the thermotolerance of RD07. Besides this, the other mutations, for example, involved in DNA and protein quality control mechanisms, are expected to support the stress resistance of this strain. Further physiological experiments are required to verify the presented hypotheses derived from our system-wide analysis.

As the mutants are based on an industrial strain, which is already used in many starter cultures provided by Sacco S.r.l., they are readily applicable for cheese production. Initial cheese trials at Arla Foods, Denmark confirmed the suitability of the mutant strains for producing superior cheeses exemplified on a Havarti-style cheese made with exceptionally high cooking temperature. Summarizing, we showed how ALE could be applied for producing superior strains for the starter culture industry, while gaining important insight into the stress physiology of *L. lactis* at the same time.

3.6 Methods

3.6.1 Adaptive Laboratory Evolution

L. lactis subsp. *lactis* biovar diacetylactis SD96 was cultivated on M17 agar supplemented with 0.5% lactose (LM17), and a single colony each was used for starting two separate cultures in 9 ml UHT milk. The two lineages referred to as lineage A and B were exposed to different temperatures, as described below. Generally, when the milk was coagulated, the culture was considered fully grown with approximately 10^{10} cells (27). Fully-grown cultures were homogenized by shaking and propagated in 9 ml fresh UHT milk. Then the procedure was repeated. Every week, the culture was saved by mixing a fully-grown culture with 50% glycerol 1:1 and storing at -80°C. When required, freshly inoculated cultures were stored at -20°C.

Lineage A: After inoculation into fresh UHT milk, the culture was incubated for 1 h at 30°C, followed by incubation at 42°C for 1 h, and finally, the strain was allowed to acidify the milk at 30°C until the milk coagulated. The temperature profile was realized using a programmable block heater, where the shift from 30°C to 42°C was done in 10 min and from 42°C to 30°C in 2 h. Initially, coagulation occurred after approximately 24 h. Then, the culture was propagated by diluting 100-fold into 9 ml fresh UHT milk, which corresponds to 6.64 generations per propagation step. After 24 propagation steps, corresponding to approx. 160 generations, a remarkable decrease in the time needed until coagulation was observed, and JA24 was isolated while screening for a protease, lactose, and citrate positive phenotype. JA24 was isolated approximately one month after the ALE of lineage A was started.

Lineage B: Here, the culture was kept continuously at high temperatures. For avoiding a long time between propagation steps, the coagulated, fully grown culture was propagated by diluting 10-fold into 9 ml fresh UHT-milk, which corresponds to 3.32 generations per propagation step. Initially, SD96 was grown at 39°C, and the coagulation of the UHT milk was observable after approximately 48 h. 20 propagation steps were conducted until coagulation was observable after approx. 24 h, and then the temperature was increased to 40°C, again resulting in coagulation after approx. 48 h. After 25 propagation steps at 40°C (45 propagation steps in total, 150 generations), coagulation was observable after 24 h, and RD01 was isolated. RD01 was isolated after approximately five months after the ALE of lineage B was started. Subsequently, the temperature was increased to 41°C, and another 75 propagation steps (120 propagation steps in

total, 400 generations) were needed until solidification was visible after 24 h, which was when RD07 was isolated and the ALE experiment was terminated. Overall, ALE of lineage B was carried out for more than one year.

When the cultures began to grow slower than before, judged by visual inspection, they were enriched for protease-positive strains by plating on UHT milk agar (1% UHT milk, 1.5% agar) and subsequent harvesting of the colonies. On the UHT agar, protease-positive strains formed larger colonies so that after harvesting, they constituted a larger fraction of the entire culture. This enrichment was done twice during the ALE at high temperatures.

3.6.2 Isolation of Mutant Strains

The mutant strains JA24, RD01, and RD07, were isolated from the propagated cultures at the time points indicated above. As plasmid loss was reported to occur frequently during propagation in milk (27), we screened for strains, which grew fast in milk and utilized citrate. Therefore, cultures were plated on medium containing 1% (g/v) skim milk powder, 0.25% peptone, 0.25 % lactose, 1.5% agar, 0.1% potassium ferricyanide, 0.025% ferric citrate and 0.02% sodium citrate as adapted from (77). The skim milk powder, peptone, lactose, and agar were mixed and dissolved. Then, the pH was adjusted to 6.5, autoclaved, and cooled down to 60°C. A 10% potassium ferricyanide solution and a solution containing 2.5% ferric citrate and 2.5% sodium citrate were steamed at 100°C for 30 min and added 1:100 into the agar solution. Large, dark blue colonies, indicating efficient lactose and citrate utilization, were re-streaked on medium containing 1% UHT milk and 1.5% agar. Protease-positive strains formed larger colonies on this agar than protease-negative strains. Several candidates were confirmed by a simple coagulation test. For this, 1 ml UHT milk was inoculated with a single colony and incubated at 30°C overnight. When the milk was fully coagulated, the strain was considered protease positive. Lastly, the thermotolerance of several isolates fulfilling the above criteria was assessed by measuring the acidification curve at the temperatures applied during ALE using 96-well microtiter plates with integrated chemical, optical pH sensors (HydroPlate HP96U by PreSens Precision Sensing GmbH, Germany), in combination with a Tecan Infinite 200 Pro microplate reader (Tecan Trading AG, Switzerland). From those measurements, the fastest strain was chosen and designated JA24, RD01, or RD07.

3.6.3 Milk Acidification

Cultures were inoculated from LM17 agar into fresh milk (UHT milk, PM, or RSM) and, as a first pre-culture, cultivated at 30°C for 16 h. A second pre-culture was started by diluting the first pre-culture 1:100 into fresh milk, followed by incubation at 30°C for 16 h. Then, the cultures were inoculated into pre-warmed fresh milk using a 1:100 dilution. The cultures were equipped with a pH-probe, and acidification was monitored for 20 h using an iCinac instrument. For cultivations applying the 42°C heat shock, the cultures were transferred between two water baths, one at

30°C and one at 42°C, so that a rapid temperature change happened. Before inoculation, the pH-probes were calibrated at the temperature applied during milk fermentation. From the acidification curve, the time needed to reach pH 5.2 was calculated and presented.

3.6.4 Phage Profiling

Phage profiling was carried out at Sacco S.r.l. using the internal comprehensive phage collection. The collection comprised over 100 phages, of which 26 were from the Félix d'Hérelle Reference Center for Bacterial Viruses, Université Laval, Canada, including ϕbIL170, ϕP008, ϕsk1, ϕ712, ϕhp, ϕc2, ϕbil67, ϕrlt, ϕeb1, ϕQ54, ϕml3, ϕC6A, ϕTuc2009, ϕP369, ϕ949, ϕP335, ϕP270, ϕP107, ϕ1706, ϕ936, ϕul36, ϕp2, ϕP087, ϕ1483, ϕBK5-T, ϕP001. Single phages were contained in whey obtained from infected strains and filtered before use (0.4 µm). Phages were mixed in pools of five to seven phages. 5 ml RSM supplemented with 0.1% yeast extract, bromcresol green, and bromcresol purple were inoculated with 100 µl of a fully grown culture in RSM. Next, 100 µl of whey containing a phage pool or a single phage were added, followed by incubation at 30°C in a water bath. Acidification was monitored by observing the color change of the milk in a 96-well plate using a custom-made pH-scanner. When acidification failed using a phage pool, the single phages contained in the pool were tested individually. Each pool or single phage was propagated four times as follows: When the milk was acidified successfully, meaning that no phage had attacked or the attack happened late during the fermentation, the culture was centrifuged (5000 x g, 10 min), and the whey was collected and used as above for infecting a new culture. In this manner, attacking phages were concentrated and identified, even if they required a higher titer for attacking than initially present.

3.6.5 DNA Isolation and Genome Resequencing

De novo sequencing of SD96 has been described elsewhere (see **Chapter 2**, (35)). Further bioinformatics analysis was carried out by homology search using BLAST, and phage defense genes were identified using a database containing lactococcal phage defense proteins, which were mapped to the genome of SD96 (78,79). Genome resequencing of the mutant strains was performed as described previously with minor modifications (see **Chapter 2**, (35)). Cells from the early-stationary phase were harvested from LM17 broth. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). The cell wall was digested for 16 h at 22°C using 0.5 mg/ml lysozyme in Tris/NaCl-buffer (10 mM each, pH 8.0) with 1% Triton X-100 and 10 mM EDTA. Then, DNA was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, product number K0512) according to the standard protocol with minor modifications. The chloroform precipitated cell lysate was centrifuged for 15 min at 20.000 x g, instead of 2 min. Sequencing was carried out by BGI China on an Illumina HiSeq 4000 platform using PE150 PCR-free libraries. The raw paired-end 150-bp reads were processed using Geneious Prime (version 2019.1.3) (80). First, adapter sequences and low-quality sequences were trimmed from both ends and, finally,

reads shorter than 50 bp were discarded. The reads were assembled to the chromosome and plasmids of SD96 (accession numbers NZ_CP043518 – NZ_CP043528) using Bowtie2 (version 2.3.0) in Geneious (81). Next, variant calling was performed using the respective function in Geneious accepting variants with > 95% variant frequency. For identifying false-positive mutations, variant calling was also performed using sequencing data from SD96 using the same methods as for the mutants. Mutations occurring in both the mutants and SD96 were not considered in the analysis.

3.6.6 RNA Isolation, Sequencing, and Differential Expression Analysis

RNA sequencing was carried out for SD96, JA24, RD01, and RD07 grown in RSM at 30°C and 39°C. Those conditions were chosen to guarantee proper growth for all strains. At 39°C, the growth of SD96 was severely inhibited but measurable after a more extended lag-phase compared to the other strains. In praxis, 10 ml RSM were inoculation from LM17 using a single colony and incubated for 16 h at 30°C (first pre-culture). A second pre-culture was inoculated from the first using a 100-fold dilution. The second pre-culture was used to inoculate 50 ml pre-warmed RSM. Growth was monitored spectrophotometrically (OD_{600}) after 10-fold dilution in EDTA solution (2 g/L, pH 12.0), and the pH was monitored simultaneously using an iCinac instrument as described above. When the cultures reached the exponential phase (OD_{600} 0.7-0.9), cells were harvested. For this, 10 ml of the culture were withdrawn, immediately cooled on ice, and centrifuged at 4°C (5000 x g, 10 min). The resulting pellet was quickly resuspended in RNA*later* solution (Invitrogen) and stored at 4°C. Half of the collected sample was used for isolating RNA using the RNeasy Mini Kit (Qiagen) according to the protocol “Purification of total RNA from bacteria (RY28 Nov-06)”. The following steps, including RNA sequencing, were carried out at BGI China. Ribosomal RNA was removed using the Ribo-off rRNA Depletion Kit (Bacteria) from Vazyme. RNA sequencing was carried out using a PE100 library on a BGISEQ (DNBseq tech) platform resulting in paired-end 100 bp reads. Next, quality trimming and mapping to the SD96 genome were carried out in Geneious, as described above. Raw-read counts, RPKM (Reads Per Kilobase Million), FPKM (Fragments Per Kilobase Million), and TPM (Transcripts Per Kilobase Million) were calculated in Geneious using the built-in “Calculate Expression Levels” feature. For differential gene expression analysis, raw-read counts were exported and analyzed manually using the DESeq2 package in RStudio (82). Log2-fold changes and corresponding log2-fold standard errors were obtained. The adjusted p-value (p_{adj}) was considered for evaluating the confidence of the differential expression values. Clusters-of-Orthologous-Groups analysis was carried out by annotating all CDS of SD96 using the eggNOG-mapper v2 (83), which is based on eggNOG v5.0 clusters and phylogenies (84). CDS, which were not assigned to a COG category, were excluded from the analysis. Furthermore, CDS located on lost genetic elements in RD01 and RD07 were treated separately during the analysis.

3.7 Acknowledgment

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3.8 Availability of Data and Materials

All experimentally generated data are included in this article. Sequencing data will be publicly available upon publication, including DNA-sequencing results of JA24, RD01, and RD07 and the RNA-sequencing dataset.

3.9 References

1. Kindstedt PS. The history of cheese. In: Global Cheesemaking Technology: Cheese Quality and Characteristics. Editors: Papademas P, Bintsis T. John Wiley & Sons Ltd. **2017**, 3–19.
2. United States Department of Agriculture (USDA). Dairy: World Markets and Trade. **2019**.
3. Food and Agriculture Organization on the United Nations (FAO). OECD-FAO Agricultural Outlook 2019-2028 - Dairy and dairy products. **2019**.
4. Bachmann H, Pronk JT, Kleerebezem M, Teusink B. Evolutionary engineering to enhance starter culture performance in food fermentations. *Curr Opin Biotechnol* **2015**, 32:1–7.
5. Johansen E. Use of Natural Selection and Evolution to Develop New Starter Cultures for Fermented Foods. *Annu Rev Food Sci Technol* **2018**, 9:411–28.
6. Fox PF, Mcsweeney PLH. Cheese: An Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 5–21.
7. Kelly AL, Huppertz T, Sheehan JJ. Pre-treatment of cheese milk: Principles and developments. *Dairy Sci Technol* **2008**, 88:549–72.
8. Panthi RR, Jordan KN, Kelly AL, Sheehan JJD. Selection and Treatment of Milk for Cheesemaking. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 23–50.
9. Fagan CC, O'Callaghan DJ, Mateo MJ, Dejmek P. The Syneresis of Rennet-Coagulated Curd. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 145–77.
10. Parente E, Cogan TM, Powell IB. Starter Cultures: General Aspects. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 201–26.
11. Chen J, Shen J, Solem C, Jensen PR. Oxidative Stress at High Temperatures in *Lactococcus lactis* Due to an Insufficient Supply of Riboflavin. *Appl Environ Microbiol* **2013**, 79(19):6140–7.
12. Ong L, Lawrence RC, Gilles J, Creamer LK, Crow VL, Heap HA, Honoré CG, Johnston KA, Samal PK, Powell IB, Gras SL. Cheddar Cheese and Related Dry-Salted Cheese Varieties. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 829–63.
13. Stokes D, Ross RP, Fitzgerald GF, Coffey A. Application of *Streptococcus thermophilus* DPC1842 as an adjunct to counteract bacteriophage disruption in a predominantly lactococcal Cheddar cheese starter: Use in bulk starter culture systems. *Lait* **2001**, 81:327–34.

14. Michel V, Martley FG. *Streptococcus thermophilus* in Cheddar cheese - Production and fate of galactose. *J Dairy Res* **2001**, 68(2):317–25.
15. Rehn U, Petersen MA, Saedén KH, Ardö Y. Ripening of extra-hard cheese made with mesophilic DL-starter. *Int Dairy J* **2010**, 20(12):844–51.
16. Papadimitriou K, Alegría Á, Bron PA, Angelis M De, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, Sinderen D Van, Varmanen P, Ventura M, Zúñiga M, Tsakalidou E. Stress Physiology of Lactic Acid Bacteria. *Microbiol Mol Biol Rev* **2016**, 80(3):837–90.
17. Tsakalidou E, Papadimitriou K. Stress Responses of Lactic Acid Bacteria. Editors: Tsakalidou E, Papadimitriou K. Springer **2011**,.
18. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **2002**, 82(1–4):187–216.
19. Sanders JW, Venema G, Kok J. Environmental stress responses in *Lactococcus lactis*. *FEMS Microbiol Rev* **1999**, 23(4):483–501.
20. Potrykus K, Cashel M. (p)ppGpp: Still Magical? *Annu Rev Microbiol* **2008**, 62(1):35–51.
21. Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL, Berninghausen O, Hauryliuk V, Beckmann R, Wilson DN. The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Res* **2016**, 44(13):6471–81.
22. Geiger T, Wolz C. Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. *Int J Med Microbiol* **2014**, 304(2):150–5.
23. Sandberg TE, Salazar MJ, Weng LL, Palsson BO, Feist AM. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. *Metab Eng* **2019**, 56:1–16.
24. Bachmann H, Molenaar D, Branco F, Teusink B. Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria. *FEMS Microbiol Rev* **2017**, 24:1–19.
25. Dragosits M, Mattanovich D. Adaptive laboratory evolution – principles and applications for biotechnology. *Microb Cell Fact* **2013**, 12(64):1–17.
26. Chen J, Shen J, Ingvar Hellgren L, Ruhdal Jensen P, Solem C. Adaptation of *Lactococcus lactis* to high growth temperature leads to a dramatic increase in acidification rate. *Sci Rep* **2015**, 5:1–15.
27. Bachmann H, Starrenburg MJC, Molenaar D, Kleerebezem M, Vlieg JETVH. Microbial domestication signatures of *Lactococcus lactis* can be reproduced by experimental evolution. *Genome Res* **2012**, 22:115–24.
28. Smith WM, Pham TH, Lei L, Dou J, Soomro AH, Beatson SA, Dykes GA, Turner MS. Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of llmg_1816 (*gdpP*) induced by high-temperature growth. *Appl Environ Microbiol* **2012**, 78(21):7753–9.
29. Spus M, Liu H, Wels M, Abee T, Smid EJ. Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **2017**, 241:173–80.
30. Dijkstra AR, Starrenburg MJC, Todt T, Van Hijum SAFT, Hugenholtz J, Bron PA. Transcriptome analysis of a spray drying-resistant subpopulation reveals a zinc-dependent mechanism for robustness in *L. lactis* SK11. *Front Microbiol* **2018**, 9:1–10.
31. Ryssel M, Meisner Hviid AM, Dawish MS, Haaber J, Hammer K, Martinussen J, Kilstrup M. Multi-stress resistance in *Lactococcus lactis* is actually escape from purine-induced stress sensitivity. *Microbiology* **2014**, 160(2014):2551–9.
32. Rallu F, Gruss A, Ehrlich SD, Maguin E. Acid- and multistress-resistant mutants of *Lactococcus lactis*: Identification of intracellular stress signals. *Mol Microbiol* **2000**, 35(3):517–28.
33. Johansen E. Challenges when transferring technology from *Lactococcus* laboratory strains to industrial strains. *Genet Mol Res* **2003**, 2(1):112–6.

34. Pedersen MB, Iversen SL, Sørensen KI, Johansen E. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiol Rev* **2005**, 29:611–24.
35. Dorau R, Chen J, Jensen PR, Solem C. Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis SD96. *Microb Resour Announc* **2020**, 9(3):1–2.
36. Deveau H, Labrie SJ, Chopin MC, Moineau S. Biodiversity and classification of lactococcal phages. *Appl Environ Microbiol* **2006**, 72(6):4338–46.
37. Oliveira J, Mahony J, Hanemaaijer L, Kouwen TRHM, van Sinderen D. Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *J Dairy Sci* **2018**, 101(1):96–105.
38. Neighbors - Genome - NCBI.
39. Van Heel AJ, De Jong A, Song C, Viel JH, Kok J, Kuipers OP. BAGEL4: A user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res* **2018**, 46:278–81.
40. Den Hengst CD, Van Hijum SAFT, Geurts JMW, Nauta A, Kok J, Kuipers OP. The *Lactococcus lactis* CodY regulon: Identification of a conserved cis-regulatory element. *J Biol Chem* **2005**, 280(40):34332–42.
41. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* **2016**, 44:16–21.
42. Kilstrup M, Hammer K, Jensen PR, Martinussen J. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* **2005**, 29:555–90.
43. Dijkstra AR, Setyawati MC, Bayjanov JR, Alkema W, Van Hijum SAFT, Bron PA, Hugenholtz J. Diversity in robustness of *Lactococcus lactis* strains during heat stress, oxidative stress, and spray drying stress. *Appl Environ Microbiol* **2014**, 80(2):603–11.
44. van Hylckama Vlieg JE, Rademaker JL, Bachmann H, Molenaar D, Kelly WJ, Siezen RJ. Natural diversity and adaptive responses of *Lactococcus lactis*. *Curr Opin Biotechnol* **2006**, 17(2):183–90.
45. Weidmann S, Maitre M, Laurent J, Coucheney F, Rieu A, Guzzo J. Production of the small heat shock protein Lo18 from *Oenococcus oeni* in *Lactococcus lactis* improves its stress tolerance. *Int J Food Microbiol* **2017**, 247:18–23.
46. Desmond C, Fitzgerald GF, Stanton C, Ross RP, Al DET, Icrobiol APPLENM. Improved Stress Tolerance of GroESL-Overproducing *Lactococcus lactis* and Probiotic Lactobacillus paracasei NFBC 338. *Appl Environ Microbiol* **2004**, 70(10):5929–36.
47. Abdullah-Al-Mahin, Sugimoto S, Higashi C, Matsumoto S, Sonomoto K. Improvement of multiple-stress tolerance and lactic acid production in lactococcus lactis NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli dnaK*. *Appl Environ Microbiol* **2010**, 76(13):4277–85.
48. Stulova I, Kabanova N, Kriščiunaite T, Laht TM, Vilu R. The effect of milk heat treatment on the growth characteristics of lactic acid bacteria. *Agron Res* **2011**, 9:473–8.
49. Douglas FW, Greenberg R, Farrell HM, Edmondson LF. Effects of Ultra-High-Temperature Pasteurization on Milk Proteins. *J Agric Food Chem* **1981**, 29(1):11–5.
50. Zittle CA, Thompson MP, Custer JH, Cerbulis J. κ-Casein-β-Lactoglobulin Interaction in Solution When Heated. *J Dairy Sci* **1962**, 45(7):807–10.
51. Sadovskaya I, Vinogradov E, Courtin P, Armalyte J, Meyrand M, Giaouris E, Palussière S, Furlan S, Péchoux C, Ainsworth S, Mahony J, van Sinderen D, Kulakauskas S, Guérardel Y, Chapot-Chartier MP. Another brick in the wall: A rhamnan polysaccharide trapped inside peptidoglycan of *Lactococcus lactis*. *MBio* **2017**, 8(5):1–16.
52. Codon usage table. “http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=416870&aa=9&style=N”. Cited **2020**.
53. Brinsmade SR. CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. *Curr Genet* **2017**, 63(3):417–25.

54. Han AR, Kang HR, Son J, Kwon DH, Kim S, Lee WC, Song HK, Song MJ, Hwang KY. The structure of the pleiotropic transcription regulator CodY provides insight into its GTP-sensing mechanism. *Nucleic Acids Res* **2016**, 44(19):9483–93.
55. Guédon E, Serror P, Ehrlich SD, Renault P, Delorme C. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Mol Microbiol* **2001**, 40(5):1227–39.
56. Trevors JT. Plasmid curing in bacteria. *FEMS Microbiol Rev* **1986**, 32:149–57.
57. Bachmann H, Molenaar D, Kleerebezem M, Van Hylckama Vlieg JE. High local substrate availability stabilizes a cooperative trait. *ISME J* **2011**, 5(5):929–32.
58. Seegers JFML, Van Sinderen D, Fitzgerald GF. Molecular characterization of the lactococcal plasmid pCIS3: Natural stacking of specificity subunits of a type I restriction/modification system in a single lactococcal strain. *Microbiology* **2000**, 146(2):435–43.
59. Kushwaha GS, Bange G, Bhavesh NS. Interaction studies on bacterial stringent response protein RelA with uncharged tRNA provide evidence for its prerequisite complex for ribosome binding. *Curr Genet* **2019**, 65(5):1173–84.
60. Loveland AB, Bah E, Madireddy R, Zhang Y, Brilot AF, Grigorieff N, Korostelev AA. Ribosome-RelA structures reveal the mechanism of stringent response activation. *Elife* **2016**, 5:1–23.
61. Barreteau H, Kovač A, Boniface A, Sova M, Gobec S, Blanot D. Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol Rev* **2008**, 32(2):168–207.
62. Chapot-Chartier M-P, Kulakauskas S. Cell wall structure and function in lactic acid bacteria. *Microb Cell Fact* **2014**, 13:1–23.
63. Emanuele JJ, Jin H, Jacobson BL, Chang CY, Einspahr HM, Villafranca JJ. Kinetic and crystallographic studies of *Escherichia coli* UDP-N-acety-muramate:L-alanine ligase. *Protein Sci* **1996**, 5:2566–74.
64. Hao P, Liang D, Cao L, Qiao B, Wu H, Caiyin Q, Zhu H, Qiao J. Promoting acid resistance and nisin yield of *Lactococcus lactis* F44 by genetically increasing D-Asp amidation level inside cell wall. *Appl Microbiol Biotechnol* **2017**, 101(15):6137–53.
65. Wu H, Xue E, Zhi N, Song Q, Tian K, Caiyin Q, Yuan L, Qiao J. D-Methionine and D-Phenylalanine Improve *Lactococcus lactis* F44 Acid Resistance and Nisin Yield by Governing Cell Wall Remodeling. *Appl Environ Microbiol* **2020**, 86(9):1–12.
66. Hove-Jensen B, Andersen KR, Kilstrup M, Martinussen J, Switzer RL, Willemoës M. Phosphoribosyl Diphosphate (PRPP): Biosynthesis, Enzymology, Utilization, and Metabolic Significance. *Microbiol Mol Biol Rev* **2017**, 81(1):1–83.
67. Duwat P, Ehrlich SD, Gruss A. Effects of metabolic flux on stress response pathways in *Lactococcus lactis*. *Mol Microbiol* **1999**, 31(3):845–58.
68. Steiner RE, Kyle AM, Ibba M. Oxidation of phenylalanyl-tRNA synthetase positively regulates translational quality control. *Proc Natl Acad Sci* **2019**, 116(20):10058–63.
69. Bullwinkle TJ, Ibba M. Translation quality control is critical for bacterial responses to amino acid stress. *Proc Natl Acad Sci* **2016**, 113(8):2252–7.
70. Nilsson D, Lauridsen AA, Tomoyasu T, Ogura T. A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli ftsH* gene product. *Microbiology* **1994**, 140(10):2601–10.
71. Ito K, Akiyama Y. Cellular Functions, Mechanism of Action, and Regulation of FtsH Protease. *Annu Rev Microbiol* **2005**, 59(1):211–31.
72. Narberhaus F, Obrist M, Führer F, Langklotz S. Degradation of cytoplasmic substrates by FtsH, a membrane-anchored protease with many talents. *Res Microbiol* **2009**, 160(9):652–9.
73. Martínez B, Zomer AL, Rodríguez A, Kok J, Kuipers OP. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. *Mol Microbiol* **2007**, 64(2):473–86.

74. Bove P, Capozzi V, Garofalo C, Rieu A, Spano G, Fiocco D. Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: Effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiol Res* **2012**, 167(4):187–93.
75. Roces C, Campelo AB, Escobedo S, Wegmann U, García P, Rodríguez A, Martínez B. Reduced binding of the endolysin LysTP712 to *Lactococcus lactis* Δ*ftsH* contributes to phage resistance. *Front Microbiol* **2016**, 7:1–10.
76. El Karoui M, Ehrlich D, Gruss A. Identification of the lactococcal exonuclease/recombinase and its modulation by the putative Chi sequence. *Proc Natl Acad Sci* **1998**, 95(2):626–31.
77. Kempler GM, McKay LL. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. *Appl Environ Microbiol* **1980**, 39(4):926–7.
78. Zhang Y, Zhang Z, Zhang H, Zhao Y, Zhang Z, Xiao J. PADS Arsenal: a database of prokaryotic defense systems related genes. *Nucleic Acids Res* **2020**, 48:590–8.
79. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* **1990**, 215(3):403–10.
80. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, 28(12):1647–9.
81. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie2. *Nat Methods* **2012**, 9(4):357–9.
82. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **2014**, 15(12):1–21.
83. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, Von Mering C, Bork P. Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. *Mol Biol Evol* **2017**, 34(8):2115–22.
84. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, Mende DR, Letunic I, Rattei T, Jensen LJ, Von Mering C, Bork P. EggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res* **2019**, 47:309–14.

3.10 Supplementary files

Table S 3.1 Mutations of the mutant strain JA24, RD01, and RD07.

Mutation ID	Locus Tag	Position	Variant Frequency	Polymorphism type	Change	CDS	Codon Change	Protein Effect
JA24-1	FTN78_RS07905	1531408	100%	SNP	G → T	LCP family protein	ACC → ACA	None
JA24-2	FTN78_RS09305	1814364	100%	SNP	G → T	Pleiotropic transcriptional regulator *codY*	CGT → AGT	R → S
RD01-1	FTN78_RS03040	533440	100.0%	SNP	C → T	EamA family transporter	CCC → CCT	None
RD01-2	FTN78_RS09305	1815136	94.7%	Deletion	(A)6 → (A)5	Upstream of *codY*		None
RD01-3	FTN78_RS10010	1943775	100.0%	SNP	C → G	RelA, Bifunctional (p)ppGpp synthetase	GTT → CTT	V → L
RD01-4	FTN78_RS11940	2310414	100.0%	SNP	T → G	UDP-N-acetylmuramate-L-alanine ligase	TTT → TTG	F → L
RD01-Gap	**Table S 3.2**	1823878 - 1897135		Deletion				
RD07-1	FTN78_RS00265	4892	99.6%	SNP	C → A	Phenylalanine-tRNA ligase subunit beta	CGT → AGT	R → S
RD07-2	FTN78_RS00795	88575	99.2%	SNP	T → A	Hypothetical protein	GTA → GAA	V → E
RD07-3	FTN78_RS03040	533440	98.9%	SNP	C → T	EamA family transporter	CCC → CCT	None
RD07-4	FTN78_RS10440	2013099	99.3%	SNP	G → T	DNA primase	GCT → TCT	A → S
RD07-5	FTN78_RS10530	2025377	99.8%	SNP	C → G	Upstream of *hflB*/*ftsH*, encoding FtsH		None
RD07-6	FTN78_RS10615	2043810	99.4%	SNP	C → A	RexA, ATP-dependent exonuclease subunit A	GAC → TAC	D → Y
RD07-7	FTN78_RS10700	2065547	99.1%	SNP	G → A	Pur operon repressor	GGC → AGC	G → S
RD07-8	FTN78_RS11940	2310414	96.8%	SNP	T → G	UDP-N-acetylmuramate-L-alanine ligase	TTT → TTG	F → L
RD07-Gap	**Table S 3.2**	1823878 - 1897135		Deletion				

Table S 3.2 CDS which have been lost in RD01 and RD07 because of a large deletion in the chromosome. The deletion starts at 1823878 and ends at 1897135.

Locus Tag	Start	End	Length	Product	Protein ID
FTN78_RS09360	1824746	1824946	201	hypothetical protein	WP_021214808.1
FTN78_RS09365	1824924	1826054	1131	site-specific integrase	WP_021214807.1
FTN78_RS09370	1826054	1826257	204	DUF3173 domain-containing protein	WP_021214806.1
FTN78_RS09375	1826335	1826826	492	antirestriction protein ArdA	WP_021214805.1
FTN78_RS09380	1826848	1827891	1044	CHAP domain-containing protein	WP_021216403.1
FTN78_RS09385	1827906	1830068	2163	hypothetical protein	WP_021214802.1
FTN78_RS09390	1830068	1832671	2604	conjugal transfer protein	WP_032398941.1
FTN78_RS09395	1832675	1833085	411	hypothetical protein	WP_021214799.1
FTN78_RS09400	1833099	1833362	264	hypothetical protein	WP_012897131.1
FTN78_RS09405	1833371	1834621	1251	hypothetical protein	WP_021214798.1
FTN78_RS09410	1834608	1834790	183	hypothetical protein	WP_021214797.1
FTN78_RS09415	1834915	1835118	204	hypothetical protein	WP_021463969.1
FTN78_RS09420	1835294	1835554	261	hypothetical protein	WP_003131790.1
FTN78_RS09425	1835682	1835993	312	hypothetical protein	WP_021214795.1
FTN78_RS09430	1836080	1836409	330	hypothetical protein	WP_021214794.1
FTN78_RS09435	1836546	1837700	1155	replication initiation factor domain-containing protein	WP_023349078.1
FTN78_RS09440	1837827	1838313	487	hypothetical protein	
FTN78_RS09445	1838300	1838509	210	helix-turn-helix transcriptional regulator	WP_021214790.1
FTN78_RS09450	1839182	1840360	1179	ImmA/IrrE family metallo-endopeptidase	WP_021214788.1
FTN78_RS09455	1840378	1841067	690	hypothetical protein	WP_021214787.1
FTN78_RS09460	1841219	1842904	1686	cell division protein FtsK	WP_021214786.1
FTN78_RS09465	1842908	1843384	477	DUF961 domain-containing protein	WP_021214785.1
FTN78_RS09470	1843402	1843701	300	hypothetical protein	WP_014570437.1
FTN78_RS09475	1843854	1844926	1073	hypothetical protein	
FTN78_RS09480	1845038	1845358	321	helix-turn-helix transcriptional regulator	WP_021214781.1
FTN78_RS09490	1846023	1846367	345	XRE family transcriptional regulator	WP_017371264.1
FTN78_RS09495	1846371	1846946	576	hypothetical protein	WP_021214780.1
FTN78_RS09500	1846976	1847344	369	hypothetical protein	WP_021214779.1

Locus Tag	Start	End	Length	Product	Protein ID
FTN78_RS09505	1847382	1847599	>218	transposase	
FTN78_RS09510	1847598	1847684	>87	bacteriocin	
FTN78_RS09515	1847815	1848015	201	cold shock domain-containing protein	WP_003132324.1
FTN78_RS09520	1848292	1848492	201	cold-shock protein	WP_011669084.1
FTN78_RS09525	1848742	1848876	>135	helix-turn-helix domain-containing protein	
FTN78_RS09530	1849236	1852253	3018	HsdR family type I site-specific deoxyribonuclease	WP_150891121.1
FTN78_RS09535	1852282	1852782	501	TIR domain-containing protein	
FTN78_RS09540	1852784	1853575	792	hypothetical protein	WP_150891157.1
FTN78_RS09545	1853655	1854914	1260	restriction endonuclease subunit S	WP_150891123.1
FTN78_RS09550	1854926	1855918	993	tyrosine-type recombinase/integrase	WP_150891125.1
FTN78_RS09555	1856000	1856635	636	restriction endonuclease subunit S	WP_150891127.1
FTN78_RS09560	1856632	1857273	642	type I R/M system specificity subunit	WP_150891129.1
FTN78_RS09565	1857218	1858729	1512	N-6 DNA methylase	WP_150891131.1
FTN78_RS09570	1858751	1860553	1803	N-6 DNA methylase	WP_150891133.1
FTN78_RS09575	1860847	1861164	>318	peptidase M13	
FTN78_RS09580	1861219	1861899	681	IS6-like element ISS1N family transposase	WP_014727478.1
FTN78_RS09585	1861899	1861973	>75	DNA transposition-like protein	
FTN78_RS09590	1861973	1862179	>207	IS982 family transposase	
FTN78_RS09595	1862426	1865539	3114	ATP-dependent DNA helicase RecQ	WP_101913464.1
FTN78_RS09600	1865917	1866114	>198	IS3 family transposase	
FTN78_RS09605	1866307	1866381	>75	DNA transposition-like protein	
FTN78_RS09610	1866389	1867204	816	EbhA	WP_101913463.1
FTN78_RS09615	1867346	1867420	>75	restriction endonuclease subunit S	
FTN78_RS09620	1867525	1868109	585	recombinase family protein	WP_081213206.1

Locus Tag	Start	End	Length	Product	Protein ID
FTN78_RS09625	1868237	1868341	105	type I toxin-antitoxin system Fst family toxin	WP_127005300.1
FTN78_RS09630	1868992	1869369	378	hypothetical protein	WP_101913462.1
FTN78_RS09635	1869369	1869956	588	HNH endonuclease	WP_101913461.1
FTN78_RS09640	1869963	1873193	3231	ATP-binding protein	WP_101913460.1
FTN78_RS09645	1873193	1873753	561	hypothetical protein	WP_081213209.1
FTN78_RS09650	1873740	1874234	495	hypothetical protein	WP_081213210.1
FTN78_RS09655	1874244	1876523	2280	hypothetical protein	WP_101913459.1
FTN78_RS09660	1876520	1877821	1302	hypothetical protein	WP_101913458.1
FTN78_RS09665	1878370	1878555	186	hypothetical protein	WP_101913457.1
FTN78_RS09670	1878606	1879502	897	WYL domain-containing protein	WP_101913456.1
FTN78_RS09675	1880435	1880653	219	hypothetical protein	WP_101913472.1
FTN78_RS09680	1880673	1881569	897	zinc ribbon domain-containing protein	WP_081213214.1
FTN78_RS09685	1881579	1882289	711	zinc-ribbon domain-containing protein	WP_081213215.1
FTN78_RS09690	1882393	1882761	>369	IS6 family transposase	
FTN78_RS09695	1882844	1884000	1157	IS3 family transposase	
FTN78_RS09700	1884319	1885785	1467	Y-family DNA polymerase	WP_101913455.1
FTN78_RS09705	1885745	1886125	381	hypothetical protein	WP_101913454.1
FTN78_RS09710	1886332	1886628	297	recombinase family protein	WP_150891135.1
FTN78_RS09715	1886677	1887624	948	IS30 family transposase	WP_003130410.1
FTN78_RS09720	1887649	1887969	>321	recombinase family protein	
FTN78_RS09725	1888073	1888870	798	AAA family ATPase	WP_010890656.1
FTN78_RS09730	1888884	1889150	267	hypothetical protein	WP_010890679.1
FTN78_RS09735	1889221	1889586	366	hypothetical protein	WP_021214758.1
FTN78_RS09740	1889600	1889980	381	single-stranded DNA-binding protein	WP_010890681.1
FTN78_RS09745	1890006	1890335	330	hypothetical protein	WP_021214757.1
FTN78_RS09750	1890340	1891230	891	IS982 family transposase	WP_150891137.1
FTN78_RS09755	1891380	1892279	900	EamA family transporter	WP_021216425.1
FTN78_RS09760	1892402	1893166	765	ABC transporter ATP-binding protein	WP_011835457.1
FTN78_RS09765	1893147	1894727	1581	hypothetical protein	WP_021037031.1
FTN78_RS09770	1894881	1895771	891	IS982 family transposase	WP_150891139.1
FTN78_RS09775	1895826	1896866	1041	hypothetical protein	WP_021214667.1

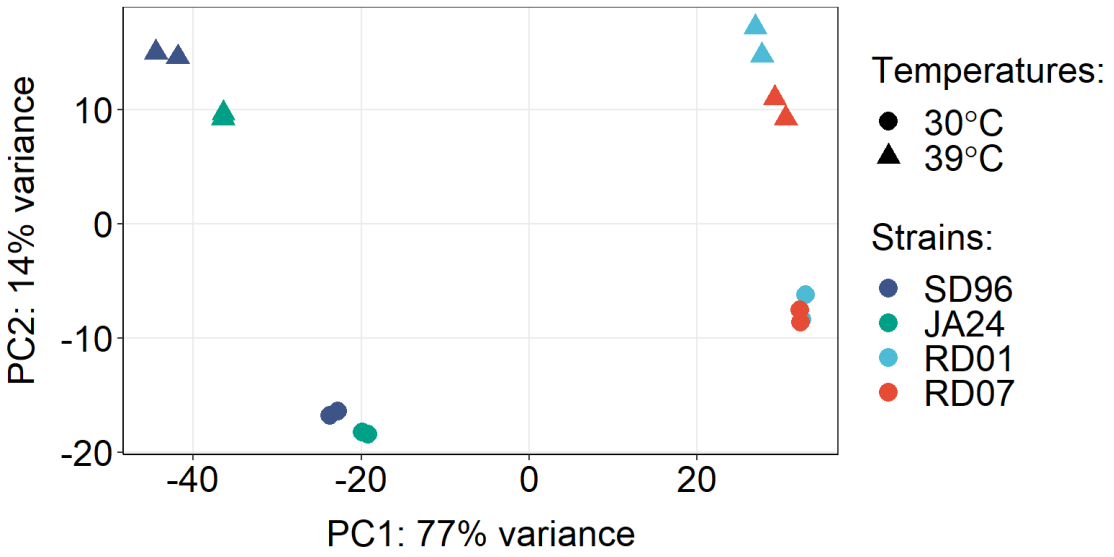

Figure S 3.1 Principal component analysis of the sample groups. Biological replicates are shown as separate dots in the same color. Strains are indicated with different colors (SD96: dark blue, JA24: green, RD01: light blue, RD07: red). Sample from different temperatures are shown with different shapes (30°C: circles, 39°C: triangles).

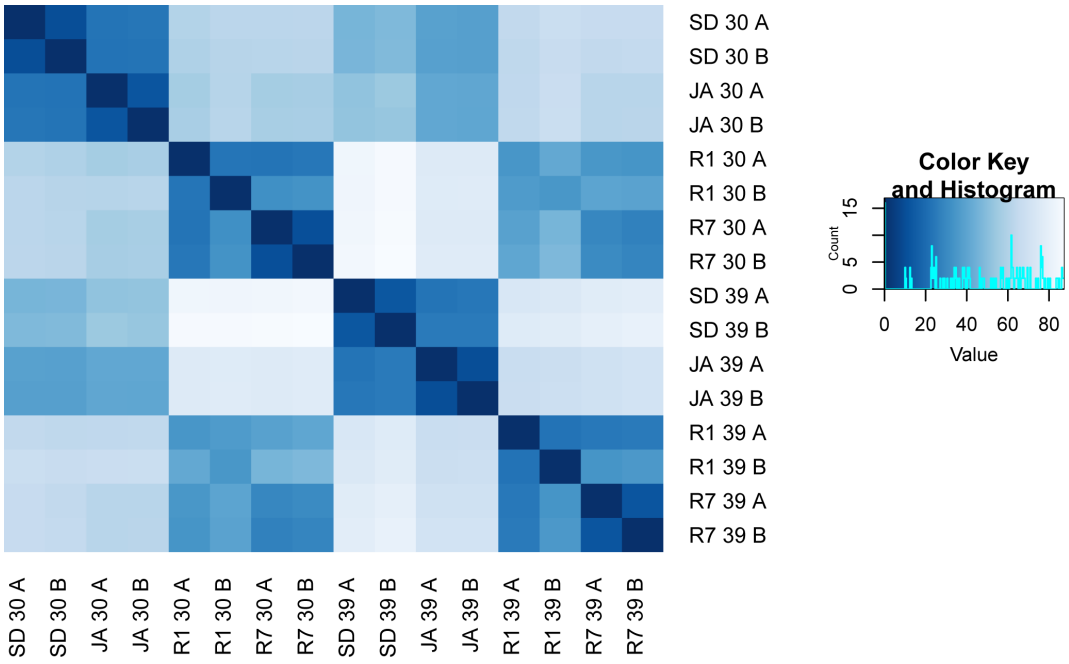

Figure S 3.2 Sample distance between biological replicates (A and B) and conditions (30°C and 39°C). Abbreviations are as follows: SD: SD96, JA: JA24, R1: RD01, R7: RD07. Labels should be read as follows: strain - temperature - replicate. The darker the shape, the higher the similarity between the samples.

4. Towards Single Strain Starter Cultures Characterization of a Robust *Lactococcus lactis* subsp. *lactis* Strain with Superior Autolytic Properties

This chapter serves as a manuscript for publication in an international scientific journal.

4.1 Abstract

Autolysis of the starter lactic acid bacterium *Lactococcus lactis* (*L. lactis*) is crucial for flavor development in cheese. Typically, autolysis begins in early ripening, and results in the release of intracellular enzymes, such as peptidases, into the cheese curd. In an attempt to isolate *L. lactis* subsp. *lactis* strains less affected by high cooking temperatures, we carried out adaptive laboratory evolution (ALE), exposing *L. lactis* subsp. *lactis* biovar diacetylactis SD96 to gradually increasing growth temperatures. The outcome of the ALE was successful, and we obtained three mutants that grew fast in milk at elevated temperatures. Surprisingly, the autolytic properties of the strains had changed, and they all displayed more rapid autolysis when compared to the parent strain SD96, in particular one isolate RD07, which autolyzed 2-3 times faster than its parent strain. Analysis of the transcriptome of RD07 revealed what appeared to be the underlying cause for the enhanced autolytic property, namely an altered pyrimidine metabolism. The *L. lactis* strains, which have been isolated, are unique and of great value to the dairy industry, and when applied, could lead to a more controlled and faster cheese manufacturing process, without compromising overall cheese quality.

4.2 Background

Lactic acid bacteria (LAB) are of immense importance for the dairy industry (1,2). Approximately 852 million tons of milk were produced worldwide in 2019, from which approximately 21 million tons of cheese were made (3,4). In the coming decade, global milk production is expected to increase continually by circa 1.7% per year, thereby reaching approximately 981 million tons by 2028 with a constant fraction devoted to cheese manufacturing (5). Enzymatically-coagulated cheeses, such as Gouda or Cheddar, are by far the most popular cheese variants and constitute approximately 75% of the world production (6). The production process for these two types of cheese can be divided into two phases, the manufacturing (up to 24 h) and the ripening phase (up to several months).

In the manufacturing phase, the milk is standardized, coagulated, and acidified, resulting in the cheese curd, which is cooked, salted, and processed depending on the desired final product (6).

Coagulation is usually achieved at 30°C by the enzymatic degradation of casein by aspartyl proteinases, traditionally isolated from calf stomachs (rennet) but increasingly obtained through microbial processes (7). Briefly, casein micelles form a stable emulsion, which is mainly stabilized by κ-casein. Most proteases specifically cleave κ-casein, causing a chain reaction leading to casein aggregation and formation of a solid protein gel, the cheese curd (8). Prior or simultaneous to the addition of the coagulating proteases, the starter culture is added, the function of which is to acidify the curd, by converting lactose into lactic acid, and to generate flavor compounds. Typical mesophilic starter culture for producing semi-hard cheese is mainly composed of *Lactococcus lactis* (*L. lactis*) subsp. *lactis* and *L. lactis* subsp. *cremoris*. The *lactis* subspecies is considered more robust and the driver for acidification, while the *cremoris* subspecies is essential for flavor formation during cheese ripening due to its superior autolytic properties (9,10). Often other LAB are supplemented in lower amounts, which are essential for ripening, flavor, or gas formation. Such strains are not involved in acidification of the milk and therefore called adjunct cultures (adjuncts) or nonstarter LAB (NSLAB). Both defined and undefined starter cultures are used nowadays, composed either of a few selected strains or an undefined mixture of strains originally obtained through spontaneous fermentation of raw milk (11).

After coagulation, the curd is cooked to expel whey, thus increasing curd firmness. Cooking involves exposing the curd to a moderate increase in temperature, typically 35-40°C, which promotes contraction of the curd gel structure, a process termed syneresis (12). Cooking is a challenging step for mesophilic starter culture, which grows optimally at 30°C, and elevated temperatures can significantly hamper growth, acidification, and subsequent flavor formation (13-16). After the cooking step, the curd is allowed to cool down to 30°C, and acidification proceeds until pH 5.4 - 5.2 is reached. With the emergence of next-generation sequencing technology, we just now start to understand the composition and dynamics of mesophilic starter cultures in detail (17,18), and more research is required to understand the compositional shifts under different cooking temperatures.

The ripening phase is essential for flavor development, and the most costly part of cheese production as ripening is a slow process. Extensive, specialized facilities ensuring a stable environment regarding temperature and humidity are required for storing the young cheeses for up to several months or years. For example, the costs for a nine-month ripening period were estimated to be 500-800 € per tonne of cheese. During this time, complex biochemical reactions occur and facilitate flavor formation (19). It is widely recognized that the lysis of LAB facilitates cheese ripening through the release of intracellular enzymes (11,20,21). Until recently, this was believed to be the main driver for cheese ripening, but since then, it has been shown that also intact cells remain throughout prolonged ripening times and likely contribute to flavor formation (17,22–24). During ripening, undesired bitter peptides are degraded enzymatically into well-tasting amino acids (25–29). Those bitter peptides derive from α- or β-casein and are formed due to excessive proteolysis by the coagulant or the cell-wall bound protease of *L. lactis* during the

early ripening phase (30–32). The degradation of those bitter peptides is mediated by a range of peptidases, of which proline iminopeptidase (PepI), prolidase (PepQ), and X-prolyl dipeptidyl aminopeptidase (PepX) are most important because they degrade proline-rich substrates, which are particularly bitter (32,33). Furthermore, the enzymatic degradation of milk fat into fatty acids and volatile flavor compounds happens during the ripening period (34).

As ripening is facilitated by intracellular enzymes released by lysed starter bacteria, autolysis is considered a rate-limiting step for cheese ripening (10,35–37). Generally, autolysis is highly strain-dependent, and traditionally *L. lactis* subsp. *cremoris* is more autolytic than *L. lactis* subsp. *lactis* (9,10). Consequently, cheese starter cultures contain many LAB strains, typically *L. lactis* subsp. *cremoris*, which autolyze well under the processing conditions (11,37–39). Autolysis is usually initiated by environmental factors such as nutrient depletion (40), heat shock (41), or salt-stress (42), which induce the expression of peptidoglycan (PG) hydrolases, so-called autolysins, that degrade the bacterial cell wall. In *L. lactis*, two major autolysins have been reported: AcmA and AcmB, whereas the former is considered most important (43,44). Besides that, three autolysins exist in *L. lactis*, which share homology with AcmA: AcmC, AcmD, and YlgB (45,46). Furthermore, a comparative study of several autolytic strains revealed a remarkable correlation between autolysis and the presence of temperate prophages in the genome (47). Accordingly, many highly autolytic strains were shown to express prophage encoded PG hydrolases that become active upon phage proliferation (48,49).

Due to the high costs of cheese ripening, many efforts have been undertaken to accelerate the ripening process. One straight forward way is the inclusion of highly autolytic strains in the starter cultures, but the accurate prediction of the lytic behavior during cheese production can be challenging (21,50). Alternatively, bacteriocin-producing cultures such as nisin-producing cultures are commonly used to prevent clostridial growth during cheese ripening, and to some extent, for accelerating cheese ripening as well (Per Dedenroth Pedersen, Sacco S.r.l., Italy). It has been shown independently, that nisin producing cultures are suitable for accelerating cheese production (51,52). The supplementation of enzymes is another possibility of accelerating ripening for which commercial solutions are available. It has also been suggested to use genetic engineering for accelerating cheese ripening, but low consumer acceptance has limited those approaches (38,39). All proposed methods generally face the difficulty of achieving the right type of autolysis, as it has been shown that both too weak and too excessive starter autolysis are detrimental for cheese production (53). Particularly when (pro)phages or *trans*-acting PG hydrolases are involved, excessive lysis of the starter bacteria during the acidification phase might occur and lead to fermentation failure (54).

Autolytic strains have been created through genetic engineering for controlling and accelerating cheese ripening. In 1992, a series of *L. lactis* strains was created expressing lysin from bacteriophage ϕ ML3, which grew well during exponential growth but lysed quickly in the stationary phase (55,56). By cloning and overexpressing the main lactococcal autolysin (AcmA),

autolysis was controlled in an inducible manner (44). A slightly different strategy was presented by expressing Metalloendopeptidase Enterolysin A from *Enterococcus faecalis* in *L. lactis*. This enzyme efficiently lysed several gram-positive bacteria and was overexpressed after induction with nisin, resulting in complete lysis after 2 h. The authors successfully tested this approach in model cheese experiments (57). Similarly, the food-grade expression of LytR, an amidase from phage ϕ r1t, was shown to enhance lysis of *L. lactis*, but only moderate improvements of the cheese taste were detected (54). Furthermore, it was shown that the deletion of alanine racemase (*alr*) or *dltD*, which encodes a protein that facilitates the ligation of D-alanine and lipoteichoic acids in the cell wall, increased autolysis (58). Such studies are useful for understanding the stress physiology of LAB. However, the resulting strains are not applicable in food production, as they are considered genetically-modified. Interestingly, a strain with the mentioned *dltD* mutation was obtained after UV-mutagenesis of *L. lactis* subsp. *lactis* MG1363 resulting in a natural mutant, which is not considered genetically-modified. However, this strain was not suitable for cheese production due to the lack of protease and lactose utilization genes (59).

For isolating naturally highly autolytic strains from culture collections, various screening methods were suggested (60). As pointed out previously, those methods have their limitations and are not well suited for high-throughput screening as autolysis strongly depends on the growth phase (61,62). Nevertheless, screening efforts were successful, and several highly autolytic strains have been isolated and described (63–65). So far, the targeted optimization of the autolytic properties has only been described in two studies, where heat-treatment favored the survival of autolytic strains, showcased on *L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *cremoris* ASCC892185, which is a commercial dairy strain, and *Lactobacillus helveticus* DSM 20075 (66,67). Generally, many of the published strain optimization campaigns have been carried out using laboratory strains of LAB, such as *L. lactis* MG1363 or IL1403, and thus resulting strains are not immediately transferrable to the industry (68,69).

Previously, we have carried out high-temperature ALE to increase the thermotolerance of the mesophilic *L. lactis* subsp. *lactis* biovar diacetylactis strain SD96, a phage resistant dairy strain provided by starter culture manufacturer Sacco S.r.l. (see **Chapter 2**, (70)). The purpose was to generate stains less sensitive to heat-stress imposed during the cooking step. We isolated three mutant strains with improved thermotolerance: JA24, RD01, and RD07. Those mutants have been fully sequenced, and RNA-sequencing of exponentially growing cells was carried out for understanding changes to the transcriptome. As high temperature imposes stress on the cell membranes, we hypothesized that adaptation to high temperatures might influence the autolytic properties of SD96. Here, we demonstrate that those thermotolerant mutants and one mutant in particular (RD07) have indeed improved autolytic properties and possess essential peptidase activity required for cheese ripening. Possible explanations for the autolytic phenotype of RD07 are discussed. In conclusion, the mutant strains and RD07, in particular, have the potential for accelerating cheese production and ripening.

4.3 Results

4.3.1 Adaption of *L. lactis* SD96 to Elevated Growth Temperature Leads to Enhanced Autolysis

L. lactis subsp. *lactis* biovar diacetylactis SD96 is a phage resistant and comparably thermotolerant strain, which has been developed by Sacco S.r.l. and is used in several of their starter cultures. In order to increase the robustness of SD96 towards temperature variations during cheese production, SD96 was gradually adapted to growth under temperature stress. SD96 was grown in UHT milk at constant high temperatures, and three thermotolerant strains (JA24, RD01, and RD07) were isolated (see **Chapter 3**). When characterizing growth in liquid cultures, we noticed a rapid drop in cell density after depletion of lactose, and we speculated that this could result from autolysis. To examine this, we used a previously reported method for quantifying autolysis (71–73). To our surprise, we found that the mutant strains autolyzed faster than the parent strain, assessed by the decrease of optical density (OD_{600}) (**Figure 4.1**). Here, we define autolytic activity as the percent decrease in OD_{600} (compared to the initial OD_{600}) after 3 h at 30°C. The autolytic activity was highest for RD07 with 75.9 ± 0.6%, followed by RD01, JA24, and SD96 with 51 ± 0.1%, 47.7 ± 0.3% and 35.3 ± 0.5%, respectively. Similarly, the initial autolysis rates [relative OD [%] / h] during the first hour were increased, and RD07 showed the most significant change (SD96: -17 ± 4 %/h, JA24: -29 ± 4 %/h, RD01: -26 ± 1 %/h, RD07: -49 ± 3 %/h). According to the data, RD07 autolyzed 2-3 times faster than the parent strain SD96. In the assay used, a surfactant (Triton X-100) is present, which accelerates autolysis. However, the same trend was observed when the surfactant was omitted in the assay, albeit the overall autolysis activities decreased (**Figure S 4.1**).

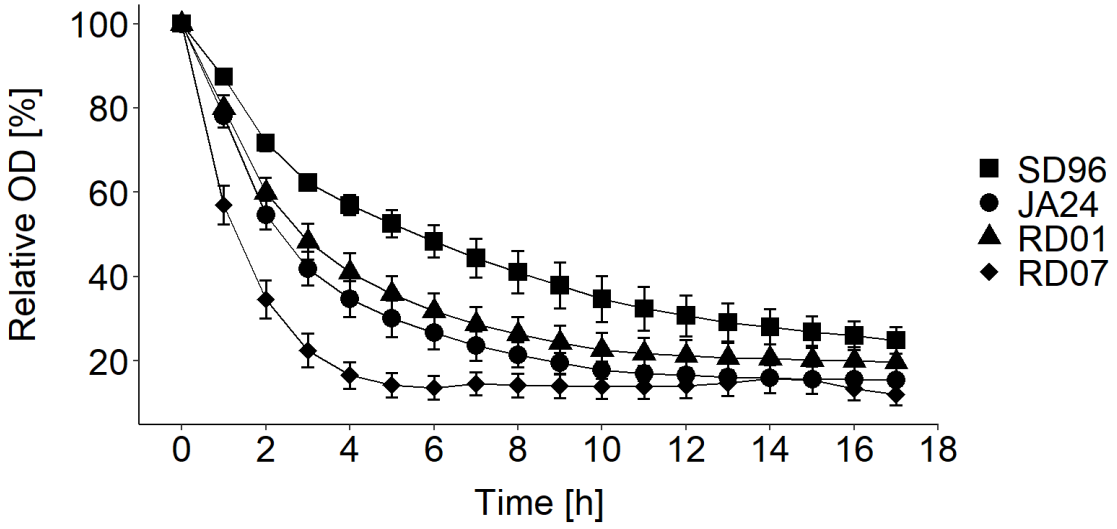

Figure 4.1 Autolysis of SD96 and the mutant strains JA24, RD01, and RD07 isolated during exponential growth in LM17 broth. The relative decrease in OD indicates cell lysis. Autolysis was induced by adding a surfactant (Triton X-100).

Next, we investigated the influence of the physiological state of the cells on autolysis by harvesting cells in the early stationary phase, at pH 5.2, during milk fermentation (**Figure 4.2**). This situation was chosen because it is similar to the environment in fresh cheese curd, even though different amounts of residual lactose are present (19). Generally, autolysis was slower compared to the cells isolated from the exponential phase in LM17, but the mutant strains showed similar behaviors. Again, the autolysis of RD07 was fastest, followed by RD01, JA24, and SD96. Autolytic activities were as follows: 14.9 ± 0.8%, 19.0 ± 1.2%, 20.8 ± 0.7% and 27.2 ± 2.7% for SD96, JA24, RD01, and RD07, respectively. Unfortunately, maximal autolysis was not observed in this assay, but after 36 h, clear differences were observed. Only an 18 ± 3% drop in cell density was observed for SD96 after 36 h, whereas a massive drop of 71 ± 3% was observed for RD07. JA24 and RD01 showed intermediate autolysis with a drop in cell density of 40 ± 3% and 52 ± 2%, respectively. This experiment underlines the previous findings that the mutant strains are more autolytic compared to the parent strain SD96, with RD07 being 2-3 times faster than SD96.

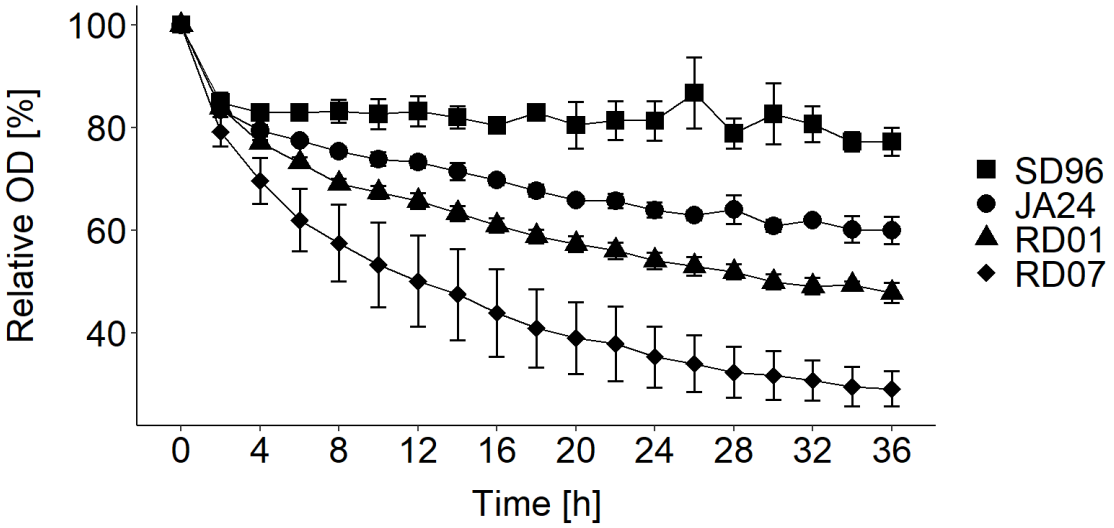

Figure 4.2 Autolysis of SD96 and the mutant strains JA24, RD01, and RD07 isolated at pH 5.2 during milk fermentation. The relative decrease in OD indicates cell lysis. Autolysis was induced by adding a surfactant (Triton X-100).

4.3.2 The Major Autolysin in *L. lactis,* AcmA, Is Moderately Overexpressed in RD07

In order to identify potential explanations for the improved autolysis of the mutant strains and RD07 in particular, we investigated the gene expression levels (transcripts per million, TPM) and differential expression of the five lactococcal PG hydrolases AcmA, AcmB, AcmC, AcmD and YjgB during exponential growth in reconstituted skim milk (RSM) (**Figure 4.3**). SD96 expressed *acmA* and *acmD* to comparably high levels (*acmA*: 378 ± 1 TPM, *acmD*: 582 ± 5 TPM), while *acmB* was expressed moderately (172 ± 9 TPM) and *acmC* and *yjgB* were expressed weakly (*acmC*: 13 ± 2 TPM, *yjgB*: 68 ± 2 TPM).

In RD07, significant (adjusted p-value (p_{adj}) < 0.05) but moderate overexpression of *acmA* (log2-fold change = 0.44 ± 0.08, corresponding to 1.35 ± 0.08-fold increase) compared to SD96 was found. At the same time, *acmB* and *acmD* expression levels were reduced (log2-fold changes: -1.13 ± 0.10 and -0.88 ± 0.08, respectively). In JA24, a slight reduction of *acmB*, *acmD,* and *yjgB* expression was observed (log2-fold changes: -0.46 ± 0.11, -0.35 ± 0.10, and -0.61 ± 0.15, respectively), whereas RD01 only showed reduced expression of *yjgB* (log2-fold changes -0.84 ± 0.27). While the moderately increased *acmA*-expression could at least partly explain the autolytic phenotype of RD07, the increase in autolysis of JA24 and RD01 remained elusive.

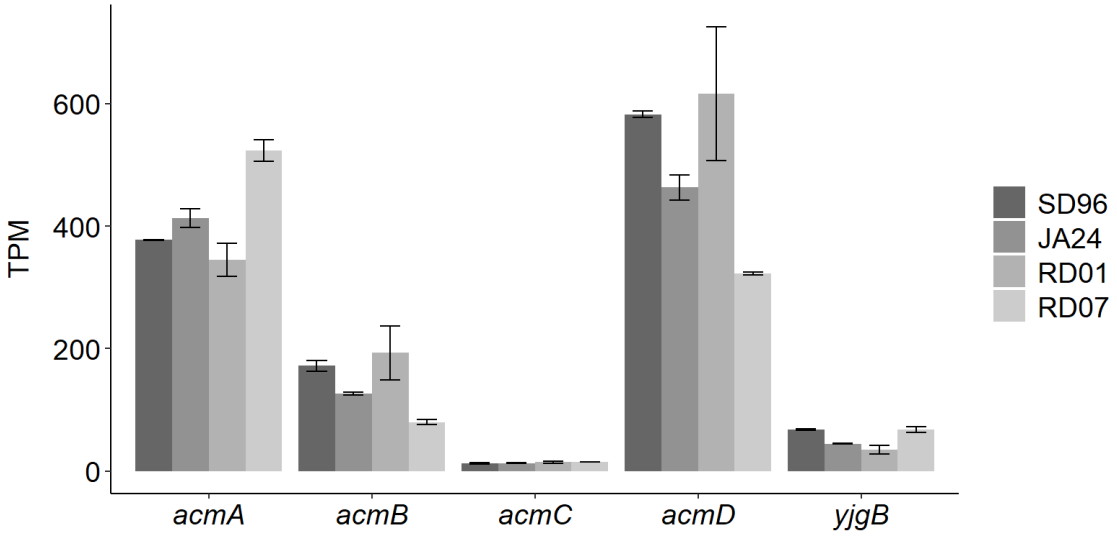

Figure 4.3 Expression levels (transcripts per million, TPM) of the five autolysins of *L. lactis*. The mayor autolysin AcmA is overexpressed in RD07.

4.3.3 Peptidase Activity in the Mutant Strains Has Not Been Affected by ALE in Milk

As a measure of the debittering potential of SD96 and derived thermotolerant mutants, we determined the activity of PepX (µmol/min/mg) in cell-free extracts using the chromogenic substrate Ala-Pro-*p*-nitro-anilide (**Figure 4.4**). PepX is a reference enzyme because it is involved in degrading bitter proline-rich peptides and is often measured when assessing autolysis (32,33). In comparison to SK11, a strain which is known to have excellent debittering properties (32), SD96 showed higher PepX activity (SK11: 74 ± 9 µmol/min/mg, SD96: 118 ± 13 µmol/min/mg). JA24 and RD07 provided similar PepX activities compared to SD96 (JA24: 131 ± 9 µmol/min/mg, RD07: 122 ± 8 µmol/min/mg), while RD01 showed a slightly lower PepX activity (90 ± 10 µmol/min/mg).

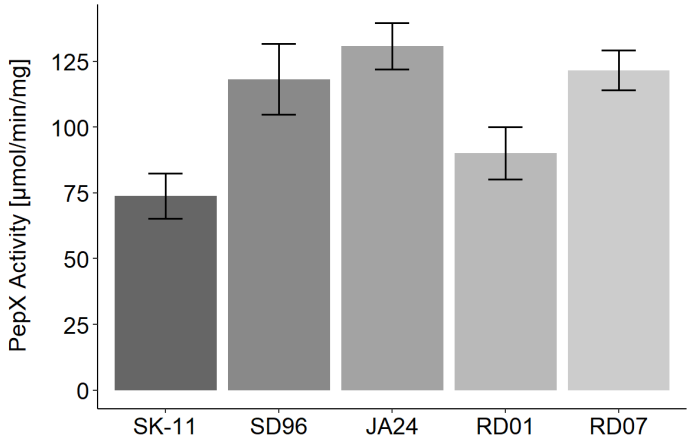

Figure 4.4 Specific PepX activity in cell-free extracts of SK11, SD96, JA24, RD01, and RD07.The results were derived from three biological replicates and the standard deviations are shown.

In order to evaluate if the expression of PepX and other peptidases was altered in the mutant strains, we investigated the expression levels of the following peptidases: PepA, PepC, PepF, PepN, PepO, PepP, PepQ, PepT, PepV and PepX (**Figure 4.5**). All of the peptidases were expressed with high TPM in SD96 (> 220 TPM), and with a few exceptions, the expression levels in the mutants had not changed significantly compared to SD96. In RD01, none of the genes was significantly differentially expressed. In JA24, three genes were expressed with slightly reduced levels (absolute log2-fold change < 0.35) and high confidence (p_{adj} < 0.05): *pepC*, *pepO,* and *pepX*. In RD07, four genes were slightly reduced with high confidence: *pepC*, *pepN*, *pepO,* and *pepQ* (log2-fold changes: -0.61 ± 0.08, -0.72 ± 0.08, -0.99 ± 0.08, and -0.29 ± 0.09, respectively). Only *pepA* was significantly overexpressed in RD07 with a log2-fold change of 1.22 ± 0.08.

This analysis showed that the peptidases, which are responsible for flavor formation during cheese ripening by degrading bitter peptides, were neither strongly negatively nor positively

affected by the ALE. This finding underlines the assumption that the highly autolytic RD07 is a promising candidate for accelerating cheese ripening by removing bitter peptides.

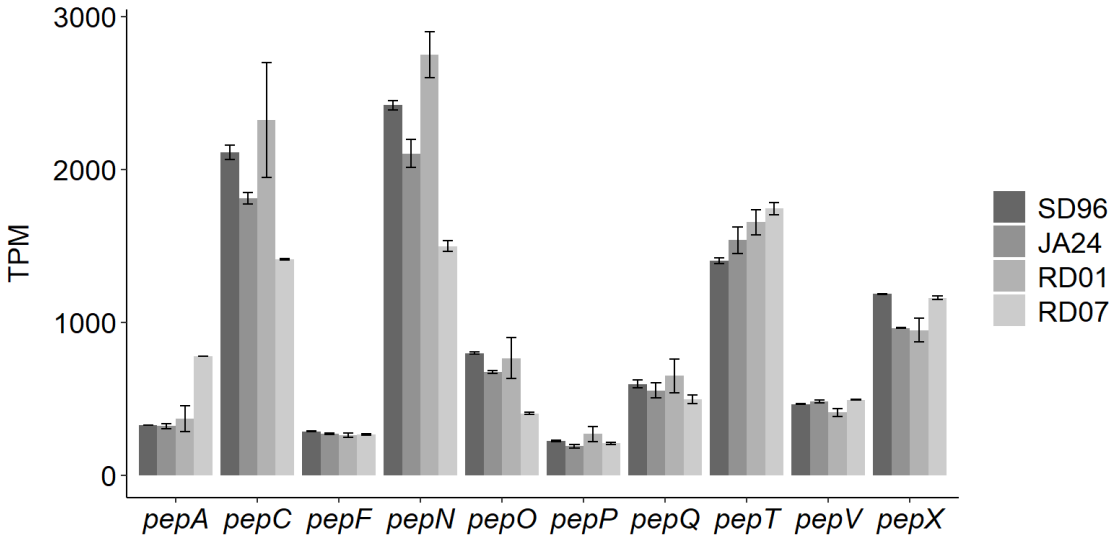

Figure 4.5 Expression level (TPM) of peptidases during early exponential growth in skim milk.

4.3.4 Purine and Pyrimidine Biosynthesis Genes are Overexpressed Exclusively in RD07

Nucleotide metabolism in *L. lactis* has been linked to cell wall biosynthesis and influences the rigidity of the PG structure (74). Therefore, we investigated the differential expression of nucleotide biosynthesis genes. In RD07, both *purR* and *pyrR*, regulating the expression of purine and pyrimidine biosynthesis genes, respectively, were significantly overexpressed (log2-fold change = 0.97 and 1.58, respectively), and nearly all other genes involved in purine and pyrimidine biosynthesis were up-regulated as well. Contrary, in JA24 and RD01, those genes were not differentially expressed (**Figure 4.6**). The purine biosynthesis genes are expressed as four operons, and the following average log2-fold changes have been observed in RD07: 1.01 ± 0.1 (*purCSQLF*), 1.341 ± 0.002 (*purMN*), 1.05 ± 0.01 (*purH-hprT*), 1.31 ± 0.11 (*purDEK*). The pyrimidine biosynthesis genes are expressed as five operons: *carB*, *pyrRPBcarA*, *pyrEC*, *pyrKDbF,* and *pyrDa* with the following average log2-fold changes in RD07: 1.27 (*carB*), 1.59 ± 0.04 (*pyrRPBcarA*), 1.54 ± 0.06 (*pyrEC*), 2.16 ± 0.07 (*pyrKDbF*) and -0.41 (*pyrDa*).

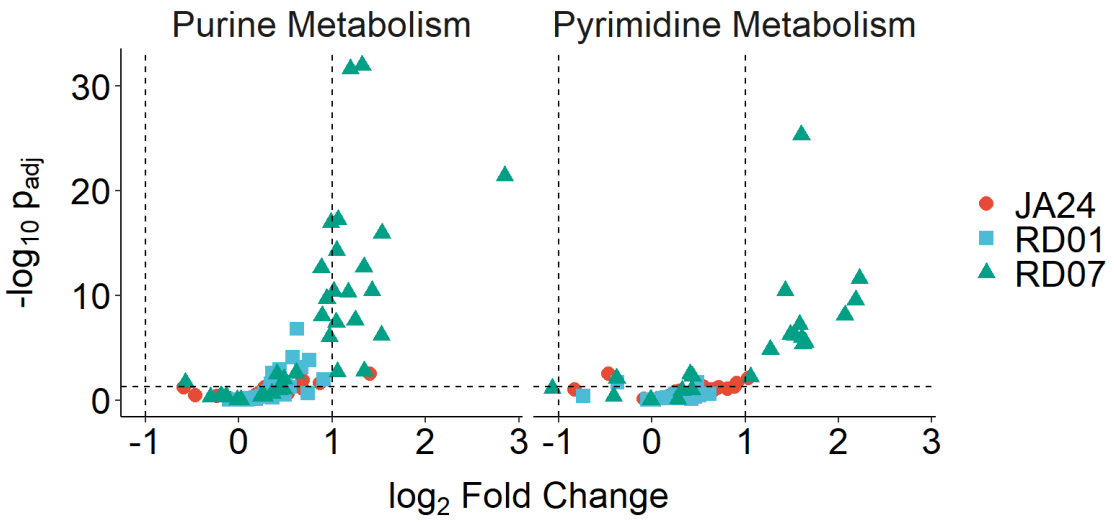

Figure 4.6 Volcano plots showing DEGs related to nucleotide biosynthesis and metabolism in JA24, RD01, and RD07 compared to SD96 at 30°C. Left panel: purine metabolism. Right panel: pyrimidine metabolism. The three mutant strains are displayed with different colors and shapes (JA24: red circles, RD01: blue squares, RD07 green triangles).

4.4 Discussion

4.4.1 RD07 Is a Unique Strain which Autolyses Efficiently

Here we report the autolytic phenotype of three thermotolerant mutants: JA24, RD01, and RD07, which were derived from *L. lactis* subsp. *lactis* biovar diacetylactis SD96 in the course of an ALE performed at gradually increasing temperatures in milk (see **Chapter 3**). One isolate, RD07, was shown to possess superior autolytic properties. Therefore, autolysis was induced in cells harvested from exponential growth using the surfactant Triton X-100 (**Figure 4.1**). Triton X-100 induces autolysis by activating autolysins (71,73,75,76), and thus facilitates the study of a process that usually takes weeks. Using this assay, RD07 was found to be 2-3 times more autolytic than SD96. To investigate the influence of the physiological state of the cell, we assessed the autolysis of cells isolated from a milk culture grown until pH 5.2 was reached, which is the typical pH in a cheese curd after fermentation (**Figure 4.2**). These experiments revealed the importance of the physiological state of the cells tested, as cells harvested at low pH autolyzed less quickly. Nevertheless, the same increased autolysis was observed for RD07, which was 2-3 times higher than SD96.

We included the strain SK11, an *L. lactis* subsp. *cremoris* strain, as a reference strain, as this particular strain is known to produce non-bitter cheese (32,41,48). However, for SK11, only a little autolysis was observed (data not shown). SK11 has been found to contain a temperate prophage, which is activated at temperatures above 37°C (41,48), and this strains thus appears not to be a

suitable reference for autolysis when using a constant low temperature. *L. lactis* subsp. *cremoris* AM2 is also known to autolyze quickly and produce non-bitter cheese and would perhaps be a better reference (10,35,36,60). The autolytic phenotype of AM2 was also assigned to the existence of a prophage, whose lysin was induced when reaching the stationary phase (49).

As autolysis has generally been shown to correlate with the presence of temperate prophages, we investigated the presence of putative prophages in SD96 (47). For this, we mined the genome of SD96 using the PHASTER tool (77,78). In total, seven prophages were identified, of which five were intact according to the tool (**Table S 4.2**). Interestingly, the incomplete prophage 4 was lost with the 80.000 kb deletion in RD01 and RD07. Besides this, no significant differences in the expression of the prophages were observed when comparing the mutant strains to SD96. Prophages 1 and 3 encode lysin genes (FTN78_RS00810 and FTN78_RS07605, respectively), which were expressed only with low levels in all strains (< 40 TPM). This data did not suggest that prophages or deriving lysins were responsible for autolysis in RD07.

4.4.2 The Autolytic Activity of RD07 Is Comparable to Other Highly Autolytic Strains

Besides SK11 and AM2, Riepe *et al.* described two highly autolytic strains in more detail (*L. lactis* subsp. *cremoris* CO and 2250). In their study, autolytic activity was defined as the percent decrease in OD_{650} (compared to the initial OD_{650}) after 3 h at 30°C and measured in different buffers without further inducing autolysis by any means (for example, by adding a surfactant). The maximal autolytic activity was detected when cells were harvested from the early-stationary phase using M17 supplemented with glucose (CO: 45%, 2250: 36%) (65). When using similar conditions, RD07 showed comparable autolytic activity (39.9 ± 0.6%), while SD96 and the other mutants showed lower autolytic activity (16.8 ± 0.3%, 17.9 ± 0.5% and 19.7 ± 0.2% respectively) (**Figure S 4.1**, left). However, different from the experiments described by Riepe *et al.*, M17 supplemented with lactose was used for cultivation, and the carbon source has been shown to influence autolytic activity, with glucose usually resulting in higher autolysis compared to lactose (79). This comparison shows that RD07 belonging to the *lactis* subspecies has similar autolytic properties as highly autolytic strains belonging to the *cremoris* subspecies, which are usually considered to be more autolytic.

Thermotolerant LAB with autolytic properties have been described first by Smith *et al.* (66). Spontaneous mutants of *L. lactis* subsp. *cremoris* MG1363 and the industrial strain *L. lactis* subsp. *cremoris* ASCC892185 were obtained during high-temperature growth, and were shown to be more thermotolerant but also more sensitive to osmotic pressure (66). Inspired by their findings, Spus *et al.* isolated spontaneous thermotolerant mutants of *Lactobacillus helveticus* DSM 20075 and revealed that temperature adaptation led to improved autolytic properties of the strain (67). In their work, a lactate buffer with high NaCl concentration was used to induce autolysis. Using the same assay condition with our strains, confirmed again that RD07 was more

autolytic compared to SD96 (**Figure S 4.1**, right). Here we presented another example where adaptation to high temperature influenced the autolytic properties of LAB. We used a different adaptation strategy, and our mutants had completely different mutations (see discussion below), as found before. In light of our findings, a conserved connection between thermotolerance and autolysis in LAB could be expected, independent of the way thermotolerance was achieved. Overall, the comparison with other autolytic *L. lactis* strains showed that RD07 is indeed a highly autolytic strain with unique mutations. Moreover, it seems that autolysis in RD07 is generally induced when its growth arrests, for example, through nutrient limitation. An induction event such as a heat shock, which is required to induce autolysis in SK11, is not required for RD07, which is beneficial for a broad application of this strain for producing various cheese products.

4.4.3 The Expression of Peptidases Was Not Altered During ALE

To strengthen the assumption that the mutant strains are well suited for producing tasty cheeses, we determined the peptidase expression in SD96 and the mutant strains, as well as the PepX activity in cell lysates (**Figure 4.4**). PepX was chosen as a representative peptidase as it plays a crucial role in degrading proline-rich peptides derived from casein breakdown (30). Those peptides were shown to be particularly bitter and are usually not degraded by other aminopeptidases (32). SD96 contained all reported lactococcal peptidases (PepA, PepC, PepF, PepN, PepO, PepP, PepQ, PepT, PepV, PepX), which were also expressed during exponential growth in milk (**Figure 4.5**). The mutant strains were shown to have similar expression levels for the peptidases, with only minor deviations. Thus, we concluded that the ALE had not altered the expression of those peptidases. By comparing the PepX activity of our strains with that of SK11, a strain which was reported to be able to remove bitterness from cheese (32), we found higher activity, indicating that SD96 and the mutant strains are indeed well suited for removal of bitter peptides. This finding is in line with previous reports, in which several strains of *L. lactis* were investigated for their peptidase activity and found suitable for degrading bitter peptides (80–82).

4.4.4 Changes in Pyrimidine Metabolism Likely Contribute to the Autolytic Phenotype of RD07

As the mutants were sequenced, we were able to identify the mutations and potential mechanisms leading to the autolytic phenotype of RD07 (**Table S 4.1**). RD01 and RD07 share several mutations, which likely do not cause the autolytic phenotype of RD07, as RD01 is only moderately autolytic. Consequently, mutations unique to RD07 (RD07-1, RD07-2, RD07-4, RD07-5, RD07-6, and RD07-7) are considered most influential to the autolytic properties. Some of the mutations shared by RD01 and RD07 could act synergistically and therefore be relevant for the autolytic phenotype, but for simplification, we focus on the mutations unique to RD07.

RD07-2 and RD07-4 are both located in putative prophages. The former mutation (RD07-2) causes V957E in a hypothetical protein located within prophage 1 (FTN78_RS00795). This

prophage encodes a lysin gene (FTN78_RS00810), and potentially the mutation could influence the activity of the prophage and, consequently, the activity of the respective lysin. Nevertheless, no significant overexpression was observed in RD07, but instead, the lysin was significantly reduced (log2-fold change = -0.98 ± 0.19). Mutation RD07-4 causes A410S in a DNA primase of prophage 6 (FTN78_RS10440). Prophage 6 does not contain a gene encoding for a lysin. As mentioned above, the expression levels of all prophages were not changed significantly in RD07. Nevertheless, the expression of the prophages could be triggered during a later growth phase or by environmental factors, such as nutrient depletion. This possibility remains to be investigated in future studies.

In RD07, *purR*, which is mutated in this mutant, and nearly all other genes involved in purine biosynthesis were significantly up-regulated (**Figure 4.6**). Genes belonging to the purine metabolism are overexpressed during acid stress, and therefore the observed overexpression could be expected (83,84). As discussed in **Chapter 3**, changes in purine metabolism are considered to contribute to the thermotolerance of RD07. Pyrimidine biosynthesis genes were also exclusively overexpressed in RD07, and interestingly, pyrimidine metabolism was shown to influence the cell wall plasticity (74). In essence, it was shown that mutants lacking *pyrB* had a more rigid cell wall, and it was speculated that a decreased pyrimidine metabolism results in increased availability of L-Asp for PG biosynthesis (**Figure 4.7**, left). For PG biosynthesis, L-Asp is racemized by RacD into D-Asp, which forms the crosslink between two MurNAc-pentapeptide units by connecting L-Lys and D-Ala. Typically, L-Asp is also converted to N-carbamoyl-L-aspartate by PyrB and thereby channeled into pyrimidine biosynthesis. When the PyrB-reaction is reduced, for example, by down-regulation of the *pyrB* gene, L-Asp is increasingly incorporated into the PG structure, allowing more PG cross-bridges. As a consequence, PG thickness and rigidity are increased (74).

This situation reassembles the opposite of what we observed here. Instead of a reduction of *pyrB* expression, *pyrB* was significantly overexpressed in RD07 (log2-fold change = 1.62 ± 0.32). Consequently, this overexpression would lead to the depletion of the L-Asp pool resulting in decreased cross-bridging in the PG structure, thereby contrasting the above-described mechanism. Accordingly, this would lead to a more flexible cell wall, which would be prone to degradation by autolysins, and lead to an autolytic phenotype (**Figure 4.7**, right).

The reason why the pyrimidine biosynthesis genes were overexpressed in RD07 is not apparent because no related mutation was identified. Compared to SD96, RD07 grows faster in milk (see **Chapter 3**), and this faster growth could potentially be the reason for an increased demand for nucleotides resulting in overexpression of the pyrimidine genes. Alternatively, mutation RD07-7 located in *purR* might be involved indirectly in the overexpression of pyrimidine metabolism. RD07-7 caused G206S mutation in the phosphoribosyl pyrophosphate (PRPP) binding pocket of PurR and thereby likely affected the expression of the purine biosynthesis genes. Potentially, the observed enhancement of the *pur* genes in RD07 might have caused a decrease of intracellular

PRPP levels because PRPP is used for purine biosynthesis (85). The expression of the pyrimidine biosynthesis genes was shown to correlate negatively with intracellular PRPP levels (85). Consequently, the overexpression of purine biosynthesis genes, resulting in a decreased PRPP pool, could thus lead to the overexpression of the pyrimidine biosynthesis genes.

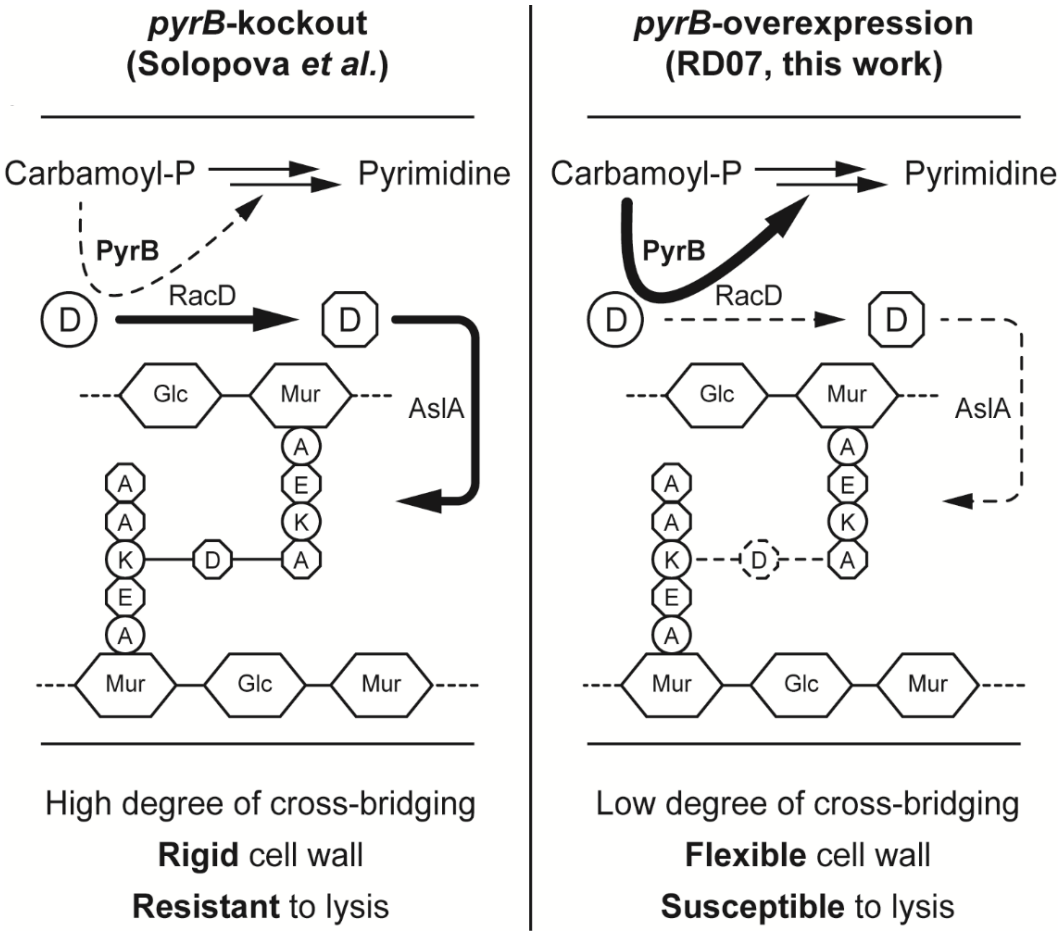

Figure 4.7 Possible relationship between pyrimidine metabolism and cell wall structure in RD07. L-amino acids are shown as circles with one-letter code. D-amino acids are shown as diamond-squares. Thick arrows indicate an increased metabolite flux, while dashed arrows indicate a reduced flux. Left: in a *pyrB*-knockout strain described by Solopova *et al.*, it was suggested that increased L-Asp availability resulted in cells resistant to lysis and a higher degree of crosslinking in the peptidoglycan part of the cell wall (74). Right: in RD07, increased expression of *pyrB* would consequently result in the drainage of the L-Asp pool. Then, L-Asp would not be sufficiently available for cell wall biosynthesis via RacD and AslA, resulting in a lower degree of crosslinking and a more flexible cell wall, which is more susceptible to autolysis. Abbreviations are as follows: PyrB: aspartate transcarbamoylase, RacD: aspartate racemase, AslA: aspartate ligase, Glc: N-acetylglucosamine, Mur: N-acetylmuramic acid.

4.4.5 Other Mutations in RD07 Might Contribute to Its Autolytic Phenotype

Mutation RD07-5 is located upstream of *hflB*/*ftsH*, which encodes an ATP-dependent zinc metalloprotease (FTN78_RS10530). The expression of this gene was reduced at high temperature in RD07 (log2-fold change -0.8 ± 0.17). In the other two mutants, this gene was weakly enhanced but with low confidence. This pattern is surprising, as FtsH was shown to be important for stress resistance, and therefore its down-regulation in RD07 was unexpected (86,87). Recently, a link between cellular lysis and FtsH was established, where an *ftsH*-deletion mutant of *L. lactis* became insensitive to the action of a phage endolysin (88). Here, an autolytic mutant was presented, having reduced *ftsH* expression, which seems contradictory to previous observations. Consequently, it would be interesting to investigate the interaction between FtsH and endogenous PG hydrolases such as AcmA.

As described in **Chapter 3**, in UHT milk, SD96 most likely starved for amino acids, which likely affected PG biosynthesis. This hypothesis was built on the observation that SD96 initially grew slowly in UHT milk, and mutations affecting CodY in JA24 and RD01 seemed to have overcome this limitation. Amino acid starvation and cellular lysis are linked to one another, as was demonstrated previously. Huang *et al.* showed that the adverse effects of amino acid starvation could be released by mutations in CodY. In this study, the deletion of the peptidase PepV resulted in L-Ala starvation, causing an autolytic phenotype, most likely due to the incorporation of other amino acids in the PG structure (89). These results strengthen the assumption that the amino acid composition of the PG structure strongly affects the autolytic properties of *L. lactis*, as discussed above in the case of L-Asp (see **Section 4.4.4**). Surprisingly, RD07 did not have mutations affecting amino acid metabolism and therefore seemed to have found a different strategy to thrive in the UHT milk environment. It is possible that in RD07, a more flexible but stable cell wall structure established, which allowed fast growth even though building blocks were limited or changed. Another indication for this hypothesis is that RD01 and RD07 both have the same mutation in MurC, the enzyme responsible for incorporating L-Ala into the PG structure. This mutation might influence the PG structure by allowing the incorporation of other building blocks than L-Ala and thereby changing the properties of the cell wall, as observed previously (90). Summarizing in RD07, potentially, the enhanced pyrimidine metabolism in combination with the MurC-mutation, and the moderate overexpression of AcmA resulted in the unique fast-growing and autolytic strain.

4.5 Conclusion

The thermotolerant mutant strains JA24, RD01, and particularly RD07, were shown to be more autolytic than the parent strain SD96, while they contain all required peptidases for removing bitterness from cheese. To the best of our knowledge, this is the first report about the conversion of a mesophilic, poorly autolytic *L. lactis* subsp. *lactis* strain into a thermotolerant, fast autolyzing strain. The underlying cause for RD07s unique phenotype was investigated using genomics and

transcriptomics analysis. The latter revealed an up-regulation of the pyrimidine biosynthesis genes in RD07 compared to SD96 and the other two mutants, which might cause the loss of rigidity of the PG layer and thus be responsible for the autolytic phenotype of this mutant. Other factors and mutations potentially supporting the autolytic phenotype of RD07, such as the moderate overexpression of the mayor autolysin AcmA, were also discussed. Overall, the numerous mutations in RD07 suggest a complicated interplay of effects, which finally caused the increased autolysis. Unfortunately, without genetic engineering, a genotype-phenotype relation could not be established unambiguously. However, our systems biology analysis provides essential guidance and inspiration for future experiments to reveal the mechanisms of how thermotolerance and autolysis are connected in *L. lactis*.

In summary, the mutant strains grew fast in milk even at elevated temperatures and autolyzed readily, which makes them attractive candidates for accelerating cheese production. After all, the commonly spread assumption that autolytic *L. lactis* subsp. *cremoris* strains are required in mesophilic starter cultures for removing bitterness in the cheese, might need revision because RD07 seems to combine the best of both subspecies: fast acidification and fast autolysis.

4.6 Methods

4.6.1 Bacterial Strains and Cultivation

L. lactis subsp. *cremoris* SK11 was purchased from ATCC (ATCC® BAA-493™), and Sacco S.r.l. kindly provided *L. lactis* subsp. *lactis* biovar diacetylactis SD96. The mutant strains JA24, RD01, and RD07 were obtained by high-temperature ALE, as described in detail in **Chapter 3**. Briefly, the strains were cultivated in sequential batch fermentations using commercially available UHT milk (3.5% fat), applying high temperature as selection pressure. JA24 was cultivated at 30°C and, after 1 h of incubation, exposed to a 42°C heat shock for 1 h, followed by incubation at 30°C. RD01 and RD07 are from the same lineage, which was cultivated at constant high temperatures, starting from 39°C and ending at 41°C. RD01 was isolated at 40°C, and RD07 was isolated at 41°C. All bacteria were routinely cultivated in LM17 (M17-broth with 0.5% lactose) medium for handling in the laboratory. Repeated subcultivation using LM17 was avoided to avoid plasmid loss.

4.6.2 Surfactant-Induced Autolysis

All strains were incubated in LM17 using three independent biological replicates for 16 h at 30°C. Then, 100 ml cultures were inoculated using a 100-fold dilution. Cell density was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer. When the culture reached mid-exponential phase at OD_{600} = 0.5-0-8, cells were harvested and washed twice using cold sodium phosphate buffer (50 mM, pH 7.0). Then OD_{600} was determined again, and cells were resuspended to OD_{600} = 1 in sodium phosphate buffer (50 mM, pH 7.0) supplemented with 0.05% Triton X-100. Autolysis was determined by observing the decrease of intact cells by measuring

OD_{600} over time. 200 µl of each suspension was transferred into a honeycomb microplate, and OD_{600} was measured every 20 min using a Bioscreen C Analyzer while incubating at 30°C. Two technical replicates were analyzed. Initial autolysis rates [relative OD/h] within the first hour and the autolytic activity, defined as a decrease in OD_{600} after the first 3 h (in percent), were derived from the data.

Milk fermentation was carried out using RSM with 10% milk solids after autoclaving at 121°C for 5 min. From an LM17-plate, the first pre-culture in RSM was inoculated using a single colony and incubated at 30°C for 16 h. This pre-culture was used to inoculate a second pre-culture in RSM using 100-fold dilution, followed by incubation at 30°C for 16 h. The second pre-culture was used to inoculate 50 ml RSM 1:100, followed by incubation at 30°C. The pH was monitored using an iCinac instrument. When pH 5.20 was reached, the cultures were put on ice to stop the fermentation. Then, the cultures were diluted 10-fold into a cold EDTA-solution (2 g/L, pH 12.0) to dissolve the casein. Cells were harvested by centrifugation at 10.000 x g for 10 min at 4°C and washed again using EDTA-solution to remove residual casein. The obtained pellets were washed twice using cold sodium phosphate buffer (50 mM, pH 7.0), and OD_{600} was determined. Finally, the cells were resuspended to OD_{600} = 1 in sodium phosphate buffer (50 mM, pH 7.0) supplemented with 0.05% Triton X-100. Autolysis was monitored as described above for 36 h.

4.6.3 Salt-Induced Autolysis

For determining salt-induced autolysis, cells were harvested at pH 5.2 from RSM, as described above. After the removal of casein using EDTA, the cells were washed twice in a lactate buffer, reconstituting conditions in the cheese matrix, as described by Spus *et al.* (67). This buffer contained 100 mM potassium lactate, 10 mM KCl, 1 mM $MgSO_4$, and 5 mM KH_2PO_4 at pH 5.0. For inducing autolysis, cells were resuspended in lactate buffer supplemented with 0.5 M NaCl. OD_{600} was determined after 48 h using a spectrophotometer. Three independent biological replicates were analyzed.

4.6.4 PepX Activity

For determining PepX activity, cells were harvested from an early stationary phase culture in LM17, washed twice with cold lactate buffer, and kept on ice until measurement. Immediately before proceeding, the cells were resuspended in 900 µl lactate buffer supplemented with 0.5 M NaCl and transferred into a 2 ml safe-lock tube containing approx. 500 µl glass beads (< 106 µm). Cells were lysed using a TissueLyser II instrument (Qiagen) at maximum speed (30 Hz) for 3 min. Treatment longer than 3 min resulted in decreased PepX activity. The mixture was centrifuged at 20.000 x g for 5 min, and the supernatant was used for determining PepX activity. Protein concentration in the lysate was determined using Coomassie Plus (Bradford) Protein Assay (Thermo Fischer Scientific). PepX activity was determined using Ala-Pro-pNa (Ala-Pro-*para*-nitroanilide, Bachem) as a substrate, which was dissolved in Tris-Buffer (50 mM, pH 7.5) to

a final concentration of 2 mM. In a dark 96-well microtiter plate with a transparent bottom, 75 µl lysate was mixed with 75 µl Tris-Buffer (50 mM, pH 7.5) and 50 µl Ala-Pro-pNa solution. The increase in absorbance at 405 nm was measured in a plate reader at 30°C (Tecan Infinite 200 Pro). PepX activity was determined by linear regression. This experiment was carried out using three biological replicates and two technical replicates for each assay.

4.6.5 RNA-Sequencing Analysis

RNA-sequencing of SD96 and the mutants JA24, RD01, and RD07 has been carried out previously (see **Chapter 3**). Briefly, the four strains were grown RSM, and total RNA was isolated during the exponential phase. RNA was sequenced using an Illumina platform with a PE150 library resulting in paired-end reads with 150 bp. Geneious Prime was used for handling the sequencing data (91). After quality trimming, the reads were mapped to the genome of SD96 (70) using Bowtie2 (92). Raw read counts were calculated in Geneious. Those were used for differential expression analysis using DESeq2 in R (93).

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4.8 References

1. Gänzle MG. Lactic metabolism revisited: Metabolism of lactic acid bacteria in food fermentations and food spoilage. *Curr Opin Food Sci* **2015**, 2:106–17.
2. Wittmann C, Liao JC, M G. Industrial Biotechnology: Microorganisms. Editors: Wittmann C, Liao JC. Wiley-VCH Verlag GmbH & Co. **2017**.
3. United States Department of Agriculture (USDA). Dairy: World Markets and Trade. **2019**.
4. Food and Agriculture Organization on the United Nations (FAO). Dairy Market Review - March 2020. **2020**.
5. Food and Agriculture Organization on the United Nations (FAO). OECD-FAO Agricultural Outlook 2019-2028 - Dairy and dairy products. **2019**.
6. Fox PF, Mcsweeney PLH. Cheese: An Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 5–21.
7. Uniacke-Lowe T, Fox PF. Chymosin, Pepsins and Other Aspartyl Proteinases: Structures, Functions, Catalytic Mechanism and Milk-Clotting Properties. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 69–113.
8. Sandra S, Alexander M, Dalgleish DG. The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *J Colloid Interface Sci* **2007**, 308(2):364–73.
9. Crow VL, Coolbear T, Holland R, Pritchard GG, Martley FG. Starters as finishers: Starter properties relevant to cheese ripening. *Int Dairy J* **1993**, 3:423–60.
10. Chapot-Chartier MP, Deniel C, Rousseau M, Vassal L, Gripon JC. Autolysis of two strains of *Lactococcus lactis* during cheese ripening. *Int Dairy J* **1994**, 4(3):251–69.

11. Parente E, Cogan TM, Powell IB. Starter Cultures: General Aspects. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 201–26.
12. Fagan CC, O'Callaghan DJ, Mateo MJ, Dejmek P. The Syneresis of Rennet-Coagulated Curd. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 145–77.
13. Rehn U, Petersen MA, Saedén KH, Ardö Y. Ripening of extra-hard cheese made with mesophilic DL-starter. *Int Dairy J* **2010**, 20(12):844–51.
14. Chen J, Shen J, Ingvar Hellgren L, Ruhdal Jensen P, Solem C. Adaptation of *Lactococcus lactis* to high growth temperature leads to a dramatic increase in acidification rate. *Sci Rep* **2015**, 5:1–15.
15. Chen J, Shen J, Solem C, Jensen PR. Oxidative Stress at High Temperatures in *Lactococcus lactis* Due to an Insufficient Supply of Riboflavin. *Appl Environ Microbiol* **2013**, 79(19):6140–7.
16. Ardö Y. Characterizing ripening in low-fat, semi-hard round-eyed cheese made with undefined mesophilic DL-starter. *Int Dairy J* **1993**, 3:343–57.
17. Erkus O, de Jager VC, Spus M, van Alen-Boerrigter IJ, van Rijswijck IM, Hazelwood L, Janssen PW, van Hijum SA, Kleerebezem M, Smid EJ. Multifactorial diversity sustains microbial community stability. *ISME J* **2013**, 7(11):2126–36.
18. Frantzen CA, Kleppen HP, Holo H. *Lactococcus lactis* Diversity in Undefined Mixed Dairy Starter Cultures as Revealed by Comparative Genome Analyses and Targeted Amplicon Sequencing of *epsD*. *Appl Environ Microbiol* **2018**, 84(3):1–16.
19. McSweeney PLH. Biochemistry of Cheese Ripening: Introduction and Overview. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 379–87.
20. Blaya J, Barzideh Z, LaPointe G. Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. *J Dairy Sci* **2018**, 101(4):3611–29.
21. Crow VL, Coolbear T, Gopal PK, Martley FG, McKay LL, Riepe H. The role of autolysis of lactic acid bacteria in the ripening of cheese. *Int Dairy J* **1995**, 5(8):855–75.
22. Ruggirello M, Cocolin L, Dolci P. Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models. *Food Microbiol* **2016**, 59:112–8.
23. Ruggirello M, Dolci P, Giordano M, Doyle C, Osullivan O, Cotter P, Cocolin L. Study of *Lactococcus lactis* during advanced ripening stages of model cheeses characterized by GC-MS. *Food Microbiol* **2016**, 74:132–42.
24. van Mastrigt O, Abee T, Lillevang SK, Smid EJ. Quantitative physiology and aroma formation of a dairy *Lactococcus lactis* at near-zero growth rates. *Food Microbiol* **2018**, 73:216–26.
25. Van Kranenburg R, Kleerebezem M, Van Hylckama Vlieg J, Ursing BM, Boekhorst J, Smit BA, Ayad EHE, Smit G, Siezen RJ. Flavour formation from amino acids by lactic acid bacteria: Predictions from genome sequence analysis. *Int Dairy J* **2002**, 12:111–21.
26. Smit G, Smit BA, Engels WJM. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol Rev* **2005**, 29:591–610.
27. Ganesan B, Weimer BC. Amino Acid Catabolism and Its Relationship to Cheese Flavor Outcomes. In: Cheese: Chemistry, Physics and Microbiology: Fourth Edition. Editors: McSweeney PLH, Fox PF, Cotter PD, Everett DW. Elsevier Ltd **2017**, 483–516.
28. Kneifel W, Kaufmann M, Fleischer A, Ulberth F. Screening of Commercially Available Mesophilic Dairy Starter Cultures: Biochemical, Sensory, and Microbiological Properties. *J Dairy Sci* **1992**, 75(11):3158–66.
29. Fox PF. Proteolysis During Cheese Manufacture and Ripening. *J Dairy Sci* **1989**, 72(6):1379–400.
30. Taylor P, Dagal M Al, Fung DYC, Bennett RW. Bitterness in cheese: A review. *Crit Rev Food Sci Nutr* **1996**, 36(5):37–41.

31. Bumberger E, Belitz HD. Bitter taste of enzymic hydrolysates of casein - Isolation, structural and sensorial analysis of peptides from tryptic hydrolysates of β-casein. *Z Lebensm Unters Forsch* **1993**, 197:14–9.
32. Lee KD, Lo CG, Warthesen JJ. Removal of Bitterness from the Bitter Peptides Extracted from Cheddar Cheese with Peptidases from *Lactococcus lacfis* ssp. *cremoris* SK11. *J Dairy Sci* **1996**, 79(9):1521–8.
33. Broadbent JR, Steele JL. Proteolytic enzymes of lactic acid bacteria and their influence on bitterness in bacterial-ripened cheeses. In: Flavor of Dairy Products. Editors: Cadwallader KR, Drake MA, McGorrin RJ. **2007**, 193–203.
34. Collins YF, McSweeney PLH, Wilkinson MG. Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *J Dairy Res* **2003**, 70:105–13.
35. Wilkinson MG, Guinee TP, O'Callaghan DM, Fox PF. Autolysis and proteolysis in different strains of starter bacteria during Cheddar cheese ripening. *J Dairy Res* **1994**, 61(2):249–62.
36. O'Donovan CM, Wilkinson MG, Guinee TP, Fox PF. An investigation of the autolytic properties of three lactococcal strains during cheese ripening. *Int Dairy J* **1996**, 6:1149–65.
37. Hannon JA, Wilkinson MG, Delahunty CM, Wallace JM, Morrissey PA, Beresford TP. Use of autolytic starter systems to accelerate the ripening of Cheddar cheese. *Int Dairy J* **2003**, 13(4):313–23.
38. Fox PF, Wallace JM, Morgan S, Lynch CM, Niland EJ, Tobin J. Acceleration of cheese ripening. *Antonie Van Leeuwenhoek* **1996**, 70:271–97.
39. Upadhyay VK, McSweeney PLH. Acceleration of cheese ripening. In: Dairy Processing: Improving Quality. Editor: Smit G. Woodhead Publishing Limited **2003**, 419–47.
40. Ganesan B, Stuart MR, Weimer BC. Carbohydrate starvation causes a metabolically active but nonculturable state in *Lactococcus lactis*. *Appl Environ Microbiol* **2007**, 73(8):2498–512.
41. Feirtag JM, McKay LL. Thermoinducible Lysis of Temperature-Sensitive *Streptococcus cremoris* Strains. *J Dairy Sci* **1987**, 70(9):1779–84.
42. Søndergaard L, Ryssel M, Svendsen C, Høier E, Andersen U, Hammershøj M, Møller JR, Arneborg N, Jespersen L. Impact of NaCl reduction in Danish semi-hard Samsoe cheeses on proliferation and autolysis of DL-starter cultures. *Int J Food Microbiol* **2015**, 213:59–70.
43. Pillidge CJ, Rallabhandi PSVS, Tong XZ, Gopal PK, Farley PC, Sullivan PA. Autolysis of *Lactococcus lactis*. *Int Dairy J* **2002**, 12(2–3):133–40.
44. Buist G, Karsens H, Nauta A, Van Sinderen D, Venema G, Kok J. Autolysis of *Lactococcus lactis* caused by induced overproduction of its major autolysin, AcmA. *Appl Environ Microbiol* **1997**, 63(7):2722–8.
45. Huard C, Miranda G, Wessner F, Bolotin A, Hansen J, Foster SJ, Chapot-Chartier MP. Characterization of AcmB, an N-acetylglucosaminidase autolysin from *Lactococcus lactis*. *Microbiology* **2003**, 149(3):695–705.
46. Huard C, Miranda G, Redko Y, Wessner F, Foster SJ, Chapot-Chartier MP. Analysis of the peptidoglycan hydrolase complement of *Lactococcus lactis*: Identification of a third N-acetylglucosaminidase, AcmC. *Appl Environ Microbiol* **2004**, 70(6):3493–9.
47. O'Sullivan D, Paul Ross R, Fitzgerald GF, Coffey A. Investigation of the relationship between lysogeny and lysis of *Lactococcus lactis* in cheese using prophage-targeted PCR. *Appl Environ Microbiol* **2000**, 66(5):2192–8.
48. Meijer W, Dobbelaar C, Hugenholtz J. Thermoinducible Lysis in *Lactococcus lactis* subsp. *cremoris* SK110 : Implications for Cheese Ripening. *Int Dairy J* **1998**, 6946(98):1–6.
49. Lepeuple AS, Van Gemert E, Chapot-Chartier MP. Analysis of the bacteriolytic enzymes of the autolytic *Lactococcus lactis* subsp. *cremoris* strain AM2 by renaturing polyacrylamide gel electrophoresis: Identification of a prophage-encoded enzyme. *Appl Environ Microbiol* **1998**, 64(11):4142–8.

50. Lortal S, Chapot-Chartier MP. Role, mechanisms and control of lactic acid bacteria lysis in cheese. *Int Dairy J* **2005**, 15:857–71.
51. Sallami L, Kheadr EE, Fliss, Vuillemard JC. Impact of autolytic, proteolytic, and nisin-producing adjunct cultures on biochemical and textural properties of cheddar cheese. *J Dairy Sci* **2004**, 87(6):1585–94.
52. Garbowska M, Pluta A, Berthold-Pluta A. Impact of Nisin-Producing Strains of *Lactococcus lactis* on the Contents of Bioactive Dipeptides, Free Amino Acids, and Biogenic Amines in Dutch-Type Cheese Models. *Materials* **2020**, 13(8):1835.
53. Crow VL, Martley FG, Coolbear T, Roundhill SJ. The influence of phage-assisted lysis of *Lactococcus lactis* subsp. *lactis* ML8 on cheddar cheese ripening. *Int Dairy J* **1995**, 5(5):451–72.
54. Steen A, van Schalkwijk S, Buist G, Twigt M, Szeliga M, Meijer W, Kuipers OP, Kok J, Hugenholtz J. Lytr, a phage-derived amidase is most effective in induced lysis of *Lactococcus lactis* compared with other lactococcal amidases and glucosaminidases. *Int Dairy J* **2007**, 17(8):926–36.
55. Shearman CA, Jury K, Gasson MJ. Autolytic *Lactococcus lactis* expressing a lactococcal bacteriophage lysin gene. *Nat Biotechnol* **1992**, 10:196–9.
56. Shearman C, Underwood H, Jury K, Gasson M. Cloning and DNA sequence analysis of a *Lactococcus* bacteriophage lysin gene. *MGG Mol Gen Genet* **1989**, 218(2):214–21.
57. Hickey RM, Ross RP, Hill C. Controlled Autolysis and Enzyme Release in a Recombinant Lactococcal Strain Expressing the Metalloendopeptidase Enterolysin A. *Appl Environ Microbiol* **2004**, 70(3):1744–8.
58. Steen A, Palumbo E, Deghorain M, Cocconcelli PS, Delcour J, Kuipers OP, Kok J, Buist G, Hols P. Autolysis of *Lactococcus lactis* is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids. *J Bacteriol* **2005**, 187(1):114–24.
59. Duwat P, Cochu A, Ehrlich SD, Gruss A. Characterization of *Lactococcus Lactis* UV-sensitive mutants obtained by ISS1 transposition. *J Bacteriol* **1997**, 179(14):4473–9.
60. Boutrou R, Sepulchre A, Gripon JC, Monnet V. Simple Tests for Predicting the Lytic Behavior and Proteolytic Activity of Lactococcal Strains in Cheese. *J Dairy Sci* **1998**, 81:2321–8.
61. Lortal S, Lemée R, Valence F. Autolysis of thermophilic lactobacilli and dairy propionibacteria: A review. *Lait* **1997**, 77(1):133–50.
62. Pillidge CJ, Govindasamy-Lucey S, Gopal PK, Crow VL. The major lactococcal cell wall autolysin AcmA does not determine the rate of autolysis of *Lactococcus lactis* subsp. *cremoris* 2250 in Cheddar cheese. *Int Dairy J* **1998**, 8:843–50.
63. Kozáková D, Solichová K, Ondrácková I, Šviráková E, Plocková M. Effect of some environmental factors on autolysis of lactococci used for cheese production. *J Food Nutr Res* **2010**, 49(1):1–9.
64. Hazır Dalca S, Şimşek Ö, Gursoy O, Yilmaz Y. Selection of autolytic lactic acid bacteria as potential adjunct cultures to accelerate ripening of white-brined cheeses. *Mljekarstvo* **2018**, 68(4):320–30.
65. Riepe HR, Pillidge CJ, Gopal PK, Mckay LL. Characterization of the highly autolytic *Lactococcus lactis* subsp. *cremoris* strains CO and 2250. *Appl Environ Microbiol* **1997**, 63(10):3757–63.
66. Smith WM, Pham TH, Lei L, Dou J, Soomro AH, Beatson SA, Dykes GA, Turner MS. Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of llmg_1816 (*gdpP*) induced by high-temperature growth. *Appl Environ Microbiol* **2012**, 78(21):7753–9.
67. Spus M, Liu H, Wels M, Abee T, Smid EJ. Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *Int J Food Microbiol* **2017**, 241:173–80.
68. Johansen E. Challenges when transferring technology from *Lactococcus* laboratory strains to industrial strains. *Genet Mol Res* **2003**, 2(1):112–6.

69. Pedersen MB, Iversen SL, Sørensen KI, Johansen E. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiol Rev* **2005**, 29:611–24.
70. Dorau R, Chen J, Jensen PR, Solem C. Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis SD96. *Microb Resour Announc* **2020**, 9(3):1–2.
71. Cornett JB, Shockman GD. Cellular lysis of *Streptococcus faecalis* induced with Triton X-100. *J Bacteriol* **1978**, 135(1):153–60.
72. López-gonzález MJ, Escobedo S, Rodríguez A, Neves AR, Janzen T, Martínez B. Adaptive Evolution of Industrial *Lactococcus lactis* Under Cell Envelope Stress Provides Phenotypic Diversity. *Front Microbiol* **2018**, 9:1–17.
73. Meyrand M, Boughammoura A, Courtin P, Mézange C, Guillot A, Chapot-Chartier MP. Peptidoglycan N-acetylglucosamine deacetylation decreases autolysis in *Lactococcus lactis*. *Microbiology* **2007**, 153(10):3275–85.
74. Solopova A, Formosa-Dague C, Courtin P, Furlan S, Veiga P, Péchoux C, Armalyte J, Sadauskas M, Kok J, Hols P, Dufrêne YF, Kuipers OP, Chapot-Chartier MP, Kulakauskas S. Regulation of cell wall plasticity by nucleotide metabolism in *Lactococcus lactis*. *J Biol Chem* **2016**, 291(21):11323–36.
75. Tsuchido T, Svarachorn A, Soga H, Takano M. Lysis and aberrant morphology of *Bacillus subtilis* cells caused by surfactants and their relation to autolysin activity. *Antimicrob Agents Chemother* **1990**, 34(5):781–5.
76. Rolain T, Bernard E, Courtin P, Bron PA, Kleerebezem M, Chapot-Chartier MP, Hols P. Identification of key peptidoglycan hydrolases for morphogenesis, autolysis, and peptidoglycan composition of *Lactobacillus plantarum* WCFS1. *Microb Cell Fact* **2012**, 11:1–15.
77. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: A Fast Phage Search Tool. *Nucleic Acids Res* **2011**, 39:347–52.
78. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* **2016**, 44:16–21.
79. Moustafa HH, Collins EB. Role of galactose or glucose-1-phosphate in preventing the lysis of *Streptococcus diacetilactis*. *J Bacteriol* **1968**, 95(2):592–602.
80. Dako E, El Soda M, Vuillemard JC, Simard RE. Autolytic properties and aminopeptidase activities of lactic acid bacteria. *Food Res Int* **1995**, 28(5):503–9.
81. Picon A, García-Casado MA, Nuñez M. Proteolytic activities, peptide utilization and oligopeptide transport systems of wild *Lactococcus lactis* strains. *Int Dairy J* **2010**, 20:156–62.
82. Tomoko S, Taichi N, Atsushi I, Sachiko O, Ryozo A. Degradation of a Bitter Peptide Derived from Casein by Lactic Acid Bacterial Peptidase. *Food Sci Technol Res* **2009**, 15(2):191–4.
83. Raynaud S, Cocaign-bousquet M, Loubiere P, Biotechnologie-bioproce L. Metabolic and Transcriptomic Adaptation of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis in Response to Autoacidification and Temperature Downshift in Skim Milk. *Appl Environ Microbiol* **2005**, 71(12):8016–23.
84. Zhu Z, Yang J, Yang P, Wu Z, Zhang J, Du G. Enhanced acid-stress tolerance in *Lactococcus lactis* NZ9000 by overexpression of ABC transporters. *Microb Cell Fact* **2019**, 18(136):1–14.
85. Kilstrup M, Hammer K, Jensen PR, Martinussen J. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol Rev* **2005**, 29:555–90.
86. Nilsson D, Lauridsen AA, Tomoyasu T, Ogura T. A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli ftsH* gene product. *Microbiology* **1994**, 140(10):2601–10.
87. Bove P, Capozzi V, Garofalo C, Rieu A, Spano G, Fiocco D. Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: Effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiol Res* **2012**, 167(4):187–93.

88. Roces C, Campelo AB, Escobedo S, Wegmann U, García P, Rodríguez A, Martínez B. Reduced binding of the endolysin LysTP712 to *Lactococcus lactis* Δ*ftsH* contributes to phage resistance. *Front Microbiol* **2016**, 7:1–10.
89. Huang C, Hernandez-Valdes JA, Kuipers OP, Kok J. Lysis of a *Lactococcus lactis* Dipeptidase Mutant and Rescue by Mutation in the Pleiotropic Regulator CodY. *Appl Environ Microbiol* **2020**, 86(8):1–15.
90. Wu H, Xue E, Zhi N, Song Q, Tian K, Caiyin Q, Yuan L, Qiao J. D-Methionine and D-Phenylalanine Improve *Lactococcus lactis* F44 Acid Resistance and Nisin Yield by Governing Cell Wall Remodeling. *Appl Environ Microbiol* **2020**, 86(9):1–12.
91. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, 28(12):1647–9.
92. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **2012**, 9(4):357–9.
93. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **2014**, 15(12):1–21.

4.9 Supplementary Information

Table S 4.1 Mutations of the mutant strain JA24, RD01, and RD07. Mutations unique to RD07 are marked with an asterisk.

Mutation ID	Position	Change	CDS	Codon Change	Protein Effect
JA24-1	1531408	G → T	LCP family protein	ACC → ACA	None
JA24-2	1814364	G → T	Pleiotropic transcriptional regulator CodY	CGT → AGT	R237S
RD01-1	533440	C → T	EamA family transporter	CCC → CCT	-
RD01-2	1815136	(A)6 → (A)5	Upstream of *codY*		-
RD01-3	1943775	C → G	Bifunctional (p)ppGpp synthetase	GTT → CTT	V496L
RD01-4	2310414	T → G	UDP-N-acetylmuramate-L-alanine ligase	TTT → TTG	F54L
RD01-Gap	1823878 - 1897135	-	See **Chapter 3, Table S 3.2**	-	-
RD07-1*	4892	C → A	Phenylalanine-tRNA ligase subunit beta	CGT → AGT	R546S
RD07-2*	88575	T → A	Hypothetical protein	GTA → GAA	V957E
RD07-3	533440	C → T	EamA family transporter	CCC → CCT	-
RD07-4*	2013099	G → T	DNA primase	GCT → TCT	A410S
RD07-5*	2025377	C → G	Upstream of *hflB*/*ftsH* encoding FtsH		-
RD07-6*	2043810	C → A	RexA, ATP-dependent exonuclease subunit A	GAC → TAC	D311Y
RD07-7*	2065547	G → A	Pur operon repressor	GGC → AGC	G206S
RD07-8	2310414	T → G	UDP-N-acetylmuramate-L-alanine ligase	TTT → TTG	F54L
RD07-Gap	1823878 - 1897135	-	See **Chapter 3, Table S 3.2**	-	-

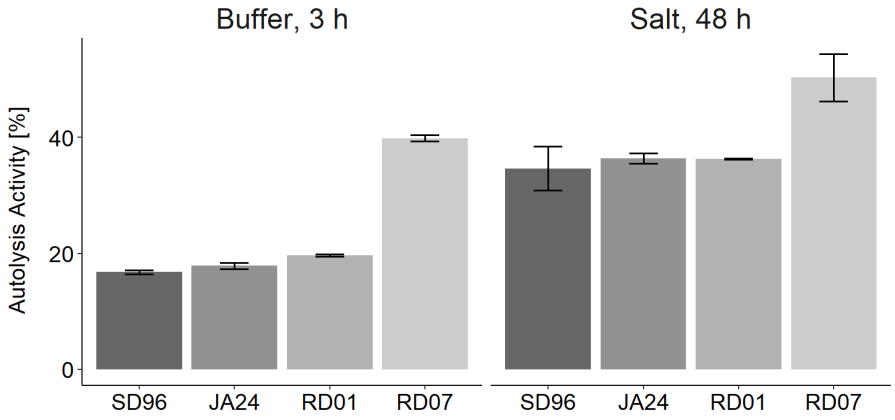

Figure S 4.1 Autolytic activity of SD96 and mutants JA24, RD01, and RD07 under two conditions. Left: Autolysis after 3 h induced by nutrient depletion in 50 mM sodium phosphate buffer at pH 7.0 using cells harvested during the exponential phase in M17. Right: Autolysis after 48 h induced by salt stress (0.5 M NaCl) in lactate buffer at pH 5.0 using cells harvested during milk fermentation at pH 5.2.

Table S 4.2 Prophages encoded on the SD96 chromosome as identified using PHASTER (78).

Prophage Number	Region Length [Kb]	Completeness	Total Proteins	Region Position	Most Similar Phage (Accession no.)
1	47.1	Intact	55	53121-100233	*Lactococcus* bIL286 (NC_002667)
2	21.8	Intact	36	106834-128670	*Lactococcus* bIL312 (NC_002671)
3	43.1	Intact	65	1487673-1530792	*Lactococcus* bIL309 (NC_002668)
4	27.8	Incomplete	24	1837787-1865594	*Lactococcus* bIL312 (NC_002671)
5	27.5	Incomplete	15	1879571-1907158	*Staphylococcus* SPbeta like (NC_029119)
6	18.9	Intact	32	1997320-2016230	*Lactococcus* bIL310 (NC_002669)
7	15.5	Intact	23	2400586-2416120	*Lactococcus* bIL311 (NC_002670)

5. Summary and Conclusion of Part I

Summary

The concepts of using lactic acid bacteria (LAB) for cheese production, as well as related current limitations and challenges are introduced in **Chapter 1**. Furthermore, stress response mechanisms and their relevance during cheese production were summarized. The next three chapters deal with the characterization and optimization of the industrial *Lactococcus lactis* (*L. lactis*) subsp. *lactis* biovar diacetylactis SD96 (SD96). **Chapter 2** describes the de novo genome sequencing of SD96, which forms the basis for the systems biology analysis applied in subsequent chapters. In **Chapter 3**, the process and outcome of a long-term adaptive laboratory evolution (ALE) for increasing the thermotolerance of SD96 are reported. SD96 and three thermotolerant mutant strains are described in detail using next-generation-sequencing-based genomics and transcriptomics analysis. Lastly, **Chapter 4** shows how the autolytic capacity of the thermotolerant mutants was studied, and thereby it was revealed that the most thermotolerant strain, RD07, was also most autolytic.

Conclusion

The complete genome sequence of SD96 adds value to the scientific community as it allows genome-wide comparisons with other *L. lactis* strains. Moreover, SD96 is a unique addition to the publicly available genome-sequenced strains, because typically information about industrial strains are rarely published. Furthermore, the phage-insensitive phenotype is unique and might reveal new mechanisms of how *L. lactis* handles phage attacks.

Long-term ALE at high temperature in milk resulted in three thermotolerant mutants of SD96, which showed improved acidification of milk at both low and high temperatures. The most thermotolerant strain, RD07, acidifies milk at 41°C, which is unique for dairy lactococci. Therefore, this strain shows excellent potential as a starter strain for producing new hard cheese varieties. Furthermore, RD07 shows greatly improved autolytic properties, which is an additional benefit of this strain, as this potentially allows shorter ripening times with superior flavor formation. RD07 will be tested for hard cheese production and accelerated cheese ripening.

The system-wide analysis of SD96 and the three mutants using genome and transcriptome sequencing led to new insights of how *L. lactis* adapts to high growth temperatures in milk. Interestingly, we observed that SD96 suffered from amino acid starvation when grown in ultra-high-temperature processed (UHT) milk, which was overcome during ALE by mutations affecting amino acid metabolism. Thermotolerance in RD07 seemed to be based on many synergistically acting mutations, of which mutations affecting purine metabolism seem to play a critical role. Furthermore, the RNA-sequencing dataset is an important contribution to the scientific community as it will be freely available upon publication of **Manuscript 2**.

Part II – Metabolic Engineering of *Lactococcus lactis* for Flavor Compound Production

6. Metabolic Engineering of Lactic Acid Bacteria

*This chapter was accepted for publication in "Metabolic Engineering" as part of the Advanced Biotechnology Series (**Manuscript 5**).*

Dorau R, Liu J, Solem C, Jensen PR. Metabolic Engineering of Lactic Acid Bacteria. In: Metabolic Engineering (Advanced Biotechnology Series). Editors: Lee SY, Nielsen J, Stephanopoulos G. Wiley-VCH Verlag GmbH & Co

6.1 Abstract

Lactic acid bacteria (LAB) comprise a group of microorganisms with high industrial relevance. Many of them have a long history of safe use within the food industry, while others play essential roles in human and animal health. For these reasons, LAB are of great and increasing interest to researchers worldwide. In particular, their safe status is of crucial importance when it comes to sensitive applications. Recently developed tools for engineering LAB, have opened up new possibilities to harness the biochemical machinery of LAB, for example, for producing bio-based chemicals, food ingredients, or compounds of therapeutic value. Here, current and potential applications of LAB are reviewed with a special focus on metabolically engineered strains.

6.2 Concise Definition of the Subject

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, microaerophilic bacteria, which generally have rather small (< 5 Mbp), low GC genomes and a relatively simple metabolism, mainly resulting in the formation of lactic acid from various sugars. Some members of the LAB are extensively used in food fermentation for producing, for example, cheese or sourdough, while others have become popular because of the beneficial effects they have on health when applied as probiotics. During the last decades, LAB and especially *Lactococcus lactis* (*L. lactis*) have been studied in great detail, and many interesting applications have been derived. Nowadays, LAB are gaining increasing attention as they were shown to efficiently produce various industrially important chemicals such as functional peptides, flavor compounds, and vitamins. Metabolic engineering of LAB has been driven by the immense importance of these bacteria, and since the 1970s, genetic engineering of LAB has been possible, and today modern genome editing techniques allow tailoring and build-up of LAB for new challenges.

*(Parts were moved to **Chapter 1** and constitute **Sections 1.2** and parts of **Sections 1.4.1**)*

6.3 Overview of this Chapter

In this chapter, we will systematically review industrial applications of LAB, focusing on metabolically engineered and optimized strains and provide an elaborate overview of the field. As many of the approached topics have undergone immense investigation previously, more detailed and comprehensive reviews will be mentioned and cited when appropriate. We will introduce LAB as a versatile group of bacteria and describe their main applications and most important species. As LAB are Gram-positive, non-sporulating and low-GC microorganisms, they differ significantly from the common model bacteria *Escherichia coli* (*E. coli*) or *Bacillus subtilis,* and consequently, different genetic tools are required to engineer these organisms. We will provide an overview of the most important tools and review state-of-the-art technologies applicable to LAB. Genetically modified organisms (GMOs) are generally not accepted in food production or related processes, and therefore we will also discuss strain optimization using traditional strategies such as random mutagenesis or adaptive laboratory evolution when appropriate. Despite the fact that LAB meet many of the requirements needed for industrial applications, improvements in performance are continuously sought after. Thus, much effort has been devoted to optimizing LAB for industrial applications, for example, increasing strain robustness to various environmental stresses like temperature, pH, or oxidative stress. For each type of application, different challenges for the bacteria arise, and therefore it is not a single but several phenotypes that researchers and industries hunt after. In this context, non-targeted optimization strategies have proven extremely successful. Alternatively, this area takes advantage of the immense diversity among LAB, and candidates obtained by screening culture collections often are well-suited starting points for a particular application. Lastly, rational rerouting of the native metabolism or introduction of heterologous pathways into LAB has led to new strains, which are efficient producers of chemicals, in particular chemicals with food or other sensitive applications. As LAB still are more challenging to engineer than other industrial microorganisms, there are fewer cases where metabolically engineered LAB have been implemented in the industry. Nonetheless, with the accelerating development of molecular tools for genetic engineering of LAB, more cases are expected in the coming years.

6.4 Genetic Engineering Strategies for LAB

6.4.1 Transformation of LAB and Shuttle Vectors

Since the early 1980s, it has been possible to genetically modify LAB. This development was mainly driven by the urge to improve starter culture performance for food production, especially in the dairy industry (1). A key accelerator of the development in this research field was the discovery that many industrially important traits, such as lactose or citrate metabolism, are encoded by genes located on plasmids (2,3). Shortly after that, evidence for conjugal transfer of

these traits in *L. lactis* was published (4), and the first transformation of in-vitro modified plasmid DNA into *L. lactis* was made possible using protoplast fusion (5). The successful and efficient transformation was then also reported for *Lactobacillus casei* (*Lb. casei*) and several other LAB (6,7). Protocols for efficient transformation by electroporation were first established for *L. lactis* and later expanded into a wide variety of LAB (6,8). Often when developing transformation protocols and tools needed for genetic manipulation, the focus is on LAB strains for which the tools work particularly well, for example, the laboratory strains MG1363 or IL1403 (*L. lactis*). Industrial strains often have different properties and can be more resistant to genetic manipulation (9,10). Despite this, protocols are continuously being improved, and new approaches to overcome these limitations have been developed, for example, selecting mutants that are more amenable to genetic manipulation (11,12).

As cloning and DNA manipulation are most conveniently performed in *E. coli*, there has been great interest in developing shuttle vectors that are able to replicate in both *E. coli* and LAB. Many early research efforts revolved around finding suitable vectors that could be used in a broad range of organisms (13). Many cloning vectors have been derived from cryptic plasmids (14), for example, the pWV01 plasmid from *L. lactis* subsp. *cremoris* Wg2 (15), the pSH71 from *L. lactis* subsp. *cremoris* NCDO712 (16) or pAMß1 from *Enterococcus faecalis* (*E. faecalis*) ATCC14508 (17–19). Furthermore, replicating plasmids used for genetic engineering are usually based on broad-host-range vectors. For example, the pG$^+$host plasmids are based on a mutated version of plasmid pVE6002, which in turn is based on the pWV01 plasmid mentioned above (20). Still, new shuttle vectors are being developed, especially for species and strains that have not received as much attention as the *L. lactis* model strains (21–23). It has also become more popular to avoid intermediate cloning hosts and pursue direct cloning in LAB. This can sometimes be advantageous, as in some industrial strains restriction-modification system could degrade DNA from other organisms (18,24). Furthermore, altered copy numbers or promoter strengths in *E. coli* compared to LAB can create challenges during the cloning process, particularly if the expressed protein is toxic in high concentrations. Moreover, leaving out an *E. coli* replicon reduces the plasmid size, which in turn helps increase transformation efficiency. Some recent protocols avoid the use of intermediate cloning steps in *E. coli* and use the easily transformable model *L. lactis* MG1363 instead (25,26).

6.4.2 Gene Expression in LAB

Nisin Controlled Gene Expression (NICE)

Controlled gene expression is one of the essential molecular biology technologies in modern biotechnology and key to many exciting applications or discoveries. The nisin controlled gene expression system has been developed for controlled gene expression in *L. lactis* (27). It has been a very successful tool for controlling gene expression in LAB due to its robustness and broad host range among Gram-positive bacteria. The NICE system is based on the quorum-sensing

and self-regulating mechanism controlling nisin production in certain *L. lactis* strains. Nisin is a small peptide with anti-microbial activity, a so-called lantibiotic. *L. lactis* strains naturally producing nisin possess the *nisABTCIPR* cluster, encoding nisin biosynthetic enzymes as well as resistance machinery to nisin. The resistance genes are only induced in the presence of nisin (28). The following components are essential for the NICE system: *nisK* and *nisR*, which act as a membrane-bound extracellular sensor for nisin and as an intracellular response regulator, respectively (**Figure 6.1, A**) (27). The NisR protein will activate the *nisA* or the *nisF* promoter and initiate the expression of the downstream-encoded genes. The two regulatory genetic elements *nisK* and *nisR* can either be placed on the genome, as in the commonly used NZ9000 strain or be expressed from a plasmid (27). The latter makes the system transferable to other LAB, including *Leuconostoc* and *Lactobacillus* spp. (29). The NICE system and respective applications have been described and reviewed previously (30,31).

Synthetic Promoter Libraries (SPL)

When studying the influence of individual genes on the metabolic flux through a metabolic pathway, several options exist: a gene can be knocked out, overexpressed, or its expression can be modulated. The latter can be achieved using synthetic promoter libraries (SPL), which is an alternative to the regulated expression systems like the NICE system. SPL have been applied for metabolic control analysis or metabolic engineering. The SPL can be introduced on the genome upstream of a particular gene in the particular organism studied, and respective methods for this are described in the following sections. LAB, in particular *L. lactis*, were among the first organisms in which the SPL technology was introduced and applied (32,33). The SPL technology is based on the randomization of spacer sequences surrounding the -35 and -10 regions of bacterial promoters (**Figure 6.1, B**). Expression levels spanning up to three orders of magnitude can easily be achieved with a high frequency of active promoters (above 50%). Sequence randomization can be introduced either by error-prone PCR or by using randomized oligonucleotides (34). A simplified method using SPL (35) gave rise to many studies focusing on determining the control that glycolytic enzymes have on the glycolytic flux (36–40), which has been reviewed recently (41). SPL have also been developed for *Lb. plantarum* and have been applied for studying the effect of co-factor disturbances on the glycolytic flux (42,43). More recently, acetoin production in a metabolically engineered *L. lactis* strain was optimized by increasing ATP demand by expressing F_1-ATPase to different levels using synthetic promoters. With increasing amounts of F_1-ATPase in the cells, biomass yield was reduced, but the acetoin yield was increased (44).

Other Systems for Controlling Gene Expression

Several other methods for controlling gene expression have been developed for LAB, and these basically rely on using native promotor systems. Examples include expression systems inducible by low pH (45), heavy metals such as zinc (46), agmatine (47), sakacin A/P (a bacteriocin similar

to nisin) (48) or general stress such as heat, acid or salt stress (49). For reviews on this topic, the readers are referred to (50–53).

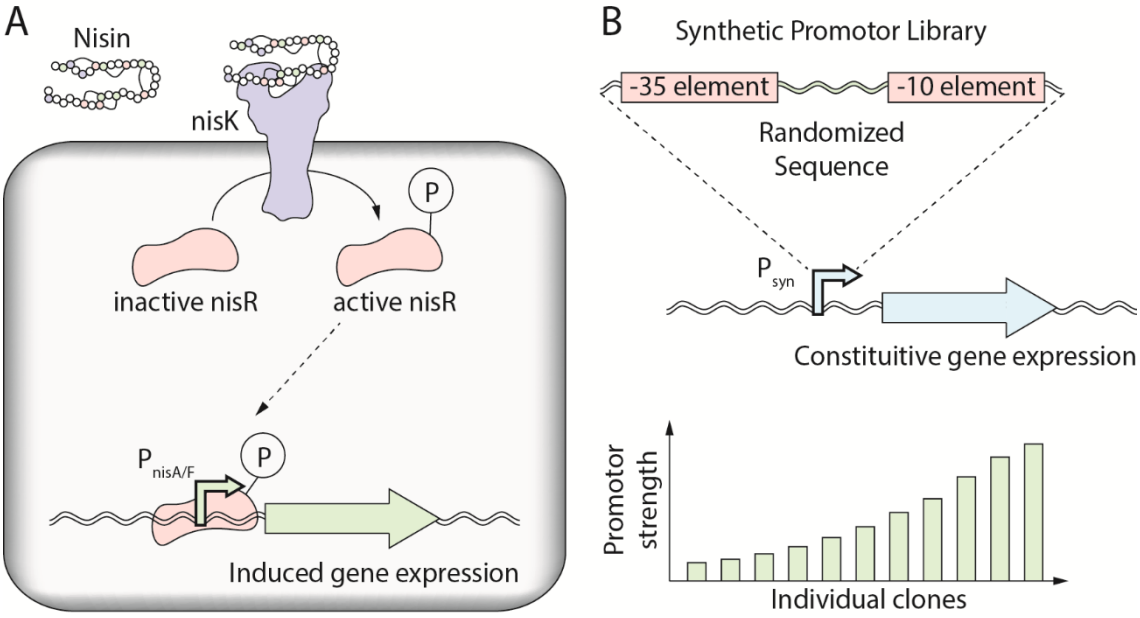

Figure 6.1. Gene expression systems in lactic acid bacteria (LAB). A: Nisin controlled gene expression (NICE) system. The expression of nisK and nisR are required for functionality. When functionally expressed, extracellularly applied nisin binds to the NisK, which in turn phosphorylates the NisR. The phosphorylated and thus activated NisR binds to the nisA or nisF promotor ($P_{nisA/F}$) and activates the transcription of the downstream gene. B: Synthetic promotor libraries (SPL) are a versatile tool for modulating gene expression. In L. lactis, the sequence between the -35- and the -10 element determines the promotor strength and, when randomized, gives rise to a promotor library spanning many different strengths. In comparison to the NICE system, SPL usually results in constitutive gene expression.

6.4.3 Genetic Engineering of LAB

Plasmid Integration *via* Homologous Recombination

A large number of genetic tools for introducing targeted changes in LAB genomes have been published and shown to be useful. Most traditional techniques are based on the same principle (**Figure 6.2**): a non- or conditionally replicating plasmid, usually carrying an antibiotic resistance marker and one or more regions homologous to the chromosomal target, is transformed into the organism. When such plasmids are unable to replicate, and selection pressure is imposed, only strains which have integrated the plasmid by homologous recombination into their genome can survive. Non-replicating plasmids have the advantage that false-positive clones are avoided since conditionally replicating plasmids can mutate spontaneously. A potential drawback when using non-replicating plasmids is the requirement of the host to be easily transformable. After plasmid integration, which takes place through a single recombination (crossover) event, a second crossover can facilitate plasmid excision with the potential outcome that the desired mutation is

introduced in the genome. The plasmids used for this are typically shuttle vectors, which replicate in several organisms, including cloning facilitators such as *E. coli*. In order to find broad use, these tools need to be robust, flexible, and easily implementable. Prominent examples that meet such criteria are pG$^+$host plasmids (54), the pOri-system (55), and the pCS1966 plasmid (56). Combinations and adaptations of the underlying concepts for various strains or species exist, and recent reviews have summed up the most relevant tools (50,57,58). The pG$^+$host system has been widely applied for genetic engineering of LAB, as it is compatible with many LAB genera and mostly independent from the host physiology. pG$^+$host is a conditionally replicating plasmid bearing a temperature-sensitive replicon, which allows the plasmid to replicate at temperatures below 30°C but not above 35°C. Consequently, the targeted host only needs to meet one criterion: it needs to be able to sustain sufficient growth at both temperatures for allowing and inhibiting plasmid replication (30-35°C). The pOri plasmid is a non-replicating plasmid, but replication can be initiated when *repA* is supplemented in *trans*, which usually is provided in a helper strain used for cloning and maintenance purposes. This system has been further developed to overcome the necessity for a high transformation efficiency, which is needed as the plasmid is unable to replicate without supplementation of *repA* in *trans*. The introduction of a temperature-sensitive expression of *repA* on the pOri plasmid helped overcome this limitation resulting in plasmid pTRK and rendered the system similar to pG$^+$host. pTRK has further been optimized by incorporation of a counterselection marker based on the *upp* gene encoding uracil phosphoribosyltransferase (UPRTase) mediating toxicity of 5-fluorouracil and has been widely used in *Lactobacillus* ssp. (59). For *L. lactis*, pCS1966 has proven to be one of the most efficient tools for manipulating the genome. The plasmid is non-replicating and therefore requires a reasonably high transformation efficiency, which usually can be achieved with relevant *L. lactis* strains. Besides the erythromycin resistance, a counter-selection marker based on the toxicity of 5-fluoroorotate mediated by the orotate transporter (*oroP*) is encoded. In pyrimidine auxotrophic strains, *oroP* can further be used as a second selection marker, when orotate is applied. One requirement is that the strain of interest lacks an orotate transporter, which is a common feature in *Lactococci*. This plasmid has been successfully applied to construct many different mutant strains of *L. lactis* (60). The search for widely applicable and flexible genetic engineering methods that can be used to introduce targeted mutations in LAB genomes is still ongoing, as indicated by recent publications covering this area (61–63). One major limitation is still that many LAB species can only be transformed with low transformation efficiencies, and editing outcomes are difficult to predict for non-model strains (11,57,64). Therefore, the development of robust molecular tools allowing for genome editing of difficult to transform hosts is needed, and so far, no universally applicable and efficient methods have been established.

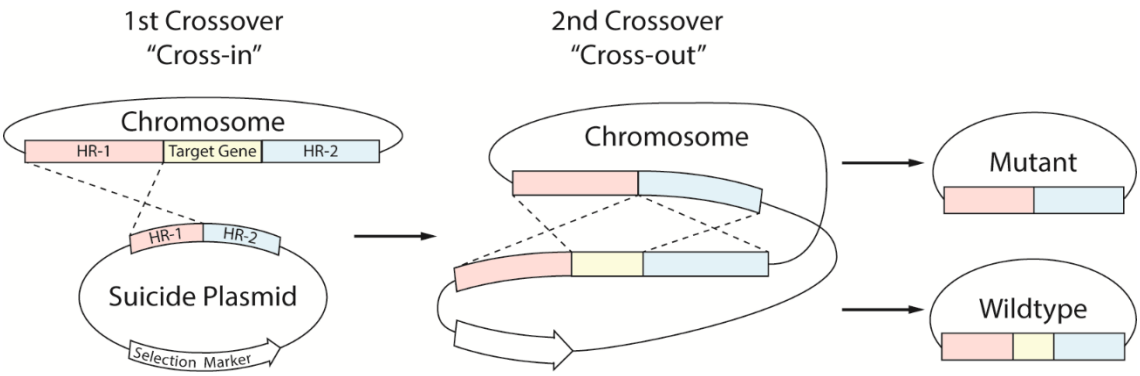

Figure 6.2. The principle behind using suicide or conditionally replicating plasmids such as pG$^+$host or pCS1966 for genetic engineering of lactic acid bacteria (LAB). Plasmids usually have one selection marker and a means for counter selection. Two homologous arms (HR-1 and HR-2, red and blue respectively) are inserted into the plasmids. In the case of pG$^+$host, the selection marker is an antibiotics resistance marker, whereas "counter selection" is based on the negative effect on growth that the plasmid replication system has when integrated into the chromosome at the permissive temperature. In the case of pCS1966 suicide plasmid, the selection marker is an erythromycin resistance gene, and the counter selection marker is the orotate transporter, which renders its host sensitive to 5'-fluoroorotate. The counter selection enables selection for the 2nd crossover event. Based on homologous recombination, the plasmid integrates into the genome at the desired locus and, subsequently, is removed from the genome via a second recombination event. This can occur in two ways, one yielding the desired mutation or removal of the target gene (yellow) (upper row), and the second is restoring the wild-type genotype (lower row).

Integration using Phage Attachment Sites

Besides integration via homologous recombination, plasmids can also be targeted towards specific loci, including chromosomal phage attachment sites. One example of site-specific integration in LAB was identified when studying *L. lactis* and its bacteriophage TP901-1 (65). The authors identified a minimal sequence containing 56 bp, which is sufficient to facilitate stable and high-frequency integration of a non-replicating plasmid into the chromosome. A requirement for the integration event to take place is the presence of an *attB*-site on the chromosome (43 bp), the minimal *attP*-site on the integrating plasmid (56 bp), and the presence of TP901-1 recombinase, which can be expressed from a helper plasmid. Although very useful, this tool always results in complete plasmid integration and does not easily allow for plasmid excision or multiple integrations. This technology was further developed into a repetitive, marker-free, and site-specific integration tool for *L. lactis*. The above-described system was combined with the Cre/*loxP* recombination system that had been shown to be suitable for multiple gene deletions or replacements in LAB earlier (66). This system is made up of several interacting parts comprising the two recombination systems, specifically the plasmid pKV6 containing the *attP*, *attB*, *lox71*, *lox66*, the *oroP* counter-selection marker and an antibiotics resistance marker, plasmid pLB95 encoding TP901-1 integrase and pKV7 encoding Cre recombinase. The pKV6 plasmid is constructed so that the two *lox*-sites are flanking all genetic elements, except for the multiple

cloning site (MCS), a transcriptional terminator, and the *attB* and *attP* sites. As a result, after integration into the genome and recombination using the Cre recombinase, only the MCS (or a genetic element of interest), including a transcriptional terminator and the plasmid derived *attB* site remain in the genome. The newly introduced *attB* site allows for iterative integration using variations of pKV6 (67). The TP901-1 integration system has been expanded by a set of vectors, readily allowing stable secretion or surface display of heterologous proteins (68). In principle, this system could also be extended to other LAB such as lactobacilli, as it has been shown that a similar integration system derived from *Lb. casei* ATCC 393 phage ϕAT3 can be applied for integrating non-replicating plasmids into an *attB* site on the chromosome (69). A limitation of this approach is the requirement for compatible *attB* sites in the chromosome of the target strain, and this can be a limiting factor. Nevertheless, one or several *attB*-sites can potentially be inserted into the chromosome prior to the plasmid integration events. Following a similar idea, an integration tool for *Lb. casei* has been described, which overcomes this limitation. Instead of relying on preexisting *attB*-sites in the chromosome, the authors firstly place a *loxP* site selectively on the chromosome by using recombineering and, secondly, exploit the Cre/*loxP* system to integrate a genetic construct into that site. This system allows for multiple and iterative integrations as several *loxP*-sites can be integrated into the chromosome, and after each recombination event, a new *loxP*-site is created by Cre recombinase (70).

Recombineering

Nowadays, especially in the popular bacterial model systems such as *E. coli*, λ-red mediated recombineering, followed by CRISPR-Cas9 counter selection, is the first choice for genetic engineering due to its high efficiency (**Figure 6.3**). Establishing a similar system in LAB has been more cumbersome, and only a few successful examples have been described (71). In contrast to integrative plasmids, where longer stretches of homologous DNA are required for successful recombination, recombination-mediated genetic engineering, or recombineering, relies on linear short single-stranded (SSDR) or double-stranded (DSDR) pieces of synthetic DNA, that are incorporated into the genome during DNA-replication. Recombineering has been demonstrated to work in *Lb. reuteri* and *L. lactis* (72). The λ-red system for *E. coli* is composed of three genes: *gam*, *exo,* and *bet*, which make the host strain recombinogenic when functionally expressed and thus allow the incorporation of linear double-stranded DNA into the chromosome. 30-50 bp are sufficient for successful recombination, which facilitates cloning and gene deletions (73). Furthermore, it has been demonstrated that for recombining single-stranded DNA into the chromosome, only the *bet* gene or Beta protein is required (74). As LAB are not readily recombinogenic, for establishing SSDR, the Beta protein or a functional homolog needs to be transiently expressed. Recently it was shown that the functional Beta protein homolog RecT from *Lb. reuteri* ATCC PTA 6475 was able to facilitate the process when expressed in *Lb. reuteri* or *L. lactis* (72). In a follow-up study, the authors evaluated several parameters for improving the

SSDR efficiency. The expression of various recombinases from different LAB in *Lb. reuteri* showed that the native RecT already is very efficient, and only RecT from *E. faecalis* CRMEN 19 was superior. For *L. lactis*, by increasing the oligonucleotide concentration up to 500 µg per transformation, and by introducing phosphorothioate-linkages to stabilize the oligonucleotides, it was possible to increase the SSDR efficiency around 10-fold. These changes, however, were not shown to be beneficial for *Lb. reuteri*. Interestingly, it was shown that in *Lb. plantarum* WCFS1, the introduction of phosphorothioate-linkages improved DSDR efficiency (75). During the last years, SSDR has been successfully applied by other research groups for introducing targeted mutations in the genomes of *L. lactis* or *Lb. reuteri* (76–80). Combining SSDR with CRISPR-Cas9 based counterselection has proven to be extremely efficient for engineering bacteria based on the lethality of double-strand breaks (81). In LAB, this has initially been shown to be feasible for lactobacilli (82). Recently, SSDR has been further optimized and combined with CRISPR-Cas9 counter selection for *L. lactis* by testing several RecT homologs for their compatibility with *L. lactis* and optimizing conditions for CRISPR-Cas9 based selection for the desired mutants (63). SSDR in *Lb. plantarum* WCFS1 was improved by overexpressing DNA adenine methylase (Dam), and more specifically, the endogenous adenine-specific methyltransferase subunit gene (lp_2243). Dam overexpression was hypothesized to disable the methyl-directed mismatch repair (MMR) systems, as Dam binds the mismatched bases faster than MMR (75).

DSDR in LAB has not received as much attention as SSDR, although the λ-red-system for *E. coli* has proven to be extremely useful for changing larger genomic regions introducing gene deletions or insertions, which is difficult using SSDR. When using the λ-red-system, homologous regions of approximately 50 bp are required for achieving recombination, when in contrast to that in *L. lactis* approx. 800-1000 bp are needed in order to achieve efficient recombination (20). In *Lb. plantarum* DSDR has been established using recombinases (Lp_0640-41-42), derived from prophage P1 in *Lb. plantarum* WCFS1, reconstituting the three λ-red genes *gam*, *exo,* and *bet*. Although recombination efficiencies could be increased, still long homologous regions are required for recombination, which limits the benefits of this system. Bioinformatics studies suggest that similar recombination systems exist in various LAB, which might be useful for establishing DSDR in other LAB (83). Recombinase-mediated DSDR has also been shown to enhance genome engineering in *Lb. casei* by expressing the heterologous prophage derived LCABL_13040-50-60 operon, that conveys the same functions as *gam*, *exo,* and *bet* (84). In combination with the Cre recombinase, a single plasmid system for iterative gene deletions was created for *Lb. casei*, which allowed the introduction of gene deletions within seven days and with efficiencies between 40 and 60% (85).

Besides the great advantages, establishing a recombineering based mutagenesis workflow for a new strain can still be challenging. Especially the high transformation efficiencies needed can be a limiting factor, and differing methyl-directed mismatch repair systems further make standardization of protocols complicated. This is particularly problematic when using industrially

or medically relevant strains, which have not undergone adaptation to the laboratory environment (71). Using the well-characterized plasmid-based systems such as pG$^+$host or pCS1966 might still be the easier choice for genetic engineering of less well-characterized LAB.

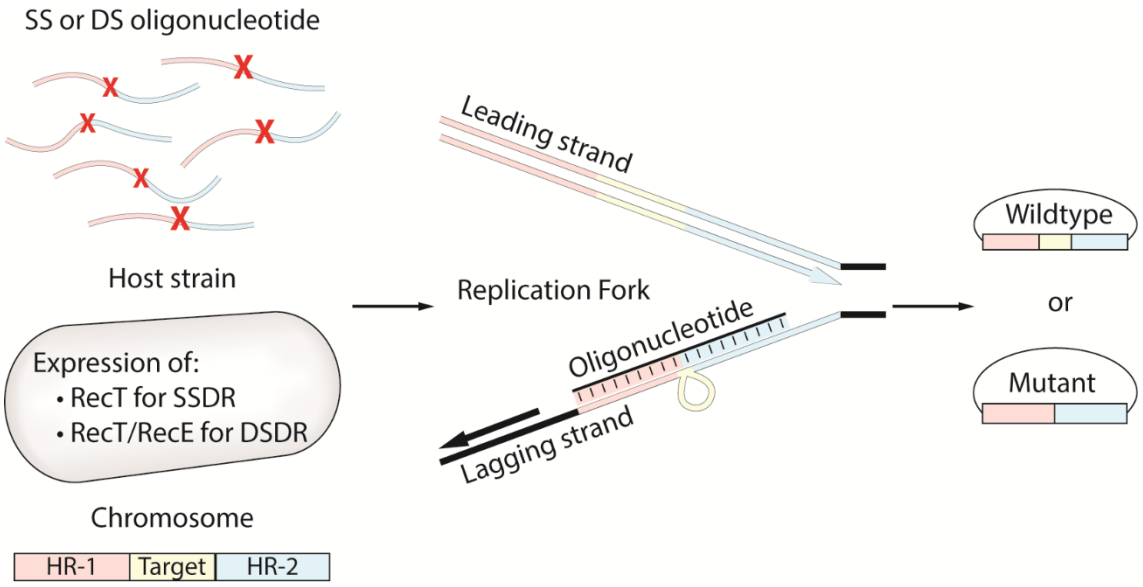

Figure 6.3. The principle behind recombineering based genetic engineering in lactic acid bacteria (LAB). In order to facilitate the recombineering, the host strain needs to express RecT, which stabilizes single-stranded oligonucleotides (SS oligos), and additionally, RecE, if double-stranded oligonucleotides (DS oligos) are used. RecE is an exonuclease, which removes the 5' end of the DS oligos, which improves recombineering efficiency. Oligonucleotides need to be homologous to the locus of interest with homologous regions (HR-1 and HR-2, red and blue, respectively). Two scenarios are shown: deletion of a target gene (yellow) (upper row) and the introduction of a point mutation (lower row). The mutant oligos are incorporated during DNA-replication, serving as Okazaki fragments. In both scenarios, a mixed mutant population is obtained, and the desired mutant can be found using PCR-screening, Cas9-counterselection, or other suitable methods.

CRISPR-Cas-Technology for Genome Editing in LAB

The discovery of clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) genes in LAB *Streptococcus thermophilus* (*S. thermophilus*) has contributed to the emergence of modern CRISPR-Cas-technology by providing the first evidence for its biological function as an adaptive immune system in bacteria (86). The subsequent explosion of respective applications as a genome-editing tool was only possible due to basic research in LAB. Especially, the involvement of CRISPR-Cas-systems in the resistance against bacteriophages was crucial for the immense success of this technology (86–91).

Besides its application as a counter-selection tool to enrich for recombinant subpopulations after genome editing (25,63,82,92), CRIPSR-technology has found other exciting applications in LAB. Direct genome editing using CRISPR-Cas in combination with DNA-repair based on homologous

recombination has not been as successful as, for example, in yeasts or *E. coli* due to the lethality of double-strand breaks in LAB. Nevertheless, it was shown that nickase-assisted genome editing is possible in *Lb. casei* with efficiencies for in-frame deletions of 25-62% (93). Most recently, DSDR assisted by RecE/T in combination with CRISPR-Cas9 was reported to be an efficient tool for genome editing in lactobacilli, and the authors claimed to achieve mutations with 50-100% efficiency within one week (**Figure 6.4**). The inability of several LAB to efficiently repair double-strand breaks or even nicks was, in this case, overcome by introducing a repair mechanism based on a repair template and a host-specific recombinase system facilitating the homologous recombination (92). Nevertheless, the great diversity among LAB complicates the establishment of universal methods, as demonstrated recently by Leenay *et al.* (64). Their comparison between SSDR based on oligonucleotides and DSDR based on a plasmid-encoded repair template followed by CRISPR-Cas9 counter selection in three different strains of *Lb. plantarum* revealed that the methods produce unpredictable editing efficiencies in the various strains. This study shows that besides the showcased examples, efficient genome editing technologies are still in the developmental phase for most LAB.

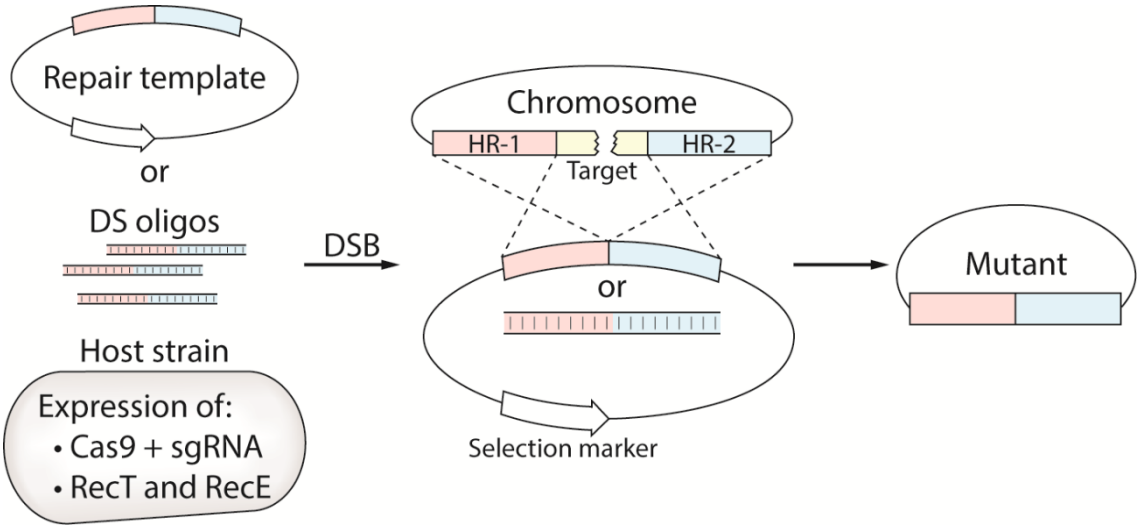

Figure 6.4. The principle behind Cas9-mediated mutagenesis. It is required that the host strain expresses a functional Cas9-enzyme and sgRNA targeting the gene of interest for introducing a double-strand break (DSB). As most lactic acid bacteria (LAB) are not readily recombinogenic, the expression of RecT and RecE are required to guarantee sufficiently high recombination frequencies. In some stains, recombination frequencies are too low for repairing a DSB, and only nickase-assisted mutagenesis can be applied. As repair templates, double-stranded oligonucleotides (DS oligos) or a plasmid can be applied, both carrying homologous regions (HR-1 and HR-2, red and blue respectively) and the desired mutations (for example, gene deletion). As the DSBs or nicks are mostly lethal to LAB, only mutant strains survive this mutagenesis protocol.

Alternative to introducing point mutations in the genome or gene deletions, CRISPR Interference (CRISPRi) based on an inactivated Cas9 (dCas9, *dead*Cas9) can be utilized for studying essential

genes in bacteria, exploiting that dCas9 still binds the targeted genes but does not cleave the DNA. For *L. lactis,* a single plasmid system was developed for simultaneously expressing two genes inducible by nisin (NICE system). This system was applied to express both Cas9 and the single-guide RNA (sgRNA) at similar levels, and it could also be demonstrated that dCas9 can be used to silence genes inducibly (94). CRISPRi was also successfully developed and applied for studying the function of several essential genes in *Lb. plantarum* WCFS1. An approach based on a two plasmid system was applied for realizing gene silencing, one expressing dCas9 and the other encoding for the single-guide RNA (95).

Another application of CRISPR-technology is the engineering of bacteriophages of *S. thermophilus* or *L. lactis*, which has been difficult before (96,97). Bacteriophage infections are a major challenge in the dairy industry, as they can cause huge production losses due to fermentation failure. Studying bacteriophages has been difficult without efficient genome editing tools, and therefore new insight into their infection mechanisms using CRISPR-technology will provide valuable knowledge. The phage engineering is based on introducing a double-strand break using Cas9, followed by repair guided by a homologous template (26). Furthermore, it has been shown that rare spontaneous mutations can be selected for using CRISPR-Cas, enabling the study and exploitation of such mutants in *S. thermophilus* (98).

Until now, most CRISPR applications are based on heterologous systems, particularly on the CRISPR-system derived from *S. pyogenes*, but similar systems are found in nearly all bacterial species (99). As many industrially LAB, including lactococci, lactobacilli, and streptococci, have endogenous CRISPR-systems (89,100–102), it might be advantageous to harness those for genome editing particularly regarding the resulting strains GMO-status (103). The first example of exploiting an endogenous CRISPR-system for genome editing in a recalcitrant strain was recently demonstrated for *Lb. crispatus* (104). This work laid the groundwork for harnessing natural CRISPR-systems for genome editing.

6.5 Traditional Applications of LAB and Optimizing Performance

*(Parts were moved to **Chapter 1** and constitute parts of **Section 1.4.5** and complete **Section 1.5**)*

6.5.1 Substrate Utilization

Metabolic engineering has been vastly used to introduce new substrate utilization routes into LAB with a strong focus on utilizing difficult-to-ferment but cheap and abundant carbohydrates. The well-known obstacle for degrading respective substrates such as starch or cellulose is their recalcitrance and complexity. Until today, no naturally occurring LAB strain has been described, that naturally degrades cellulose even though many plant-derived species have been characterized and reviewed (105). This topic has extensively been reviewed recently (106–110), and only recent examples that apply metabolic engineering are therefore elaborated here. Among others, *L. lactis* and *Lb. plantarum* have both been applied for expressing heterologous enzymes for enabling growth on cellulose. Gandini *et al.* have demonstrated that *L. lactis* supsp. *lactis*

IL1403 can be applied to produce L-lactic acid from cellodextrins (up to cellooctaose) by secreting β-glucosidase BglA and endoglucanase EngD from *C. cellulovorans* 743B (111). Similarly, Liu *et al*. used an engineered *L. lactis* subsp *cremoris* MG1363 strain to degrade paper and wheat straw by secreting Egl3 from *Trichoderma reesei* 3.3711 (112). Both studies profit from the ability of *L. lactis* to efficiently secrete proteins. *Lb. plantarum* NCIMB 8826 has been applied in a series of studies aiming at improving pentose metabolism, particularly to enable efficient arabinose utilization by substituting phosphoketolase Xpk1 with transketolase from *L. lactis* subsp. *lactis* IL1403 (113) and xylose utilization by additionally deleting Xpk2 and introducing *xylAB* operon from *Lb. pentosus* NRIC 1069 (114). Furthermore, the same starting strain secreting α-amylase from *S. bovis* 148 (AmyA) has recently been used to produce D-lactic acid solely from brown rice with competitive titers (115). All of the above-mentioned strains of *Lb. plantarum* NCIMB 8826 were deficient in L-lactate dehydrogenase and therefore produced D-lactic acid. Stern *et al*. could show that displaying a synthetic cellulosome on the surface of *Lb. plantarum* WCFS1 allowed for direct degradation of wheat straw, but the strain was unable to grow on this substrate as the sole carbon source (116).

As an alternative strategy, evolutionary engineering (**Figure 1.5**) can be applied to improve the substrate utilization of strains, which have not been characterized previously. Exposing *Lb. pentosus* CECT4023T to increasing concentrations of xylose enabled the strain to produce 1.4-fold more lactic acid from xylose when compared to the parent strain (117). When combined, such approaches could, in the future, enable large-scale fermentation of cellulose or cellulose fragments directly into enantiopure lactic acid. With appropriate re-routing of the metabolism as described in the following sections, the above-mentioned strategies will also enable the production of other compounds instead of lactic acid from cheap carbon sources.

In line with the above-mentioned examples, a systematic screening for good candidates can also yield suitable starting strains for fermenting starch or cellulosic substrates. Boguta *et al*. have characterized 296 LAB strains and evaluated their potential for growing on lignocellulosic feedstocks and tolerating various inhibitors present in this medium. The best isolates identified were *Lb. pentosus* (Strains: 10-16, LMG 17672 and LMG 17623), *Pediococcus pentosaceus* ATCC 25745 and *Pediococcus acidilactici* DSM 20284, which showed a high growth rate on xylose and arabinose and high tolerance towards various inhibitors. Consequently, these strains are ideal candidates for the conversion of lignocellulosic feedstocks into desired chemicals. Metabolic engineering of these strains would greatly facilitate their commercial application (118). A similar theoretical evaluation has recently been carried out with a smaller selection of LAB, focusing on lactobacilli and pediococci (57). Both studies come to the consensus that great potential lies in using LAB as production hosts for green chemicals, but still, major limitations are the genetic amenability of many LAB strains and their fastidious requirements.

6.6 Metabolic Engineering of LAB for Production of Chemicals or Proteins

6.6.1 Food Ingredients

Lactic acid

As the name of the group implies, all strains belonging to the LAB naturally produce lactic acid, more precisely, L-lactic acid, D-lactic acid, or a mixture thereof depending on the specific lactate dehydrogenase activities in the individual strain (**Figure 6.5, A**) (119). Besides being major contributors to the typical yogurt and cheese flavor, the potential applications of these compounds are immense and span from food ingredients to polymer-building blocks. The recent motivation for finding environmentally-friendly alternatives to petrochemical products has caused a great acceleration for enantiopure L-lactic acid production as the basis for polylactic acid (PLA) products since racemic lactic acid is not well-suited for polymerization. As chemical methods only produce racemic lactic acid, this resulted in a great drive for enhancing the biotechnological production of L-lactic acid (120). As many strains naturally produce L-lactic acid, metabolically engineering efforts have been focused much on improving either substrate utilization or stress tolerance, as described above (see **Section 1.5** and **6.6**).

Diacetyl

As mentioned above, diacetyl is spontaneously formed from α-acetolactate, a natural compound formed by *L. lactis* subsp. *lactis* biovar *diacetylactis*, which is able to metabolize the citrate that is present in milk. The production of diacetyl from α-acetolactate is a non-enzyme catalyzed reaction, which can be accelerated by metal-ions (121). Liu *et al.* exploited this approach for developing a high-level diacetyl-production strain. The strain is rerouted to overproduce α-acetolactate (Δ^3*ldh*, Δ*pta*, Δ*adhE*, Δ*butBA*, Δ*aldB*, Δ*noxE*), and when α-acetolactate levels are high enough, the non-enzymatic conversion is induced by adding metal ions (**Figure 6.5, B**). With 95 mM diacetyl produced, this is the so far most efficient LAB-based diacetyl production system reported (122). Recently, our group developed a process for obtaining even higher α-acetolactate concentrations (172 mM) from pyruvate and finally from fermentation-derived pyruvate by using a metabolically optimized whole-cell catalyst, which overexpresses a robust α-acetolactate synthase (Als) from *E. faecalis* (see **Chapter 7**, (243)). The obtained α-acetolactate can subsequently be converted into diacetyl using metal-ions.

As diacetyl is of such immense importance for many dairy products, obtaining diacetyl production strains using traditional mutagenesis methods is highly desired because of the low acceptance of GMOs by consumers. Various attempts have been made to isolate diacetyl-, or, more precisely, α-acetolactate-overproducing strains using non-GMO technologies. Consequently, different screening assays have been developed for finding such mutant strains (123,124). One successful example was described by Monnet *et al.*, who found *aldB* (acetolactate decarboxylase) negative mutants with low Ldh activity. Unfortunately, the obtained mutants were rather unstable and sensitive to oxygen, and large scale application could be difficult (125).

Alanine

Alanine is a natural sweetener and used in pharmaceutical formulations. It is naturally produced in LAB from pyruvate by alanine-dehydrogenase. *L. lactis* was successfully engineered for producing pure L-Ala by inactivating Ldh (Δ*ldh*) and alanine-racemase Alr (Δ*alr*) while expressing *Bacillus sphaericus* alanine dehydrogenase (*alaD,* Aladh[1]) (**Figure 6.5, C**) (126). The final strain was applied in a resting state to convert glucose into L-Ala, while D-Ala and an ammonium salt were supplied, and final titers of approx. 140 mM were obtained. Besides this publication, L-Ala production in LAB has not received much attention in the scientific literature, and final product titers are much lower compared to what can be achieved using engineered *E. coli* or *Corynebacterium glutamicum* (127,128).

Acetaldehyde

In yogurt, the typical flavor is due to the presence of acetaldehyde, which is naturally produced by the applied organisms *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Metabolic engineering was applied to understand and increase the production of acetaldehyde in *S. thermophilus*, which is produced from several precursors in this organism: pyruvate, acetyl-CoA, acetate, thymidine, or threonine. Besides pyruvate decarboxylase, pyruvate oxidase, pyruvate dehydrogenase, and pyruvate formate lyase, serine hydroxymethyltransferase (Shmt) were shown to be involved in acetaldehyde metabolism. By inactivating SHMT, mutant strains were obtained, which accumulated less acetaldehyde from threonine as the precursor, and by overexpressing Shmt, more acetaldehyde was obtained (129). *L. lactis* has also been modified to produce acetaldehyde, which is usually present in neglectable amounts. By overexpressing pyruvate decarboxylase from *Zymomonas mobilis* (Pdc[2]) and the native NADH oxidase, Nox (*noxE*), 21 mM of acetaldehyde was obtained under aerobic conditions from glucose, and only low amounts of other products were observed (**Figure 6.5, D**) (130). Similarly, the overexpression of pyruvate decarboxylase from *Zymobacter palmae* in *L. lactis* without overexpression of Nox resulted in 8-fold increased acetaldehyde concentrations compared to the wild-type strain with a final titer of approx. 11 mM (131).

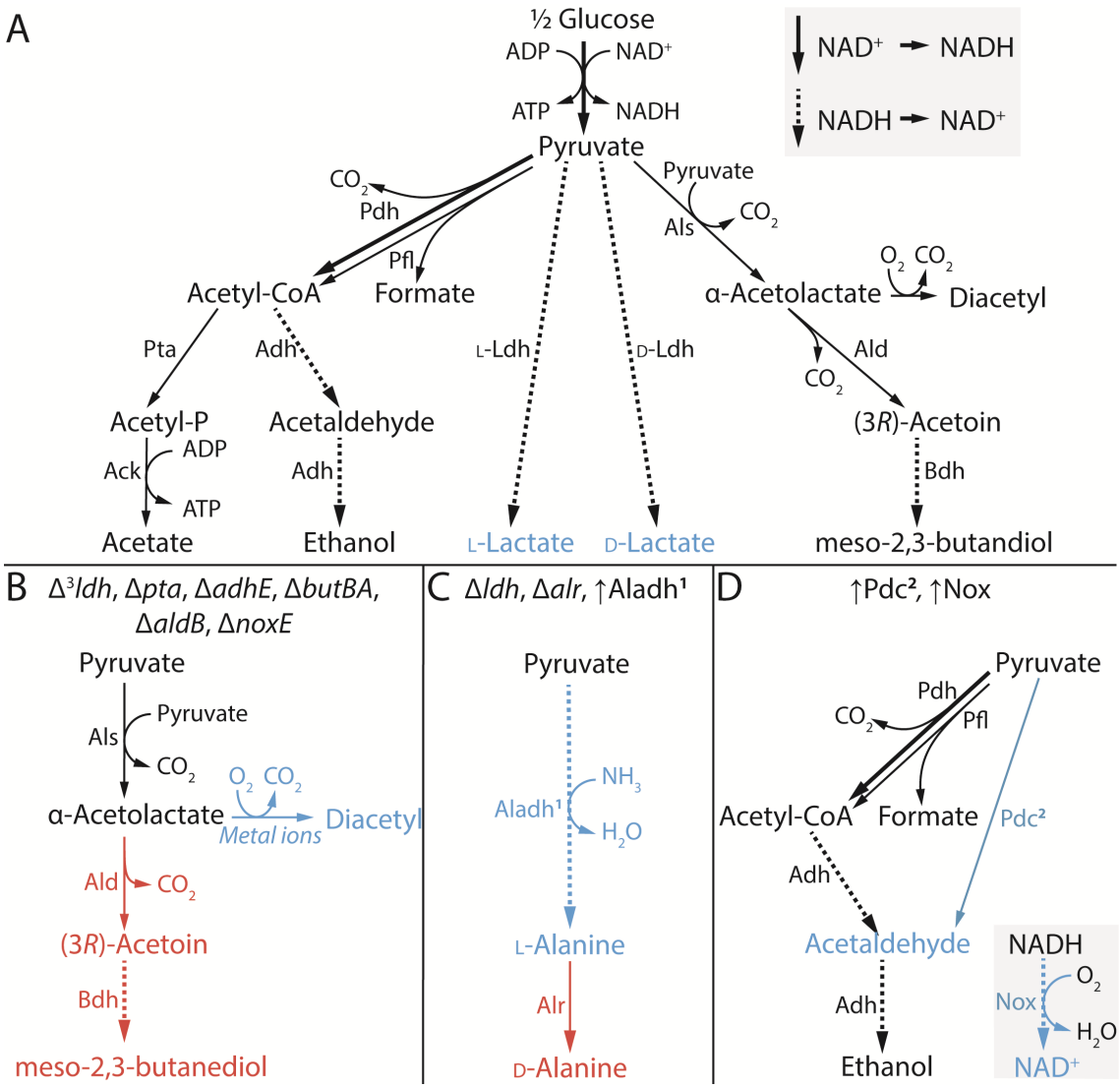

Figure 6.5. Metabolic engineering of *L. lactis* for over-producing flavor compounds. Thick arrows indicate an NADH forming reaction, and dashed arrows indicate an NADH consuming reaction. Red arrows indicate gene knock-outs for blocking the metabolite flux. Red metabolites are not produced. Blue arrows indicate the expression of a foreign gene or another non-native reaction. Metabolites with blue letters are accumulated. Pathways with no or low activity have been removed for simplification in B, C and D. Enzyme abbreviations are as follows: L-Ldh: lactate dehydrogenase forming L-lactate, Pfl: pyruvate formate lyase, Pdh: pyruvate dehydrogenase, Pta: phosphotransacetylase, Ack: acetate kinase, Adh: bifunctional alcohol and aldehyde dehydrogenase (adhE), Als: α-acetolactate synthase, Ald: α-acetolactate decarboxylase (aldB), Bdh: native butanediol dehydrogenase (butBA), Alr: alanine racemase (alr), Nox: NADH oxidase (noxE), Aladh^{1}: alanine dehydrogenase from *Bacillus sphaericus* (alaD), Pdc^{2}: pyruvate decarboxylase from *Zymomonas mobilis* (pdc). A: Pyruvate metabolism of *L. lactis*. B: Diacetyl is produced in *L. lactis* MG1363 (Δ^{3}ldh, Δpta, ΔadhE, ΔbutBA, ΔaldB, ΔnoxE) under aerobic conditions when respiration is active for cofactor regeneration. By employing non-enzymatic oxidative decarboxylation mediated by metal ions, α-acetolactate is converted to diacetyl. C: L-Ala is produced in *L. lactis* MG1363 (Δldh, Δalr), while Aladh^{1} is expressed. D-Ala is supplied in the medium. D: Acetaldehyde is produced in *L. lactis* MG1363 by overexpressing Pdc^{2} and Nox. Other pathways did not show interfering activity.

6.6.2 Bulk Chemicals

Ethanol

Due to the high glycolytic flux of LAB and its safety background, it is interesting to rewire the metabolic flux to produce other interesting chemicals instead of lactic acid. One example is ethanol production from whey, a lactose-rich side stream obtained from the production of cheese. *L. lactis* is well adapted to dairy environments and thus a natural choice for fermenting whey. By reintroducing lactose catabolism and a heterologous ethanol-producing pathway from *Zymomonas mobilis* (*pdc*, Pdc[2]**,** and *adhB*, Adh[3]), *L. lactis* MG1363 metabolism was efficiently rewired (Δ^3*ldh*, Δ*pta*, Δ*adhE*) to produce ethanol from lactose in a co-factor balanced way (**Figure 6.6, A**). Final titers of 41 g/L were obtained using a low-cost fermentation medium based on dairy waste (132). Similarly, the xylose-fermenting *L. lactis* KF147 was engineered into producing ethanol from xylose, and a yield of 83% was obtained. This approach demonstrates that a suitable LAB strain can be chosen and optimized for the production of a desired compound. The resulting strain might be better suitable for the individual fermentation medium than other laboratory strains (133).

Acetoin

Besides its importance as a flavor enhancer in dairy products, acetoin is traded as an emerging platform chemical for producing, for example, 1,3-butadiene that is used for synthetic rubber production (134). (3*R*)-acetoin is naturally produced by LAB, especially *L. lactis* subsp. *lactis* biovar *diacetylactis* via citrate fermentation (135). This trait has received much attention throughout the years because a side product from the action pathway is diacetyl, which is an important flavor compound in dairy products generating desired buttery aroma. In *L. lactis* subsp. *lactis* biovar *diacetylactis* after being transported into the cell via the citrate permease (Cp), citrate is cleaved by citrate lyase (Cl) into acetate and oxaloacetate. Oxaloacetate decarboxylase (Od) splits oxaloacetate into pyruvate and CO_2. Pyruvate is consumed via the acetoin pathway composed of α-acetolactate synthase (Als) and α-acetolactate decarboxylase (Ald), forming acetoin and small amounts of diacetyl if oxygen is present (121,136,137). High-level production of acetoin up to 27 g/L (306 mM) using cheap whey permeate as a growth medium was achieved by complete rerouting of the native metabolism. Other than exploiting the citrate metabolism, the authors inactivated several native enzymes (Δ^3*ldh*, Δ*pta*, Δ*adhE,* and Δ*butBA*) and the resulting strain produced (3*R*)-acetoin as the only fermentation product (**Figure 6.6, B**). Co-factor balance is distorted in this strain rendering it unable to grow anaerobically but growing the strain aerobically so that NADH-oxidase (Nox) can consume redundant NADH, allowed fast growth and production of acetoin. The other enantiomer of acetoin, (3*S*)-acetoin, was also produced in *L. lactis* using a different metabolic route as described below (138).

Also, other LAB, in particular *Lb. casei* were genetically modified for producing acetoin, but so far, the reported product titers are comparably low. *Lb. casei* BL23 was subjected to two gene

inactivations (*ldh*, *pdhC*) to increase acetoin production from whey permeate. Furthermore, in this strain, ALS from *L. lactis* (*ilvBN*) was overexpressed to increase the flux towards acetoin. Acetoin yields of 1.4 g/L (approx. 15 mM) were reported (139). In the same parent strain, by inactivating two additional genes: *pflB*, *ldh* (*ldh2* and *hicD3*), and *pdhC*, the titer could be increased. The resulting strain showed an 18-fold increase in acetoin production compared to the wild-type strain, and final acetoin titers of approximately 50-55 mM were reported (85).

Butanediol-Isomers

Butanediol (BDO) occurs in three stereoisomers (2*S*,3*S*)-butanediol, (2*R*,3*R*)-butanediol or *meso*-butanediol and is considered to have potential as a valuable biobased chemical. Derived products are of great importance as well, for example, methyl ethyl ketone is an important industrial solvent, and butadiene is a precursor for rubber production (140). Until now, the most efficient production of BDO in LAB was shown using *L. lactis* as a host strain. Similar to the above-mentioned acetoin production, the production strain has following genes inactivated: *ldh*, *pta*, *adhE,* and *butBA,* and therefore pyruvate is exclusively converted to (3*R*)-acetoin by acetolactate dehydrogenase (Ald*, aldB*) with α-acetolactate (ALA) as the intermediate. As the natural butanediol dehydrogenase (Bdh), which converts acetoin to BDO, is not very efficient, the authors substituted *butBA* with *ecBDH*, encoding a robust butanediol dehydrogenase (Bdh[4]) from *Enterobacter cloacae* (**Figure 6.6, C**). The resulting strain was applied to convert lactose from a dairy waste product into meso-BDO with high titer (51 g/L) and yield (0.47 g/g lactose). When *butBA* is substituted with *sadB*, encoding alcohol dehydrogenase (Sad[5]) from *Achromobacter xylosooxidants*, only (2*R*,3*R*)-BDO was obtained, with a reduced titer (32 g/L) but still with a high yield (0.4 g/g lactose). As this pathway only utilizes one mole of NADH per mole of glucose, redundant NADH is consumed by respiration, which is activated by supplementing heme to the medium. The production of (2*S*,3*S*)-butanediol in *L. lactis* was realized by integrating biocompatible chemistry (non-enzymatic chemical reaction) and metabolic engineering. A strain, which produced (2*S*,3*S*)-butanediol was obtained by overexpressing bifunctional butanediol dehydrogenase (Bdh[4]) from *Enterobacter cloacae* (122). Overexpression was carried out in a strain, which overproduced ALA because *aldB* had been inactivated (**Figure 6.6, D**). Metal-ion catalysis mediated the efficient conversion of ALA into diacetyl, which was subsequently consumed by Bdh[4] to yield (3*S*)-acetoin followed by (2*S*,3*S*)-butanediol formation. The cofactors are balanced in this strain, allowing it to grow anaerobically. The same strain was shown to be suitable to produce (3*S*)-acetoin by fine-tuning the respiration capacity of *L. lactis* using different amounts of heme (138).

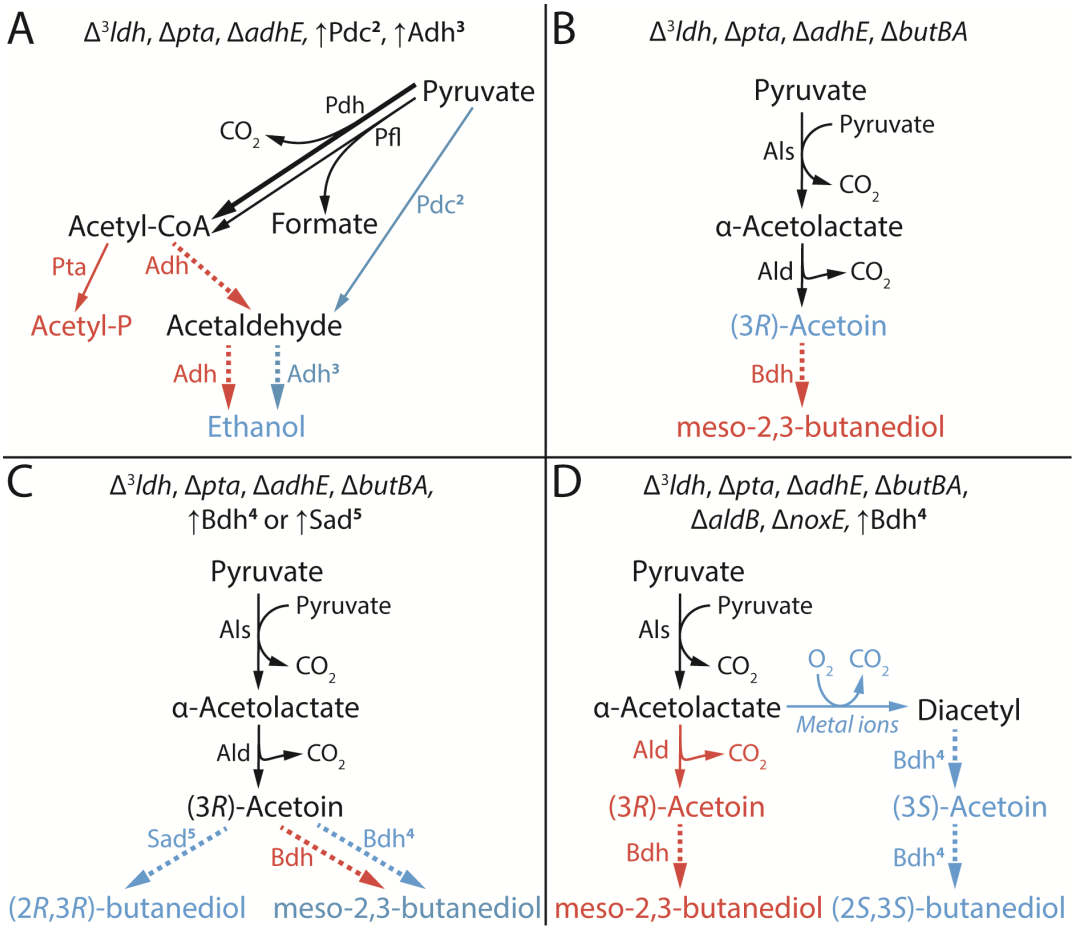

Figure 6.6. Metabolic engineering of *L. lactis* for producing bulk chemicals. The native pyruvate metabolism is shown in **Figure 6.5**, A. Thick arrows indicate an NADH forming reaction, and dashed arrows indicate an NADH consuming reaction. Red arrows indicate gene knock-outs for blocking the metabolite flux. Red metabolites are not produced. Blue arrows indicate the expression of a foreign gene. Metabolites with blue letters are accumulated. Pathways with no or low activity have been removed for simplification. Enzyme abbreviations are as follows: Pfl: pyruvate formate lyase, Pdh: pyruvate dehydrogenase, Pta: phosphotransacetylase, Ack: acetate kinase, Adh: bifunctional alcohol and aldehyde dehydrogenase (adhE), Als: α-acetolactate synthase, Ald: α-acetolactate decarboxylase (aldB), Bdh: native butanediol dehydrogenase (butBA), Pdc^{2}: pyruvate decarboxylase from *Zymomonas mobilis*, Adh^{3}: ethanol dehydrogenase from Z. mobilis, Bdh^{4}: butanediol dehydrogenase from *Enterobacter cloacae* (ecBDH), Sad^{5}: alcohol dehydrogenase from *Achromobacter xylosooxidants* (sadB). A: Ethanol is produced in *L. lactis* MG1363 (Δ^{3}ldh, Δpta, ΔadhE), while Pdc^{2} and Adh^{3} are expressed from a plasmid. B: (3R)-acetoin is produced in *L. lactis* MG1363 (Δ^{3}ldh, Δpta, ΔadhE, ΔbutBA). Cofactor balance was restored by Nox. C: (2R,3R)-butanediol or meso-2,3-butanediol are produced in *L. lactis* MG1363 (Δ^{3}ldh, Δpta, ΔadhE, ΔbutBA). Expression of Bdh^{4} resulted in the formation of meso-2,3-butanediol, and the expression of Sad^{5} resulted in the production of (2R,3R)-butanediol. The cofactor balance is restored by Nox. D: (2S,3S)-butanediol or (3S)-acetoin are produced in *L. lactis* MG1363 (Δ^{3}ldh, Δpta, ΔadhE, ΔbutBA, ΔaldB, ΔnoxE), which expressed Bdh^{4} catalyzing the two-step reduction of diacetyl to (2S,3S)-butanediol. Diacetyl is obtained from α-acetolactate using metal ions as catalysts. (3S)-acetoin was obtained using the same strain by fine-tuning the respiration machinery.

6.6.3 Vitamins and Polyols

LAB are fastidious organisms and need several externally supplied compounds for growth (141). Nevertheless, some LAB are able to produce certain vitamins important for human health, especially B-vitamins such as riboflavin (Vitamin B2), folate (Vitamin B9), or cobalamin (Vitamin B12) (142). Application of vitamin producing strains as probiotics or starter cultures for fermented food products enriched with these vitamins is desired because such products may help to reduce health problems associated with vitamin deficiencies. It is well-known that fermentation changes the vitamin content of the resulting product depending on the type of starter culture used (143,144). Nevertheless, the molecular understanding and distribution of such traits among the LAB are just increasing since more and more genetic information is made available and targeted optimization of the strains is rarely reported. The natural vitamins produced by bacteria are thought to gain better acceptance in society compared with their artificial counterparts as the latter are often derivatives of the natural vitamins (145).

Riboflavin – Vitamin B2

Riboflavin is a precursor of the coenzyme flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are both essential for many enzymatic reactions in all organisms. Several LAB were shown to produce riboflavin, as reviewed previously (142). Roseoflavin was shown to be useful for isolating riboflavin overproducing LAB as this compound is toxic for non-producers but not toxic for riboflavin producers. Using this approach, the non-producer *L. lactis* NZ9000 was converted into a riboflavin producer strain (146). Recently, Chen *et al.* optimized such a spontaneous mutant by applying mild mutagenesis in combination with high throughput screening using droplet-based microfluidics (DBM). In milk fermentations, the mutant strain increased the riboflavin content about 4-fold from 0.66 mg/L to 2.81 mg/L. This improved mutant strain was isolated after a single round of mutagenesis/screening, and further optimization is likely to result in further improvements (147). The immense potential in this approach lies in the fact that resulting strains are considered non-GMO and could, in principle, directly be applied in food products. DBM, however, requires a fluorescent readout, which limits the application to products that are either fluorescent, as riboflavin, or for which a selective fluorescent probe exists.

Folate – Vitamin B9

Folate production in LAB is a comparably common trait, as summarized previously (148). In principle, many species are able to produce folate, and among those are also commonly applied starter bacteria (*L. lactis*, *S. thermophilus,* or *Lb. plantarum*), but their abilities to produce folate are highly strain-dependent. As an example, several strains of *Lb. delbrueckii* subsp. *bulgaricus* were screened for folate production, but only a small fraction was able to do so (149). Metabolic engineering of folate producers could enhance productivity and, consequently, the industrial relevance of such strains. There are few examples in the literature describing engineering of LAB

with the purpose to increase folate production, but Sybesma *et al.* demonstrated that by overexpressing GTP cyclohydrolase I, which catalyzes the first step in the folate biosynthesis pathway starting from GTP, folate production in *L. lactis* could be increased by a factor of 10 (146). Furthermore, the simultaneous over-expression of the folate biosynthesis cluster and the *para*-aminobenzoic acid biosynthesis cluster in *L. lactis* was shown to increase the folate levels 80-fold (150). Folate overproduction in *Lb. plantarum* WCFS1, using a plasmid containing all genes for folate biosynthesis, resulted in a dramatically decreased growth rate (151). The drawback of these approaches is the GMO-status of the resulting organisms, which renders them inapplicable in food processes. A screening approach, as shown for riboflavin production (147), would probably be more suitable for obtaining non-GMO folate producers.

Cobalamin and Other Vitamins

Besides the above-mentioned vitamins, LAB have been shown to produce other nutritionally important compounds. Probably, the most important bacterially synthesized vitamin is cobalamin (vitamin B12). This compound cannot be synthesized by any higher organisms and is exclusively produced by bacteria, mainly anaerobes. Among those are *Pseudomonas* sp. or *Propionibacterium freudenreichii*, which are applied for industrial production and have undergone metabolic engineering, for example, by genome shuffling (152). Recently, members of the LAB, mainly lactobacilli, were reported to produce Vitamin B12 or derivates thereof. *Lb. reuteri* CRL1098 was the first LAB, in which Vitamin B12 production was observed (153), and other lactobacilli were also reported to produce this compound (154–156). Besides lactobacilli, only *Enterococci* have been found to produce vitamin B12 (157). These strains, however, have not been targeted for optimization yet, which would most likely result in more efficient production strains. The production of other vitamins that could potentially be produced by LAB is similarly unexploited. Among those are vitamin K2 (158), niacin, or pyridoxine (143,144).

Polyols as Natural Sweeteners

Mannitol and sorbitol are polyols, which are produced by LAB, either naturally or mediated by metabolic engineering. These compounds have importance in the food industry because they are low-calorie sweeteners, and several health benefits have been associated with them (159). They are obtained from sugars by reduction of fructose-6-phosphate catalyzed by mannitol-1P dehydrogenase or sorbitol-6P dehydrogenase, respectively. Phosphotransferase systems (PTS) are responsible for dephosphorylation and export of the polyols into the culture medium. As the responsible enzymes for the formation of polyols require NADH as cofactors, metabolic engineering has aimed at removing enzymes, which consume NADH (for example, *ldh*, *adh,* or *bdh*), and this has been shown to be beneficial for polyol production. This has been demonstrated in several studies using *L. lactis* or lactobacilli, in which *ldh* genes were inactivated (160–163). Furthermore, as several LAB are able to utilize mannitol as carbon source, the inactivation of the

mannitol transport system (phosphoenolpyruvate (PEP)-mannitol phosphotransferase system), consisting of *mtlA* and *mtlF* genes is beneficial for mannitol production if combined with knock-out of *ldh* (163). It should be noted that so far, the highest mannitol yields have been reported for wild-type lactobacilli, as summarized by Monedero *et al.* (164). Similar metabolic engineering strategies were shown to be suitable for sorbitol production when D-sorbitol 6-phosphate dehydrogenase (S6pdh) is expressed (165–167). As first demonstrated by Nissen *et al*., S6dph is encoded in the genome of *Lb. casei* but not functionally expressed. By integrating the *gutF* gene into the lactose operon, the strain was converted into a sorbitol producer (165). The same strategy was shown to be applicable to *Lb. plantarum*, but in this case, two native S6dph genes (*srlD1* and *srlD2*) were overexpressed, resulting in better sorbitol production and up to 65% conversion of glucose into sorbitol using resting cells (166). Lastly, the above mentioned metabolic engineering efforts could be transferred to sorbitol production, which was shown for *Lb. casei*, where the *ldh* gene, together with both mannitol and sorbitol transport systems, was inactivated, but the conversion was lower compared to *Lb. plantarum* (9.4%), probably due to the application of lactose instead of glucose (167). Detailed reviews further covering wild-type strains and the production of xylitol and erythritol can be consulted by the reader (164,168).

6.6.4 Therapeutic Proteins and Bacteriocins

Therapeutic Proteins

The interest in LAB as production hosts for heterologous proteins can be traced back to early studies where *L. lactis* was used as a drug delivery vehicle (DDV) to deliver Interleukin-10 for treating colitis (169) and where *L. lactis* served as a live vaccine against tetanus (170). Using *L. lactis* as DDV has the benefit that it is relatively easy to manipulate genetically, in addition to its long history of safe use in food fermentations. Recently, other LAB such as lactobacilli have gained increasing interest as they have become amenable to genetic manipulation. Most LAB can efficiently secrete proteins and are free of endotoxins, which are two important prerequisites for successful use as DDVs for therapeutic proteins to humans. Furthermore, most LAB have been shown to be able to survive the passage through the GIT. *L. lactis* does not colonize the colon normally, but many lactobacilli are able to do so. Various strategies for expressing proteins exist, as we have described above (see **Section 6.4.2**). Here, we will not provide an exhaustive review of the topic, but instead, restrict ourselves to discuss some of the metabolic engineering efforts aiming at generally optimizing LAB for the purpose of serving as DDV. For further information, the readers are referred to recent reviews (171–173).

In *L. lactis,* Usp45 is the only native protein, which is secreted in significant amounts, and for this reason, its signal peptide has been used for secreting other proteins. To optimize protein secretion in *L. lactis,* the Usp45-signal peptide has been mutagenized, and variants with up to 51% increased secretion have been obtained (174). As LAB are able to survive the passage through the GIT, one major concern, when using them for recombinant drug delivery, is that they could

end up in the environment. To address this concern, different approaches have been used. One group of researchers inactivated the thymidylate synthase (*thyA*) and showed that DNA synthesis is abolished, and these mutants only can proliferate in the presence of thymidine or thymine (175). Later another group used this approach in an Interleukin-10 producing *L. lactis*, and in this way, created a biocontainment system since it is unlikely that the strain can survive outside the GIT (176). Other biocontainment systems could be imagined, where externally triggered transcriptional activation or suppression of autolysin or toxins is relied upon (177). For more targeted approaches, for example, to eliminate certain bacteria in the GIT, positive feedback loops could be engineered into LAB. As an example, *L. lactis* was equipped with a biosensor for *E. faecalis* sex-pheromone pCF10, which was coupled to the expression of three different bacteriocins effective against enterococci, and when introduced in the GIT, the enterococci population was selectively erased (178). By using similar strategies, other pathogens or multi-drug resistant bacteria could be selectively targeted using tailored LAB.

Bacteriocins - Lantibiotics

Bacteriocins are small (< 10 kDa) ribosomally synthesized and usually post-translationally modified peptides (RiPPs). They are therefore distinct from polyketides (PKS) and non-ribosomally synthesized peptides (NRPS), which include the most common antimicrobials such as β-lactams or tetracyclines. One advantage of being ribosomally synthesized is that genome mining and structure prediction tools can be used in the search for organisms potentially producing these compounds and to determine their structure. Furthermore, side-directed mutagenesis can be applied when studying the effect of particular amino acid substitutions on activity (179). Numerous RiPPs have been identified and characterized, and these can be grouped based on their structure or nature of post-translational modification (180). Even though an immense variety exists, most RiPPs are produced via similar biosynthetic pathways. They all start as a precursor peptide, always containing the core peptide, which finally forms the backbone of the active bacteriocin, flanked with either a leader or a follower peptide. The flanking sequences are important recognition sites for the enzymes carrying out the post-translational modification and are eventually cleaved off from the modified core peptide (181). The so far most important and largest group of bacteriocins are the lanthipeptides (or lantibiotics), which include the well-studied bacteriocin nisin. Already in 1968, nisin was considered safe for human consumption in Europe and was added to the European food additive list in 1983. Later, in 1988, nisin was also approved by the FDA. Today, nisin is the only bacteriocin allowed to be used in purified form in food products (182). Most importantly, in the nearly 50 years of application as a food preservative, resistance against nisin is not emerging as quickly as for most other antimicrobial compounds, which are frequently used in the food industry. Nevertheless, spontaneous resistance to nisin has also been reported, and therefore there is an urgent need for novel, efficient and safe bacteriocins that can complement the use of nisin (183).

Lanthipeptides are characterized by the presence of one or more lanthionine bridges formed between dehydrated serine or threonine residues and a cysteine residue. These modifications are introduced by rather promiscuous enzymes (184). This feature, taken together with the modularity of RiPPs, due to their genetic encoding, makes them ideal candidates for genetic engineering. Numerous studies focusing on improving bacteriocin potency, selectivity, or production titers have been published, but only a few exemplary studies are mentioned here. For comprehensive reviews, refer to (179–182,185).

Maximizing bacteriocin production in LAB has been targeted by various means. As an example, classical mutagenesis has been applied to select mutants of the natural nisin A-producing strain *L. lactis* subsp. *lactis* ATCC 11454, which survived high concentrations of nisin and also showed more than a 3-fold increase in production. Impressively, the authors scaled up nisin production using the mutant strain from shake flasks to 10-L to 700-L and finally to 50-ton fermentations. At 50-ton scale, the mutant strain reliably produced, on average, 9960 IU/mL in 15 independent batches (186). This study is a good example of how traditional mutagenesis can be harnessed for optimizing bacteriocin production on industrial scale. The heterologous expression of bacteriocins has also been applied as exemplified with the production of enterocin A, which is naturally produced by *E. faecium* T136. *E. faecium* is not considered safe for human consumption, and therefore the heterologous expression of the gene encoding enterocin A in a food-grade host is desired. Expression was among others carried out in *L. lactis* and in different *Lactobacillus* strains. Compared to the native host, expression was improved in the heterologous hosts, approximately 2-fold for *L. lactis* (187) and 5-fold for *Lb. casei* (188). In both cases, leader and signal peptides were adjusted to the new expression host to ensure secretion and maturation.

Enhancing or altering the antimicrobial activity (AA) of bacteriocins has been undertaken in many studies. Site-directed mutagenesis is a straight forward way to study and optimize AA, which is possible in many cases thanks to the genetic encoding of the lanthipeptides. As an example, nisin Z, a derivative of nisin, has been mutagenized by site-directed mutagenesis, and two mutants (N20K and M21K) were shown to have a broader AA against some Gram-negative species and higher solubility than the wild-type nisin Z (189). Another way of changing the properties of lanthipeptides can be achieved using DNA shuffling between two or more related bacteriocins as demonstrated for pediocin PA-1 and enterocin A, which are both class IIa bacteriocins. From the resulting chimeras, 63 out of 280 tested variants were active, and a few showed increased AA against strains, which could be targeted with neither of the parental bacteriocins (190). Recently, high throughput screening technology was developed for testing AA by miniaturizing Flemings inhibition zone test, which is usually carried out on agar plates (191). Using nanoliter scale reactors based on a water-in-oil emulsion, the authors incubated a bacteriocin producer strain with a sensor strain followed by droplet sorting based on accumulated biomass. If the bacteriocin was active against the sensor strain, low biomass was present in the droplets, which resulted in low fluorescence after biomass-staining. Using this technology, the authors screened a library of

6000 combinatorial variants derived from 12 natural lantibiotics combined with synthetic modules. The library was transformed into *L. lactis*, which expressed the post-translation modification machinery required for the functional expression of the lantibiotics (*nisBTC*). In total, 205 unique variants were identified, from which 126 showed reproducible halos in a conventional inhibition zone assay. The authors demonstrated that at least 3 of the most potent variants, had an altered AA against several pathogens, which shows the suitability of their approach for exploiting the great diversity and modularity among RiPPs and lantibiotics in particular (192).

As an alternative to such untargeted approaches, rational engineering nowadays allows for high throughput optimization of biosynthesis pathways. As the gene clusters encoding for bacteriocins are quite large (for example, 11 genes for nisin) and rational genetic manipulations in some LAB are still rather difficult, a gene network engineering platform was described, which is based on an *E. coli-L. lactis* shuttle vector, which has a high copy number in *L. lactis* and only a single copy in *E. coli*. This reduced potential toxic effects of undesired transcription in the *E. coli* cells. The copy number in *E. coli* could be increased upon induction, prior to plasmid isolation and transformation into *L. lactis*. This system allows sophisticated genetic engineering in *E. coli* and subsequent evaluation in *L. lactis* (193).

6.6.5 LAB as Biocatalysts

Biotransformations involving LAB and substrates, which don't support bacterial growth, are covered in this section as for such reactions, biomass has to be prepared separately. Using LAB as whole-cell catalysts (WCCs) is a rather new approach, which is mainly deriving from LABs ability to selectively reduce prochiral ketones to enantiopure alcohols, that finds application in organic synthesis (194). The first evidence for the reducing power of LAB can be dated back to 1959 (195). The enzymes responsible for these reactions are alcohol dehydrogenases (Adh), which have been studied in great detail and are often applied in isolated forms expressed by a heterologous host. LAB, however, can also be applied as WCCs, and this type of application will be covered here as the cellular environment is relevant for such reactions, and metabolic engineering might be applicable for optimizing the conversion rates. Metabolic engineering has only been applied in a few cases, but the examples clearly demonstrate that LAB are well-suited as chassis for such novel reactions. WCCs based on LAB have been applied for many useful reactions, which will be described in the following.

Bioreduction of Ketones to Alcohols by LAB Cells

Several interesting compounds with relevance as chiral building blocks for synthesizing high-value products such as pharmaceuticals have been produced using whole cells of different *Lactobacillus* strains. Early studies have screened various LAB for their ability to reduce prochiral ketones into optically active alcohols, and among productive species were the heterofermentative *Lb. kefir*, *Lb. fermentum*, *Lb. brevis* and a few *Leuconostoc* species (194,196). It was noted that

homofermentative LAB were not able to reduce ketones. Another interesting observation was that the enantioselectivity towards the ketone substrates was highly species- and even strain-dependent (196). Later the scope of useful strains was extended to *Lb. plantarum*, *Lb. paracasei,* and *E. faecium*. *Lb. paracasei* BD101 was particularly useful for preparing (*S*)-1-phenyl ethanol from acetophenone (197). The same strain has also been applied for producing the analgesic drug (*S*)-phenyl(pyridine-2-yl)methanol from phenyl(pyridine-2-yl)methanone (198). A requirement for the successful reduction of a ketone into its deriving alcohol is the provision with reducing equivalents, which are required to regenerate NAD(P)H. NAD(P)H does not need to be added as it is present in the cell, and this greatly reduces the overall cost of the process. When provided with sugar, the WCC use their metabolism to provide reducing equivalents for the desired reaction (**Figure 6.7**). To avoid the application of expensive, reducing equivalents such as glucose or lactose, cheese whey and lignocellulosic hydrolysates were investigated for the reduction of acetophenone into (*R*)-1-phenyl ethanol using *Lb. reuteri* DSM 20016, which was found to be the best suitable strain among several *Lb. reuteri* strains for this reaction. Cheese whey or lignocellulosic hydrolysates were found to be nearly as efficient as pure lactose or glucose for providing reducing equivalents (199). The same strain was shown to be useful in the chemoenzymatic total synthesis of (*S*)-rivastigmine, a compound used for treating Alzheimer's disease (200). Because the application of sugars as reducing equivalents required an aqueous solution, this limits the substrate scope of the WCCs dramatically to water-soluble substrates. To overcome this limitation, Pfruender *et al*. developed a biphasic reaction setup based on ionic liquids (IL), which serve as an extracting agent and substrate reservoir. In contrast to common organic solvents, the IL are biocompatible and do not damage the cell membranes or the cofactor regeneration systems. In this study, *Lb. casei* was used for reducing 4- chloroacetophenone to (R)-1-(4-chlorophenyl)ethanol (201). In a similar way, increasing the performance of WCCs can be achieved by changing the cofactor regeneration system. Amidjojo *et al.* showed that *Lb. kefir* is more active when 2-propanol is used as a reducing equivalent instead of glucose. During cofactor regeneration, 2-propanol is converted to acetone. As both compounds are soluble in water, the solubility of hydrophobic compounds might be increased using this system. Overall the productivity for converting ethyl 4-chloro acetoacetate into (S)-4-chloro-3-hydroxybutanoate was improved with final titers of 1.2 M, 97% yield, and 99.5% enantiomeric excess (ee) (202). These studies show the power and flexibility of using whole cells as catalysts compared to isolated enzymes, as the intact cell provides an additional reaction compartment for protecting the enzymes, and in addition, the costs for protein purification are avoided. Overexpression of the native enzymes or heterologous expression of other alcohol dehydrogenases in combination with optimized cofactor recycling systems appears very promising for obtaining LAB optimized for their application in organic synthesis.

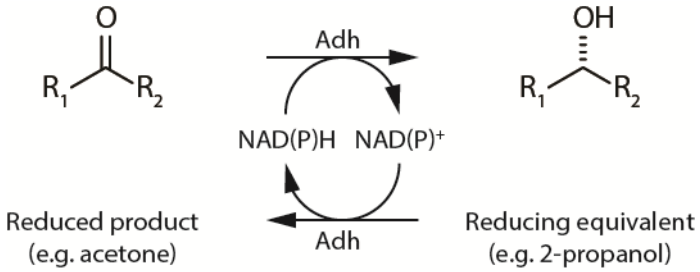

Figure 6.7. Reaction scheme of reduction of a prochiral ketone to the corresponding optically active secondary alcohol catalyzed by alcohol dehydrogenase (Adh). Adh requires the cofactor NAD(P)H, and therefore a cofactor regeneration system needs to be active, which can, for example, be another Adh, which acts in the reverse reaction or the native metabolism of the cell catalyst providing the cofactor.

Conversion of Glycerol to PDO or 3HP

Several lactobacilli have been shown to utilize glycerol and produce a mixture of 3-hydroxy propionic acid (3-HP) and 1,3-propanediol (1,3-PDO) when glycerol and glucose are supplied together. As lactobacilli lack functional dihydroxyacetone kinase, glycerol alone does not support the growth of wild-type lactobacilli but increases growth rate and biomass yield due to the gain of one ATP per glycerol (203). The natural pathway for glycerol utilization and 3-HP and 1,3-PDO production is encoded by the *pdu* (propanediol-utilization) gene cluster comprising *pduCDE* (glycerol dehydratase, Gdh), *pduP* (propionaldehyde dehydrogenase), *pduQ* (1,3-propanediol oxidoreductase), *pduL* (phosphotransacylase) and *pduW* (propionate kinase) (**Figure 6.8**). 3-hydroxypropionaldehyde (3-HPA or reuterin) is an important intermediate metabolite as it exhibits antimicrobial activity (204). The first step of the pathway catalyzed by glycerol dehydratase (Gdh) requires vitamin-B_{12} (cobalamin) as a cofactor and is considered a limiting step (205). Many attempts for optimizing 3-HP or 1,3-PDO production have been undertaken, which has been reviewed previously, and excessive work was carried out to realize this pathway in heterologous hosts such as *E. coli*, *Saccharomyces cerevisiae* or *Klebsiella pneumoniae* (206,207). Here the focus will be on applications of genetically engineered strains. As genome editing of lactobacilli has been difficult in the past, only recently have engineered strains been created and studied. One early step towards increasing the productivity of glycerol utilization through the *pdu* pathway was achieved by releasing catabolite repression, which is mediated by a catabolite repression element (*cre*) upstream of the *pdu* operon (205). By changing six bases in the *cre* locus using single-stranded DNA recombineering, a 3-fold increase in reuterin production was observed in strain RPRB3007 (208). Flux analysis in this strain revealed that in resting cells, the specific production rate of the mutant strain is 1.9-fold higher compared to the wild-type strain and 3-HP, and 1,3-PDO are produced in equimolar amounts (209). A series of process optimization steps was carried out using this mutant with the conclusion that cells from the stationary phase are better suited for biotransformation than exponentially growing cells. Furthermore, catabolite repression alone cannot explain limited final yields of either of the three

metabolites (210). This is undermined by the finding that fermentation products such as lactate or acetate at rather low concentration (20 mM) inhibit the production of 3-HP in the *cre*-mutant (RPRB3007) (211). Increasing 3-HP production was achieved by using resting cells of *Lb. reuteri*, which resulted in an equimolar production of 3-HP and 1,3-PDO. Subsequently, the 1,3-PDO was converted to 3-HP using cell extracts from *Gluconobacter oxydans* (212). A strategy for increasing 1,3-PDO production in *Lb. reuteri* is the overexpression of 1,3-propanediol oxidoreductase from *E. coli* (YqhD), which improved both productivity (34%) and yield (13%) (213). Gdh encoded by *pduCDE* in *Lb. reuteri* was further shown to accept 1,2-propanediol (1,2-PDO) as a substrate, resulting in the formation of propionic acid and propanol. *In vitro* enzyme engineering was used for widening the substrate scope of this enzyme, resulting in a variant that accepts diols with longer chains up to 6 carbons (1,2-hexanediol) but with lower activity towards glycerol or 1,2-PDO. This enzyme variant was realized in *Lb. reuteri* by mutagenesis using SSDR, and the activity towards longer diols was confirmed (214). These studies suggest that further metabolic engineering of *Lb. reuteri* could be very promising for increasing the 1,3-PDO or 3-HP yields from glycerol. Examples of successful engineering strategies were also demonstrated for *Lb. panis* and *Lb. diolivorans*. The former strain was equipped with 1,3-propanediol oxidoreductase from *E. coli* (YqhD), as previously shown to be beneficial for *Lb. reuteri* and consequently produced 8.5% more 1,3-PDO compared to the wild-type strain (215). Similarly, the homologous overexpression of the same enzyme in *Lb. diolivorans* resulted in a 20% increased 1,3-PDO production (216). Another possibility to increase the productivity of this pathway is to optimize the glycerol uptake, which is mediated by *pduF* in *Lb. diolivorans*. The transport proteins were overexpressed from a plasmid, and at the same time, Gdh expression was stimulated by supplementing 1,2-PDO into the growth medium. This combination resulted in the desired accumulation of 36 g/L 3-HPA, the intermediate metabolite, which is approximately 2-fold higher compared to the wild-type strain. Although the engineered strains show better performance compared to their parent strains, the highest reported product titers for all three products of this pathway (3-HP, 1,3-PDO and 3-HPA) have been achieved with wild-type strains using sophisticated process technologies (217,218).

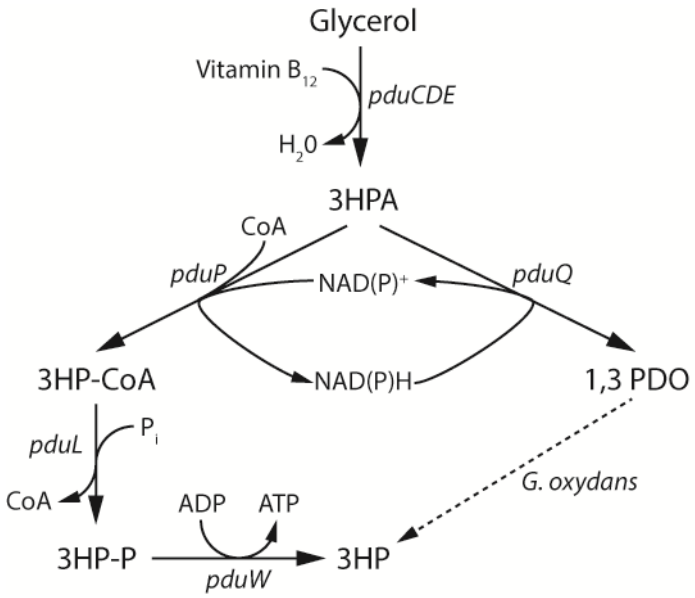

Figure 6.8. Glycerol metabolism in lactobacilli. Abbreviations as follows: 3HPA: 3-hydroxypropionaldehyde, 1,3 PDO: 1,3-propanediol, 3HP: 3-hydroxy propionic acid. Enzymes encoded by genes are the following: pduCDE: glycerol dehydratase (Gdh), pduP: propionaldehyde dehydrogenase, pduQ: 1,3-propanediol oxidoreductase, pduL: phosphotransacylase, pduW: propionate kinase. The reaction from 1,3 PDO to 3HP is catalyzed by Gluconobacter oxydans, as shown in (212).

6.6.6 More Biotransformations Using LAB

Other industrially relevant compounds, which are produced by LAB and have not received as much attention from the research community as the above-described ones, are covered in this section. Among those compounds are γ-aminobutyrate (GABA), phenyllactic acid (PLA), 2-butanol (2-BO), and isoprenoids.

GABA is naturally produced by some LAB mainly from the direct precursor L-glutamate employing glutamate decarboxylase (Gad) as catalyst (219). GABA yields by fermentation are rather low when L-glutamate is not present in the growth medium, but when supplied, promising titers are obtained as shown with the natural isolate *Lb. buchneri* WPZ001 (129 g/L) (220). Biotransformation using resting cells is a promising approach for obtaining high titers of GABA. By using resting cells of *Lb. brevis* TCCC 13007, 201 g/L GABA were obtained from L-glutamate (221). Metabolic engineering has contributed to the optimization of GABA production by LAB. By expressing glutamate decarboxylase from *Lb. plantarum* ATCC 14917 in *Lb. casei* B2-16, a 1.4-fold increased GABA production at 5-liter scale was achieved using growing cells (222). Overexpressing homologous Gad enzymes in *Lb. brevis* also showed a positive effect on the final product titer in two-stage fed-batch cultivation, reaching 105 g/L (223). By overexpressing and truncating the *gadA* gene in *Lb. brevis*, 1.2-fold higher final titers and volumetric productivities were obtained when compared to the wild-type strain. The authors also demonstrated that immobilizing the cells in gellan gum beads was beneficial for the stability and reusability of the

WCC (224). When catalase was overexpressed homologously in *Lb. brevis* CGMCC1306, a positive effect on GABA production was observed. The effect was most obvious when recycled cells were used, and a 1.5-fold higher activity after the fourth reuse was measured (225).

3-Phenyllactic acid (PLA) is a natural anti-fungal compound produced by LAB from phenylpyruvic acid (PPA) catalyzed by L- or D-lactate dehydrogenase. Similar to GABA production, co-fermentation or whole-cell catalysis are suitable for obtaining PLA from PPA (226,227). Genome shuffling was applied for increasing the antifungal activity of *Lb. plantarum*, which naturally produces PLA, and the production could be tripled from approximately 75 mg/L to 250 mg/L. Nevertheless, it was demonstrated that the antifungal activity of *Lb. plantarum* correlated better with lactic acid production, which was also increased for the shuffled strains (228). Recently, by expressing formate dehydrogenase (Fdh) from *Candida boidinii* in *Lb. plantarum*, intracellular NADH concentrations were increased in the presence of formate, which was shown to be beneficial for PLA production using resting cells. Fdh expression stabilized the activity of the WCC over repeated reaction, whereas the wild-type cells quickly lost their activity. Furthermore, the mutant strain was successfully applied in fed-batch fermentation, and 85 mM PLA with a 71% conversion rate was achieved through repeatedly feeding PPA and formate (229). In *E. coli,* it was demonstrated that L-phenylalanine could also serve as substrates for PLA synthesis when L-amino acid deaminase, L-lactate dehydrogenase, or D-lactate dehydrogenase, and formate dehydrogenase are co-expressed (230). Attempts towards using L-phenylalanine as an alternative substrate have been reported for *Lactobacillus* sp. SK007, but it was found that transamination is a limiting step for using the substrate (227).

The production of butanol has been studied in LAB. Selections of lactobacilli were screened for their ability to convert *meso*-2,3-butanediol or 2-butanone into 2-butanol, and a few *Lb. brevis* strains were identified (231,232). LAB are comparably tolerant to butanol compared to other production organisms such as yeasts or *E. coli* and would, therefore, be ideal hosts for large scale butanol production (233,234). Nevertheless, very limited research has gone into converting LAB into butanol producers, besides one proof of concept study, where the butanol pathway from was reconstructed in *Lb. brevis* by expressing genes from *Clostridium acetobutylicum* (235). The strain produced 4.1 mM 1-butanol and high amounts of ethanol and lactate when grown on glucose, showing that further metabolic engineering is required to obtain a butanol producing LAB suitable for industrial production. Recently, high-level 2-butanol production from meso-2,3-butanediol using *Lb. diolivorans* was reported. The authors supplied fermentation-derived meso-2,3-butanediol (*Serratia marcescens*) and obtained 13.4 g/L 2-butanol using a mutant overexpressing *pduQ*, which is involved in this conversion (236). This study highlights the suitability of LAB for biotransformations for obtaining toxic products in high concentrations.

Plant Metabolites

LAB are also suitable hosts for producing plant metabolites, as shown in a few examples. Anthocyanins are interesting plant metabolites, which can be used as natural colorants and antioxidants. *L. lactis* has been engineered to produce anthocyanins from flavan-3-ols by expressing two codon-optimized genes from *Arabidopsis thaliana,* anthocyanidin synthase (Ans) and UDP-glucose:anthocyanidin 3-O-glucosyltransferase (3Gt). As substrates, various flavan-3-ols, including (+)-catechin, were used to obtain anthocyanins via biotransformation. It was shown that *L. lactis* functionally expressed the heterologous genes, and various colorful products were obtained, demonstrating the suitability of LAB for such approaches. Even the utilization of commercial green tea infusions as a cheap source of flavan-3-ols was shown to be applicable (237). In another case, it has been proven to be more difficult to functionally express a plant gene in *L. lactis*, when coumarate:CoA ligase from *Arabidopsis thaliana* was expressed, which is one of two enzymes required for obtaining the valuable flavor compound vanillin from ferulic acid, a cheap and abundant chemical. The gene was functionally expressed, but the activity was too low for a useful application (238). Isoprenoids, synthesized in LAB via the mevalonate pathway, have been produced in *L. lactis* by expressing different terpene synthases, such as linalool/nerolidol synthase from *Fragassia x ananassa*, germacrene D synthase from *Vanda* Mimi Palmer and a ß-sesquiphellandrene synthase from *Persicaria minor* (239–241). Only for the former and latter enzyme *in vivo* production of linalool or ß-sesquiphellandrene, respectively, was observed, although all enzymes were shown to be functionally expressed. Metabolic engineering of the mevalonate pathway was successfully carried out by overexpressing both the *mvaA* gene coding for 3-hydroxy-3-methylglutaryl CoA reductase (Hmgr) and the *mvk* gene coding for mevalonate kinase. These enzymes were chosen as they were found to be down-regulated in a strain harboring the sesquiterpene synthases, as revealed by a transcriptomics analysis. Simultaneous overexpression of *mvaA* and *mvk* resulted in a 2.75-fold increase of β-sesquiphellandrene production compared to the unmodified sesquiterpene synthase containing strain (242).

6.6.7 Conclusion and Prospects

Today many chemicals used, for example, as high-energy fuels, building blocks for pharmaceutical production, or solvents are derived from fossil resources. As those are doomed to be depleted soon, our society needs alternative resources to sustain our current standard of living. The bioeconomy is an alternative concept for a sustainable future, in which chemicals are derived from renewable resources. Nevertheless, in order to avoid competition with human food production, the optimal starting materials for chemical production are waste or side streams from food or feed production and processing. Biological transformation through fermentation or biocatalysis for upgrading and recycling such low-value streams is going to be a key technology within the bioeconomy concept. As LAB are used extensively for food fermentations, they are

ideal candidates for fermenting respective waste streams as these often contain similar nutrients enabling fast growth and production. Already today, many successful studies were reported, where waste streams from the food industry were utilized and refined via fermentation using LAB. Extending this concept towards whole-cell catalysis is emerging as a promising asset for diversifying and improving production efficiency even further.

LAB will have a promising future as production organisms for several reasons: Firstly, many LAB have GRAS status, making them ideal candidates for producing chemicals intended for sensitive applications in food, pharma, or cosmetic products. Secondly, the high glycolytic flux of LAB makes them efficient cell factories and potential candidates for biobased production of commodity chemicals from waste materials. This is further strengthened by LAB's high resistance towards alcohols, organic acids, or other toxic or inhibitory compounds. Many examples of successfully using LAB for producing a wide range of chemicals ranging from flavor compounds over biofuels to vitamins have been reported. Thirdly, the natural diversity of LAB is an extremely promising source for new strains and has not been fully exploited yet. This reservoir might contain strains producing so far unknown metabolites, bioactive peptides, or enzymes and is waiting to be explored. Lastly, with the harnessing of CRISPR-Cas-technology into LAB, non-model strains, often having superior properties compared to the laboratory strains, will become amenable to genetic changes allowing their commercial exploitation. This has been difficult until recently due to the lack of efficient genetic engineering tools and transformation protocols. Using non-model LAB might enable efficient chemical production from cheap and abundant raw materials such as lignocellulosic materials.

Furthermore, we expect technological development, in particular high-throughput screening technology, such as droplet-based microfluidics, to become more broadly available. This technology has already been shown to be well suited for isolating superior mutants from large populations of microorganisms. The largely enhanced screening capacity provided by this technology is likely to give rise to many superior production strains. Especially within the food industry, such strains are demanded as they are not considered to be genetically modified organisms and thus are readily accepted by the consumers.

The high nutrient requirements of many LAB, limited stress resistance, or narrow substrate range for plant-derived carbohydrates, are promising targets for optimization. Increasing the productivity and titers of valuable primary or secondary metabolites could also be targeted using high-throughput screening and metabolic optimization. The natural diversity of LAB might also be a promising source of organisms, which could be further developed into profitable and beneficial production strains or probiotics. Similarly, new enzymes could be discovered for biotransformations employing LAB.

LAB are also gaining more and more attention within pharmaceutical research, especially because of their potential as probiotics or drug delivery vehicles. We expect to see more research within this field in the near future as the understanding of their roles within the human microbiome

increases rapidly. Probiotic strains sustaining the sensitive balance between beneficial and malicious microorganisms may become important for our health and well-being.

6.7 References

1. Kondo JK, McKay LL. Gene Transfer Systems and Molecular Cloning in Group N Streptococci: A Review. *J Dairy Sci* **1985**, 68(9):2143–59.
2. Kempler GM, McKay LL. Genetic evidence for plasmid-linked lactose metabolism in *Streptococcus lactis* subsp. *diacetylactis*. *Appl Environ Microbiol* **1979**, 37(5):1041–3.
3. Kempler GM, McKay LL. Characterization of plasmid deoxyribonucleic acid in *Streptococcus lactis* subsp. *diacetylactis*: Evidence for plasmid-linked citrate utilization. *Appl Environ Microbiol* **1979**, 37(2):316–23.
4. Mckay LL, Baldwin KA, Walsh PM. Conjugal Transfer of Genetic Information in Group N Streptococci. *Appl Environ Microbiol* **1980**, 40(1):84–91.
5. Kondo JK, Mckay LL. Plasmid Transformation of *Streptococcus lactis* Protoplasts: Optimization and Use in Molecular Cloning. *Appl Environ Microbiol*. **1984**, 48(2):252–9.
6. Chassy BM, Flickinger JL. Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiol Lett* **1987**, 44:173–7.
7. Luchansky JB, Muriana PM, Klaenhammer TR. Application of electroporation for transfer of plasmid DNA to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus* and *Propionibacterium*. *Mol Microbiol* **1988**, 2(5):637–46.
8. Holo H, Nes IF. High-Frequency Transformation, by Electroporation, of *Lactococcus lactis* subsp. *cremoris* Grown with Glycine in Osmotically Stabilized Media. *Appl Environ Microbiol* **1989**, 55(12):3119–23.
9. Vento JM, Crook N, Beisel CL. Barriers to genome editing with CRISPR in bacteria. *J Ind Microbiol Biotechnol* **2019**, 46:1327–41.
10. Biswas I, Gruss A, Ehrlich SD, Maguin E. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J Bacteriol*. **1993**, 175(11):3628–35.
11. Landete JM, Arqués JL, Peirotén Á, Langa S, Medina M. An improved method for the electrotransformation of lactic acid bacteria: A comparative survey. *J Microbiol Methods* **2014**, 105:130–3.
12. Ortiz-Velez L, Ortiz-Villalobos J, Schulman A, Oh JH, Pijkeren JP, Britton RA. Genome alterations associated with improved transformation efficiency in *Lactobacillus reuteri*. *Microb Cell Fact* **2018**, 17(138):1–13.
13. De Vos WM. Gene cloning and expression in lactic streptococci. *FEMS Microbiol Lett* **1987**, 46(3):281–95.
14. Shareck J, Choi Y, Lee B, Miguez CB. Cloning vectors based on cryptic plasmids isolated from lactic acid bacteria: Their characteristics and potential applications in biotechnology. *Crit Rev Biotechnol* **2004**, 24(4):155–208.
15. Kok J, Van Der Vossen JMBM, Venema G. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* **1984**, 48(4):726–31.
16. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **1983**, 154(1):1–9.
17. Clewell DB, Yagi Y, Dunny GM, Schultz SK. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. *J Bacteriol* **1974**, 117(1):283–9.
18. Simon D, Chopin A. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. *Biochimie* **1988**, 70(4):559–66.
19. Sullivan DJO, Klaenhammer TR. High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **1993**, 137:227–31.

20. Maguin E, Duwat P, Hege T, Ehrlich D, Gruss A. New thermosensitive plasmid for gram-positive bacteria. *J Bacteriol* **1992**, 174(17):5633–8.
21. Spangler JR, Caruana JC, Phillips DA, Walper SA. Broad range shuttle vector construction and promoter evaluation for the use of *Lactobacillus plantarum* WCFS1 as a microbial engineering platform. *Synth Biol* **2019**, 4(1):1–14.
22. Kaur T, Balgir PP, Kaur B. Construction of a shuttle expression vector for lactic acid bacteria. *J Genet Eng Biotechnol* **2019**, 17(1):1–13.
23. Tagliavia M, Nicosia A. Advanced strategies for food-grade protein production: A new *E. coli*/lactic acid bacteria shuttle vector for improved cloning and food-grade expression. *Microorganisms* **2019**, 7(5):1–10.
24. Spath K, Heinl S, Grabherr R. Direct cloning in *Lactobacillus plantarum*: Electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. *Microb Cell Fact* **2012**, 11(141):1–8.
25. Els S Van Der, James JK, Kleerebezem M, Bron PA. Versatile Cas9-Driven Subpopulation Selection Toolbox for *Lactococcus lactis*. *Appl Environ Microbiol* **2018**, 84(8):1–11.
26. Lemay M-L, Renaud A, Rousseau G, Moineau S. Targeted Genome Editing of Virulent Phages Using CRISPR-Cas9. *Bio-Protocols* **2018**, 8.
27. Kuipers OP, De Ruyter PGGA, Kleerebezem M, De Vos WM. Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol* **1998**, 64(1):15–21.
28. Kuipers OP, Beerthuyzen MM, De Ruyter PGGA, Luesink EJ, De Vos WM. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J Biol Chem* **1995**, 270(45):27299–304.
29. Kleerebezem M, Beerthuyzen MM, Elaine VE, de Vos WM, Kuipers OP. Controlled Gene Expression Systems for Lactic Acid Bacteria: Transferable Nisin-Inducible Expression Cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. *Appl Environ Microbiol* **1997**, 63(11):4581–4584.
30. Mierau I, Kleerebezem M. 10 Years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **2005**, 68(6):705–17.
31. Wolf D, Mascher T. The applied side of antimicrobial peptide-inducible promoters from Firmicutes bacteria: expression systems and whole-cell biosensors. *Appl Microbiol Biotechnol* **2016**, 100(11):4817–29.
32. Jensen PR, Hammer K. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl Environ Microbiol* **1998**, 64(1):82–7.
33. Andersen HW, Pedersen MB, Hammer K, Jensen PR. Lactate dehydrogenase has no control on lactate production but has a strong negative control on formate production in *Lactococcus lactis*. *Eur J Biochem* **2001**, 268(24):6379–89.
34. Hammer K, Mijakovic I, Jensen PR. Synthetic promoter libraries - Tuning of gene expression. *Trends Biotechnol* **2006**, 24(2):53–5.
35. Solem C, Jensen PR. Modulation of Gene Expression Made Easy. *Appl Environ Microbiol* **2002**, 68(5):2397–403.
36. Solem C, Koebmann B, Jensen PR. Control analysis of the role of triosephosphate isomerase in glucose metabolism in *Lactococcus lactis*. *Inst Eng Technol* **2008**, 2(2):64–72.
37. Koebmann B, Solem C, Jensen PR. Control analysis as a tool to understand the formation of the las operon in *Lactococcus lactis*. *FEBS J* **2005**, 272(9):2292–303.
38. Solem C, Koebmann BJ, Jensen PR. Glyceraldehyde-3-phosphate dehydrogenase has no control over glycolytic flux in *Lactococcus lactis* MG1363. *J Bacteriol* **2003**, 185(5):1564–71.
39. Solem C, Petranovic D, Koebmann B, Mijakovic I, Jensen PR. Phosphoglycerate mutase is a highly efficient enzyme without flux control in *Lactococcus lactis*. *J Mol Microbiol Biotechnol* **2010**, 18(3):174–80.

40. Koebmann BJ, Solem C, Jensen PR. Control analysis of the importance of phosphoglycerate enolase for metabolic fluxes in *Lactococcus lactis* subsp. *lactis* IL1403. *IEE Proc-Syst Biol* **2006**, 153(5):346–9.
41. Liu J, Chan SHJ, Chen J, Solem C, Jensen PR. Systems Biology – A Guide for Understanding and Developing Improved Strains of Lactic Acid Bacteria. *Front Microbiol* **2019**, 10:1–19.
42. Rud I, Jensen PR, Naterstad K, Axelsson L. A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. *Microbiology* **2006**, 152(4):1011–9.
43. Rud I, Solem C, Jensen PR, Axelsson L, Naterstad K. Co-factor engineering in lactobacilli: Effects of uncoupled ATPase activity on metabolic fluxes in *Lactobacillus* (*L.*) *plantarum* and *L. sakei*. *Metab Eng* **2008**, 10(5):207–15.
44. Liu J, Kandasamy V, Würtz A, Jensen PR, Solem C. Stimulation of acetoin production in metabolically engineered *Lactococcus lactis* by increasing ATP demand. *Appl Microbiol Biotechnol* **2016**, 100(22):9509–17.
45. Madsen SM, Hindré T, Le Pennec JP, Israelsen H, Dufour A. Two acid-inducible promoters from *Lactococcus lactis* require the cis-acting ACiD-box and the transcription regulator RcfB. *Mol Microbiol* **2005**, 56(3):735–46.
46. Llull D, Poquet I. New expression system tightly controlled by zinc availability in *Lactococcus lactis*. *Appl Environ Microbiol* **2004**, 70(9):5398–406.
47. Linares DM, Alvarez-Sieiro P, Rio B, Ladero V, Redruello B, Martin MC, Fernandez M, Alvarez MA. Implementation of the agmatine-controlled expression system for inducible gene expression in *Lactococcus lactis*. *Microb Cell Fact* **2015**, 14(208):1–12.
48. Sørvig E, Grönqvist S, Naterstad K, Mathiesen G, Eijsink VGH, Axelsson L. Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiol Lett* **2003**, 229:119–26.
49. Benbouziane B, Ribelles P, Aubry C, Martin R, Kharrat P, Riazi A, Langella P, Bermúdez-Humarán LG. Development of a Stress-Inducible Controlled Expression (SICE) system in *Lactococcus lactis* for the production and delivery of therapeutic molecules at mucosal surfaces. *J Biotechnol* **2013**, 168(2):120–9.
50. Landete JM. A review of food-grade vectors in lactic acid bacteria: from the laboratory to their application. *Crit Rev Biotechnol* **2017**, 37(3):296–308.
51. Klein JR, Weber B, Delorme C, Renault P, Henrich B, Wegmann U. Food-Grade Delivery System for Controlled Gene Expression in *Lactococcus lactis*. *Appl Environ Microbiol* **2009**, 68(11):5429–36.
52. Morello E, Bermúdez-Humarán LG, Llull D, Solé V, Miraglio N, Langella P, Poquet I. *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *J Mol Microbiol Biotechnol* **2007**, 14:48–58.
53. Kok J, van Gijtenbeek LA, de Jong A, van der Meulen SB, Solopova A, Kuipers OP. The Evolution of gene regulation research in *Lactococcus lactis*. *FEMS Microbiol Rev* **2017**, 41:220–43.
54. Maguin E, Prévost H, Ehrlich SD, Gruss A. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J Bacteriol* **1996**, 178(3):931–5.
55. Leenhouts K, Venema G, Kok J. A lactococcal pWV01-based integration toolbox for bacteria. *Methods Cell Sci* **1998**, 20:35–50.
56. Solem C, Koebmann B, Jensen PR. The extent of co-metabolism of glucose and galactose by *Lactococcus lactis* changes with the expression of the lacSZ operon from *Streptococcus thermophilus*. *Biotechnol Appl Biochem* **2008**, 50:35–40.
57. Bosma EF, Forster J, Nielsen AT. Lactobacilli and pediococci as versatile cell factories – Evaluation of strain properties and genetic tools. *Biotechnol Adv* **2017**, 35(4):419–42.
58. Hatti-Kaul R, Chen L, Dishisha T, Enshasy H El. Lactic acid bacteria: From starter cultures to producers of chemicals. *FEMS Microbiol Lett* **2018**, 365(20):1–20.

59. Goh YJ, Andrea Azcárate-Peril M, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR. Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* **2009**, 75(10):3093–105.
60. Solem C, Defoor E, Jensen PR, Martinussen J. Plasmid pCS1966, a New Selection / Counterselection Tool for Lactic Acid Bacterium Strain Construction Based on the oroP Gene, Encoding an Orotate Transporter from *Lactococcus lactis*. *Appl Environ Microbiol* **2008**, 74(15):4772–5.
61. Van Zyl WF, Dicks LMT, Deane SM. Development of a novel selection/counter-selection system for chromosomal gene integrations and deletions in lactic acid bacteria. *BMC Mol Biol* **2019**, 20(10):1–16.
62. Zuo F, Zeng Z, Hammarström L, Marcotte H. Inducible Plasmid Self-Destruction (IPSD) Assisted Genome Engineering in Lactobacilli and Bifidobacteria. *ACS Synth Biol* **2019**, 8:1723–9.
63. Zhang Y, Guo T, Kong J, Xin Y, Gu X. A rapid and versatile tool for genomic engineering in *Lactococcus lactis*. *Microb Cell Fact* **2019**, 18(22):1–12.
64. Leenay RT, Vento JM, Shah M, Martino ME, Leulier F, Beisel CL. Genome Editing with CRISPR-Cas9 in *Lactobacillus plantarum* Revealed That Editing Outcomes Can Vary Across Strains and Between Methods. *Biotechnol J* **2019**, 14(3):1–11.
65. Brøndsted L, Hammer K. Use of the integration elements encoded by the temperate lactococcal bacteriophage TP901-1 to obtain chromosomal single-copy transcriptional fusions in *Lactococcus lactis*. *Appl Environ Microbiol* **1999**, 65(2):752–8.
66. Lambert JM, Bongers RS, Kleerebezem M. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl Environ Microbiol* **2007**, 73(4):1126–35.
67. Petersen KV, Martinussen J, Jensen PR, Solem C. Repetitive, Marker-Free, Site-Specific Integration as a Novel Tool for Multiple Chromosomal Integration of DNA. *Appl Environ Microbiol* **2013**, 79(12):3563–9.
68. Koko I, Song AAL, Masarudin MJ, Abdul Rahim R. Engineering integrative vectors based on phage site-specific recombination mechanism for *Lactococcus lactis*. *BMC Biotechnol* **2019**, 19(82):1–12.
69. Lin CF, Lo TC, Kuo YC, Lin TH. Stable integration and expression of heterologous genes in several lactobacilli using an integration vector constructed from the integrase and attP sequences of phage ΦaT3 isolated from *Lactobacillus casei* ATCC 393. *Appl Microbiol Biotechnol* **2013**, 97(8):3499–507.
70. Xin Y, Mu Y, Kong J, Guoa T. Targeted and Repetitive Chromosomal Integration Enables High-Level Heterologous Gene Expression in *Lactobacillus casei*. *Appl Environ Microbiol* 2019**,** 85(9):1–12.
71. Van Pijkeren JP, Britton RA. Precision genome engineering in lactic acid bacteria. *Microb Cell Fact* **2014**, 13:1–10.
72. Van Pijkeren JP, Britton RA. High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Res* **2012**, 40(10):1–13.
73. Yu D, Ellis HM, Lee E-C, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci* **2000**, 97(11):5978–5983.
74. Ellis HM, Yu D, DiTizio T, Court DL. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc Natl Acad Sci* **2001**, 98(12):6742–6.
75. Zhou D, Jiang Z, Pang Q, Zhu Y, Wang Q, Qi Q. CRISPR/Cas9-assisted seamless genome editing in *Lactobacillus plantarum* and its application in N-acetylglucosamine production. *Appl Environ Microbiol* **2019**, 85(21):1–11.
76. Hemarajata P, Spinler JK, Balderas MA, Versalovic J. Identification of a proton-chloride antiporter (EriC) by Himar1 transposon mutagenesis in *Lactobacillus reuteri* and its role in histamine production. *Antonie van Leeuwenhoek* **2014**, 105(3):579–92.

77. Hemarajata P, Gao C, Pflughoeft KJ, Thomas CM, Saulnier DM, Spinler JK, Versalovic J. Lactobacillus reuteri-Specific immunoregulatory gene rsiR modulates histamine production and immunomodulation by *Lactobacillus reuteri*. *J Bacteriol* **2013**, 195(24):5567–76.
78. van der Meulen SB, Hesseling-Meinders A, De Jong A, Kok J. The protein regulator ArgR and the sRNA derived from the 3'-UTR region of its gene, ArgX, both regulate the arginine deiminase pathway in *Lactococcus lactis*. *PLoS One* **2019**, 14(6):1–18.
79. Ainsworth S, Sadovskaya I, Vinogradov E, Courtin P, Guerardel Y, Mahony J, Grard T, Cambillau C, Chapot-Chartier MP, van Sinderen D. Differences in lactococcal cell wall polysaccharide structure are major determining factors in bacteriophage sensitivity. *MBio* **2014**, 5(3):1–11.
80. Mahony J, Alqarni M, Stockdale S, Spinelli S, Feyereisen M, Cambillau C, Van Sinderen D. Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. *Sci Rep* **2016**, 6:1–10.
81. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol*. **2013**, 31(3):233–9.
82. Oh JH, Van Pijkeren JP. CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*. *Nucleic Acids Res* **2014**, 42(17):1–11.
83. Yang P, Wang J, Qi Q. Prophage recombinases-mediated genome engineering in *Lactobacillus plantarum*. *Microb Cell Fact* **2015**, 14(154):1–11.
84. Xin Y, Guo T, Mu Y, Kong J. Identification and functional analysis of potential prophage-derived recombinases for genome editing in *Lactobacillus casei*. *FEMS Microbiol Lett* **2017**, 364(24):1–9.
85. Xin Y, Guo T, Mu Y, Kong J. A single-plasmid genome editing system for metabolic engineering of *Lactobacillus casei*. *Front Microbiol* **2018**, 9:1–11.
86. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero D a, Horvath P. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* **2007**, 315:1709–12.
87. Deveau H, Barrangou R, Garneau JE, Labonté J, Fremaux C, Boyaval P, Romero DA, Horvath P, Moineau S. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. *J Bacteriol* **2008**, 190(4):1390–400.
88. Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res* **2011**, 39(21):9275–82.
89. Bolotin A, Quinquis B, Sorokin A, Dusko Ehrlich S. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **2005**, 151(8):2551–61.
90. Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S. The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **2010**, 468(7320):67–71.
91. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci* **2012**, 109(39):2579–86.
92. Huang H, Song X, Yang S. Development of a RecE/T-Assisted CRISPR–Cas9 Toolbox for *Lactobacillus*. *Biotechnol J* **2019**, 14(7):1–12.
93. Song X, Huang H, Xiong Z, Ai L, Yang S. CRISPR-Cas9D10A Nickase-Assisted Genome Editing in *Lactobacillus casei Appl Environ Microbiol*. **2017**, 83(22):1–14.
94. Berlec A, Škrlec K, Kocjan J, Olenic M, Štrukelj B. Single plasmid systems for inducible dual protein expression and for CRISPR-Cas9/CRISPRi gene regulation in lactic acid bacterium *Lactococcus lactis*. *Sci Rep* **2018**, 8:1–11.
95. Myrbråten IS, Wiull K, Salehian Z, Håvarstein LS, Straume D, Mathiesen G, Kjos M. CRISPR Interference for Rapid Knockdown of Essential Cell Cycle Genes in *Lactobacillus plantarum*. *mSphere* **2019**, 4(2):1–14.

96. Martel B, Moineau S. CRISPR-Cas: An efficient tool for genome engineering of virulent bacteriophages. *Nucleic Acids Res* **2014**, 42(14):9504–13.
97. Lemay ML, Tremblay DM, Moineau S. Genome Engineering of Virulent Lactococcal Phages Using CRISPR-Cas9. *ACS Synth Biol* **2017**, 6(7):1351–8.
98. Selle K, Klaenhammer TR, Barrangou R. CRISPR-based screening of genomic island excision events in bacteria. *Proc Natl Acad Sci* **2015**, 112(26):8076–81.
99. Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P, Moineau S, Mojica FJM, Wolf YI, Yakunin AF, Van Der Oost J, Koonin E V. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* **2011**, 9(6):467–77.
100. Millen AM, Horvath P, Boyaval P, Romero DA. Mobile CRISPR/Cas-Mediated Bacteriophage Resistance in *Lactococcus lactis*. *PLoS One* **2012**, 7(12):1–9.
101. Sun Z, Harris HMB, McCann A, Guo C, Argimón S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF, Liu W, Song Y, Salvetti E, Wrobel A, Rasinkangas P, Parkhill J, Rea MC, O'Sullivan O, Ritari J, Douillard FP, Paul Ross R, Yang R, Briner AE, Felis GE, De Vos WM, Barrangou R, Klaenhammer TR, Caufield PW, Cui Y, Zhang H, O'Toole PW. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nat Commun* **2015**, 6:1–13.
102. Horvath P, Coûté-Monvoisin AC, Romero DA, Boyaval P, Fremaux C, Barrangou R. Comparative analysis of CRISPR loci in lactic acid bacteria genomes. *Int J Food Microbiol* **2009**, 131:62–70.
103. Hidalgo-Cantabrana C, O'Flaherty S, Barrangou R. CRISPR-based engineering of next-generation lactic acid bacteria. *Curr Opin Microbiol* **2017**, 37:79–87.
104. Hidalgo-Cantabrana C, Goh YJ, Pan M, Sanozky-Dawes R, Barrangou R. Genome editing using the endogenous type I CRISPR-Cas system in *Lactobacillus crispatus*. *Proc Natl Acad Sci* **2019**, 116(32):15774–83.
105. Filannino P, Di Cagno R, Gobbetti M. Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. *Curr Opin Biotechnol* **2018**, 49:64–72.
106. Mazzoli R, Bosco F, Mizrahi I, Bayer EA, Pessione E. Towards lactic acid bacteria-based biorefineries. *Biotechnol Adv* **2014**, 32(7):1216–36.
107. Lübeck M, Lübeck PS. Application of lactic acid bacteria in green biorefineries. *FEMS Microbiol Lett* **2019**, 366(3):1–8.
108. Tarraran L, Mazzoli R. Alternative strategies for lignocellulose fermentation through lactic acid bacteria: The state of the art and perspectives. *FEMS Microbiol Lett*. **2018**, 365:1–14.
109. Sauer M, Russmayer H, Grabherr R, Peterbauer CK, Marx H. The Efficient Clade: Lactic Acid Bacteria for Industrial Chemical Production. *Trends Biotechnol* **2017**, 35(8):756–69.
110. Juturu V, Wu JC. Microbial production of lactic acid: the latest development. *Crit Rev Biotechnol* **2016**, 36(6):967–77.
111. Gandini C, Tarraran L, Kalemasi D, Pessione E, Mazzoli R. Recombinant *Lactococcus lactis* for efficient conversion of cellodextrins into L-lactic acid. *Biotechnol Bioeng* **2017**, 114(12):2807–17.
112. Liu Q, Shao T, Dong Z, Bai Y. Solution for promoting *egl3* gene of *Trichoderma reesei* high-efficiency secretory expression in Escherichia coli and *Lactococcus lactis*. *Process Biochem* **2017**, 62:135–43.
113. Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H, Kondo A. Homo-D-lactic acid fermentation from arabinose by redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Appl Environ Microbiol* **2009**, 75(15):5175–8.
114. Okano K, Yoshida S, Yamada R, Tanaka T, Ogino C, Fukuda H, Kondo A. Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Appl Environ Microbiol* **2009**, 75(24):7858–61.

115. Okano K, Hama S, Kihara M, Noda H, Tanaka T, Kondo A. Production of optically pure D-lactic acid from brown rice using metabolically engineered *Lactobacillus plantarum*. *Appl Microbiol Biotechnol* **2017**, 101(5):1869–75.
116. Stern J, Moraïs S, Ben-David Y, Salama R, Shamshoum M, Lamed R, Shoham Y, Bayer EA, Mizrahi I. Assembly of synthetic functional cellulosomal structures onto the cell surface of *Lactobacillus plantarum*, a potent member of the gut microbiome. *Appl Environ Microbiol* **2018**, 84(8):1–14.
117. Cubas-Cano E, González-Fernández C, Tomás-Pejó E. Evolutionary engineering of *Lactobacillus pentosus* improves lactic acid productivity from xylose-rich media at low pH. *Bioresour Technol* **2019**, 288:1–9.
118. Boguta AM, Bringel F, Martinussen J, Jensen PR. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. *Microb Cell Fact* **2014**, 13(97):1–16.
119. Garvie EI. Bacterial lactate dehydrogenases. *Microbiol Rev* **1980**, 44(1):106–39.
120. Patel M, Crank M, Dornburg V, Hermann B, Roes L, Hüsling B, Overbeek L, Terragni F, Recchia E. Medium and Long-term Opportunities and Risks of the Biotechnological Production of Bulk Chemicals from Renewable Resources - The BREW Project. **2006**.
121. de Man JC. The formation of diacetyl and acetoin from α-acetolactic acid. *Recl des Trav Chim des Pays-Bas* **1959**, 78(7):480–6.
122. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. *Metab Eng* **2016**, 36:57–67.
123. Monnet C, Schmitt P, Divies C. Development and Use of a Screening Procedure for Production of α-Acetolactate by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis Strains. *Appl Environ Microbiol* **1997**, 63(2):793–5.
124. Goupil N, Corthier G, Ehrlich SD, Renault P. Imbalance of leucine flux in *Lactococcus lactis* and its use for the isolation of diacetyl-overproducing strains. *Appl Environ Microbiol* **1996**, 62(7):2636–40.
125. Monnet C, Aymes F, Corrieu G. Diacetyl and α-acetolactate overproduction by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis mutants that are deficient in α-acetolactate decarboxylase and have a low lactate dehydrogenase activity. *Appl Environ Microbiol* **2000**, 66(12):5518–20.
126. Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholtz J, Delcour J, De Vos WM. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. *Nat Biotechnol* **1999**, 17(6):588–92.
127. Zhou L, Deng C, Cui WJ, Liu ZM, Zhou ZM. Efficient L-Alanine Production by a Thermo-Regulated Switch in *Escherichia coli*. *Appl Biochem Biotechnol* **2016**, 178(2):324–37.
128. Jojima T, Fujii M, Mori E, Inui M, Yukawa H. Engineering of sugar metabolism of *Corynebacterium glutamicum* for production of amino acid L-alanine under oxygen deprivation. *Appl Microbiol Biotechnol* **2010**, 87(1):159–65.
129. Chaves ACSD, Fernandez M, Lerayer ALS, Mierau I, Kleerebezem M, Hugenholtz J. Metabolic Engineering of Acetaldehyde Production by *Streptococcus thermophilus*. *Appl Environ Microbiol* **2002**, 68(11):135–53.
130. Bongers RS, Hoefnagel MHN, Kleerebezem M. High-Level Acetaldehyde Production in *Lactococcus lactis* by Metabolic Engineering. *Appl Environ Microbiol* **2005**, 71(2):1109–13.
131. Liu S, Dien BS, Cotta MA. Functional expression of bacterial Zymobacter palmae pyruvate decarboxylase gene in *Lactococcus lactis*. *Curr Microbiol* **2005**, 50(6):324–8.
132. Liu J, Dantoft SH, Würtz A, Jensen PR, Solem C. A novel cell factory for efficient production of ethanol from dairy waste. *Biotechnol Biofuels* **2016**, 9:33.
133. Petersen KV, Liu J, Chen J, Martinussen J, Jensen PR, Solem C. Metabolic characterization and transformation of the non-dairy *Lactococcus lactis* strain KF147, for production of ethanol from xylose. *Biotechnol J* **2017**, 12:1–10.

134. Zhu C, Shen T, Liu D, Wu J, Chen Y, Wang L, Guo K, Ying H, Ouyang P. Production of liquid hydrocarbon fuels with acetoin and platform molecules derived from lignocellulose. *Green Chem* **2016**, 18:2165–74.
135. Long HF, Hammer BW. Classification of the organisms important in dairy products I. *Streptococcus liquefaciens*. *Res Bull - Iowa Agric Home Econ Exp Stn* **1936**, 18(206):219–50.
136. Starrenburg MJC, Hugenholtz J. Citrate fermentation by *Lactococcus* and *Leuconostoc* spp. *Appl Environ Microbiol* **1991**, 57(12):3535–40.
137. Laëtitia G, Pascal D, Yann D. The Citrate Metabolism in Homo- and Heterofermentative LAB: A Selective Means of Becoming Dominant over Other Microorganisms in Complex Ecosystems. *Food Nutr Sci* **2014**, 5(10):953–69.
138. Liu J, Solem C, Jensen PR. Integrating biocompatible chemistry and manipulating cofactor partitioning in metabolically engineered *Lactococcus lactis* for fermentative production of (3S)-acetoin. *Biotechnol Bioeng* **2016**, 113(12):2744–8.
139. Nadal I, Rico J, Pérez-Martínez G, Yebra MJ, Monedero V. Diacetyl and acetoin production from whey permeate using engineered *Lactobacillus casei*. *J Ind Microbiol Biotechnol* **2009**, 36(9):1233–7.
140. Ji XJ, Huang H, Ouyang PK. Microbial 2,3-butanediol production: A state-of-the-art review. *Biotechnol Adv* **2011**, 29(3):351–64.
141. Jensen PR, Hammer K. Minimal Requirements for Exponential-Growth of *Lactococcus lactis*. *Appl Environ Microbiol* **1993**, 59(12):4363–6.
142. Leblanc JG, Laiño JE, del Valle MJ, Vannini V, van Sinderen D, Taranto MP, de Valdez GF, de Giori GS, Sesma F. B-Group vitamin production by lactic acid bacteria - current knowledge and potential applications. *J Appl Microbiol* **2011**, 111(6):1297–309.
143. Alm L. Effect of Fermentation on B-Vitamin Content of Milk in Sweden. *J Dairy Sci* **1982**, 65(3):353–9.
144. Shahani KM, Chandan RC. Nutritional and Healthful Aspects of Cultured and Culture-Containing Dairy Foods. *J Dairy Sci* **1979**, 62(10):1685–94.
145. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Curr Opin Biotechnol* **2013**, 24(2):160–8.
146. Sybesma W, Burgess C, Starrenburg M, Van Sinderen D, Hugenholtz J. Multivitamin production in *Lactococcus lactis* using metabolic engineering. *Metab Eng* **2004**, 6(2):109–15.
147. Chen J, Vestergaard M, Glasdam Jensen T, Shen J, Dufva M, Solem C, Ruhdal Jensen P. Finding the Needle in the Haystack — the Use of Microfluidic Droplet Technology to Identify Vitamin-Secreting Lactic Acid Bacteria. *MBio* **2017**, 8(3):1–12.
148. Saubade F, Hemery YM, Guyot JP, Humblot C. Lactic acid fermentation as a tool for increasing the folate content of foods. *Crit Rev Food Sci Nutr* **2017**, 57(18):3894–910.
149. Laiño JE, LeBlanc JG, de Giori GS. Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal argentinean yogurts. *Can J Microbiol* **2012**, 58(5):581–8.
150. Wegkamp A, Van Oorschot W, De Vos WM, Smid EJ. Characterization of the role of para-aminobenzoic acid biosynthesis in folate production by *Lactococcus lactis*. *Appl Environ Microbiol* **2007**, 73(8):2673–81.
151. Wegkamp A, Mars AE, Faijes M, Molenaar D, de Vos RCH, Klaus SMJ, Hanson AD, de Vos WM, Smid EJ. Physiological responses to folate overproduction in *Lactobacillus plantarum* WCFS1. *Microb Cell Fact* **2010**, 9(100):1–14.
152. Zhang Y, Liu JZ, Huang JS, Mao ZW. Genome shuffling of *Propionibacterium shermanii* for improving vitamin B12 production and comparative proteome analysis. *J Biotechnol* **2010**, 148(2–3):139–43.
153. Taranto MP, Vera JL, Hugenholtz J, Valdez GF De, Sesma F. *Lactobacillus reuteri* CRL1098 Produces Cobalamin. *J Bacteriol* **2003**, 185(18):5643–7.

154. Torres AC, Vannini V, Font G, Saavedra L, Taranto MP. Novel Pathway for Corrinoid Compounds Production in *Lactobacillus*. *Front Microbiol* **2018**, 9:1–9.
155. Bhushan B, Tomar SK, Chauhan A. Techno-functional differentiation of two vitamin B12producing *Lactobacillus plantarum* strains: an elucidation for diverse future use. *Appl Microbiol Biotechnol* **2017**, 101(2):697–709.
156. Li P, Gu Q, Yang L, Yu Y, Wang Y. Characterization of extracellular vitamin B12 producing *Lactobacillus plantarum* strains and assessment of the probiotic potentials. *Food Chem* **2017**, 234:494–501.
157. Li P, Gu Q, Wang Y, Yu Y, Yang L, Chen J V. Novel vitamin B12-producing *Enterococcus* spp. and preliminary in vitro evaluation of probiotic potentials. *Appl Microbiol Biotechnol* **2017**, 101(15):6155–64.
158. Liu Y, van Bennekom EO, Zhang Y, Abee T, Smid EJ. Long-chain vitamin K2 production in *Lactococcus lactis* is influenced by temperature, carbon source, aeration and mode of energy metabolism. *Microb Cell Fact* **2019**, 18(129):1–14.
159. Livesey G. Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutr Res Rev* **2003**, 16(2):163–91.
160. Aarnikunnas J, Von Weymarn N, Rönnholm K, Leisola M, Palva A. Metabolic engineering of Lactobacillus fermentum for production of mannitol and pure L-lactic acid or pyruvate. *Biotechnol Bioeng* **2003**, 82(6):653–63.
161. Ferain T, Schanck AN, Delcour J. 13C nuclear magnetic resonance analysis of glucose and citrate end products in an ldhL-ldhD double-knockout strain of *Lactobacillus plantarum*. *J Bacteriol* **1996**, 178(24):7311–5.
162. Neves AR, Ramos A, Shearman C, Gasson MJ, Almeida JS, Santos H. Metabolic characterization of *Lactococcus lactis* deficient in lactate dehydrogenase using in vivo 13C-NMR. *Eur J Biochem* **2000**, 267(12):3859–68.
163. Gaspar P, Neves AR, Ramos A, Gasson MJ, Shearman CA, Santos H. Engineering *Lactococcus lactis* for Production of Mannitol: High Yields from Food-Grade Strains Deficient in Lactate Dehydrogenase and the Mannitol Transport System. *Appl Environ Microbiol* **2004**, 70(3):1466–74.
164. Monedero V, Pérez-Martínez G, Yebra MJ. Perspectives of engineering lactic acid bacteria for biotechnological polyol production. *Appl Microbiol Biotechnol* **2010**, 86(4):1003–15.
165. Nissen L, Pérez-Martínez G, Yebra MJ. Sorbitol synthesis by an engineered *Lactobacillus casei* strain expressing a sorbitol-6-phosphate dehydrogenase gene within the lactose operon. *FEMS Microbiol Lett* **2005**, 249(1):177–83.
166. Ladero V, Ramos A, Wiersma A, Goffin P, Schanck A, Kleerebezem M, Hugenholtz J, Smid EJ, Hols P. High-level production of the low-calorie sugar sorbitol by *Lactobacillus plantarum* through metabolic engineering. *Appl Environ Microbiol* **2007**, 73(6):1864–72.
167. De Boeck R, Sarmiento-Rubiano LA, Nadal I, Monedero V, Pérez-Martínez G, Yebra MJ. Sorbitol production from lactose by engineered *Lactobacillus casei* deficient in sorbitol transport system and mannitol-1-phosphate dehydrogenase. *Appl Microbiol Biotechnol* **2010**, 85(6):1915–22.
168. Ortiz ME, Bleckwedel J, Raya RR, Mozzi F. Biotechnological and in situ food production of polyols by lactic acid bacteria. *Appl Microbiol Biotechnol* **2013**, 97(11):4713–26.
169. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **2000**, 289(5483):1352–5.
170. Robinson K, Chamberlain LM, Schofield KM, Wells JM, Page RWF Le. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat Biotechnol* **1997**, 15:653–7.
171. Plavec TV, Berlec A. Engineering of lactic acid bacteria for delivery of therapeutic proteins and peptides. *Appl Microbiol Biotechnol* **2019**, 103(5):2053–66.

172. Del Rio B, Redruello B, Fernandez M, Cruz Martin M, Ladero V, Alvarez MA. Lactic acid bacteria as a live delivery system for the in situ production of nanobodies in the human gastrointestinal tract. *Front Microbiol* **2019**, 9:1–16.
173. Mays ZJ, Nair NU. Synthetic biology in probiotic lactic acid bacteria: At the frontier of living therapeutics. *Curr Opin Biotechnol* **2018**, 53:224–31.
174. Ng DTW, Sarkar CA. Engineering signal peptides for enhanced protein secretion from *Lactococcus lactis*. *Appl Environ Microbiol* **2013**, 79(1):347–56.
175. Pedersen MB, Jensen PR, Janzen T, Nilsson D. Bacteriophage resistance of a ΔthyA mutant of *Lactococcus lactis* blocked in DNA replication. *Appl Environ Microbiol* **2002**, 68(6):3010–23.
176. Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat Biotechnol* **2003**, 21(7):785–9.
177. Chan CTY, Lee JW, Cameron DE, Bashor CJ, Collins JJ. “Deadman” and “Passcode” microbial kill switches for bacterial containment. *Nat Chem Biol* **2016**, 12(2):82–6.
178. Borrero J, Chen Y, Dunny GM, Kaznessis YN. Modified lactic acid bacteria detect and inhibit multiresistant enterococci. *ACS Synth Biol*. **2015**, 4(3):299–306.
179. Hudson GA, Mitchell DA. RiPP antibiotics: biosynthesis and engineering potential. *Curr Opin Microbiol* **2018**, 45:61–9.
180. Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP. Bacteriocins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol* **2016**, 100(7):2939–51.
181. Repka LM, Chekan JR, Nair SK, Van Der Donk WA. Mechanistic Understanding of Lanthipeptide Biosynthetic Enzymes. *Chem Rev* **2017**, 117(8):5457–520.
182. López-Cuellar M del R, Rodríguez-Hernández AI, Chavarría-Hernández N. LAB bacteriocin applications in the last decade. *Biotechnol Biotechnol Equip* **2016**, 30(6):1039–50.
183. Ahmad V, Khan MS, Jamal QMS, Alzohairy MA, Al Karaawi MA, Siddiqui MU. Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *Int J Antimicrob Agents* **2017**, 49:1–11.
184. Yu Y, Mukherjee S, Van Der Donk WA. Product formation by the promiscuous lanthipeptide synthetase ProcM is under kinetic control. *J Am Chem Soc* **2015**, 137(15):5140–8.
185. Juturu V, Wu JC. Microbial production of bacteriocins: Latest research development and applications. *Biotechnol Adv* **2018**, 36(8):2187–200.
186. Zheng Z, Zhu Y, Zhou B, Chen M. Screening of High-Yield Nisin-Producing *Lactococcus lactis* Mutants Using Adaptive Mutation Methods. *Ind Biotechnol* **2016**, 12(6):350–6.
187. Martín M, Gutiérrez J, Criado R, Herranz C, Cintas LM, Hernández PE. Cloning, production and expression of the bacteriocin enterocin a produced by *Enterococcus faecium* PLBC21 in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **2007**, 76(3):667–75.
188. Jiménez JJ, Diep DB, Borrero J, Gútiez L, Arbulu S, Nes IF, Herranz C, Cintas LM, Hernández PE. Cloning strategies for heterologous expression of the bacteriocin enterocin A by *Lactobacillus sakei* Lb790, *Lb. plantarum* NC8 and *Lb. casei* CECT475. *Microb Cell Fact* **2015**, 14(166):1–11.
189. Yuan J, Zhang ZZ, Chen XZ, Yang W, Huan LD. Site-directed mutagenesis of the hinge region of nisinZ and properties of nisinZ mutants. *Appl Microbiol Biotechnol* **2004**, 64(6):806–15.
190. Tominaga T, Hatakeyama Y. Development of innovative pediocin PA-1 by DNA shuffling among class IIa bacteriocins. *Appl Environ Microbiol* **2007**, 73(16):5292–9.
191. Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *Br J Exp Pathol* **1929**, 10(3):226–36.
192. Schmitt S, Montalbán-López M, Peterhoff D, Deng J, Wagner R, Held M, Kuipers OP, Panke S. Analysis of modular bioengineered antimicrobial lanthipeptides at nanoliter scale. *Nat Chem Biol* **2019**, 15(5):437–43.

193. Kong W, Kapuganti VS, Lu T. A gene network engineering platform for lactic acid bacteria. *Nucleic Acids Res* **2015**, 44(4):1–10.
194. Maconi E, Aragozzini F. Stereoselective reduction of non-cyclic ketones with lactic acid bacteria. *Appl Microbiol Biotechnol* **1989**, 31:29–31.
195. Wallen LL, Stodola FH, Jackson RW. Type reactions in fermentation chemistry. United States Department of Agriculture. **1959**.
196. Aragozzini F, Valenti M, Santaniello E, Ferraboschi P, Grisenti P. Biocatalytic, enantioselective preparations of (*R*)- and (*S*)-ethyl 4-chloro-3-hydroxybutanoate, a useful chiral synthon. *Biocatal Biotransformation* **1992**, 5(4):325–32.
197. Yilmaz D, Şahin E, Dertli E. Highly Enantioselective Production of Chiral Secondary Alcohols Using *Lactobacillus paracasei* BD101 as a New Whole Cell Biocatalyst and Evaluation of Their Antimicrobial Effects. *Chem Biodivers* **2017**, 14(11):1–8.
198. Şahin E, Serencam H, Dertli E. Production of enantiomerically pure (*S*)-phenyl(pyridin-2-yl)methanol with *Lactobacillus paracasei* BD101. *Biocatal Biotransformation* **2019**, 37(6):448–54.
199. Perna FM, Ricci MA, Scilimati A, Mena MC, Pisano I, Palmieri L, Agrimi G, Vitale P. Cheap and environmentally sustainable stereoselective arylketones reduction by *Lactobacillus reuteri* whole cells. *J Mol Catal B Enzym* **2016**, 124:29–37.
200. Vitale P, Perna FM, Agrimi G, Pisano I, Mirizzi F, Capobianco RV, Capriati V. Whole-cell biocatalyst for chemoenzymatic total synthesis of rivastigmine. *Catalysts* **2018**, 8(2):1–12.
201. Pfruender H, Amidjojo M, Kragl U, Weuster-Botz D. Efficient whole-cell biotransformation in a biphasic ionic liquid/water system. *Angew Chemie - Int Ed* **2004**, 43(34):4529–31.
202. Amidjojo M, Weuster-Botz D. Asymmetric synthesis of the chiral synthon ethyl (*S*)-4-chloro-3- hydroxybutanoate using *Lactobacillus kefir*. *Tetrahedron Asymmetry* **2005**, 16(4):899–901.
203. Pflügl S, Marx H, Mattanovich D, Sauer M. 1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*. *Bioresour Technol* **2012**, 119:133–40.
204. Talarico TL, Casas IA, Chung TC, Dobrogosz WJ. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrob Agents Chemother* **1988**, 32(12):1854–8.
205. Sriramulu DD, Liang M, Hernandez-Romero D, Raux-Deery E, Lünsdorf H, Parsons JB, Warren MJ, Prentice MB. *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. *J Bacteriol* **2008**, 190(13):4559–67.
206. Saxena RK, Anand P, Saran S, Isar J. Microbial production of 1,3-propanediol: Recent developments and emerging opportunities. *Biotechnol Adv* **2009**, 27(6):895–913.
207. Russmayer H, Egermeier M, Kalemasi D, Sauer M. Spotlight on biodiversity of microbial cell factories for glycerol conversion. *Biotechnol Adv* **2019**, 37:1–15.
208. van Pijkeren JP, Neoh KM, Sirias D, Findley AS, Britton RA. Exploring optimization parameters to increase ssDNA recombineering in *Lactococcus lactis* and *Lactobacillus reuteri*. *Bioengineered* **2012**, 3(4):209–17.
209. Dishisha T, Pereyra LP, Pyo S, Britton RA, Hatti-kaul R. Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of acid and 1,3-propanediol from glycerol. *Microb Cell Fact* **2014**, 13(76):1–10.
210. Gopal Ramakrishnan G, Nehru G, Suppuram P, Balasubramaniyam S, Gulab BR, Subramanian R. Bio-transformation of glycerol to 3-hydroxypropionic acid using resting cells of *Lactobacillus reuteri*. *Curr Microbiol* **2015**, 71(4):517–23.
211. Gopi GR, Ganesh N, Pandiaraj S, Sowmiya B, Brajesh RG, Ramalingam S. A study on enhanced expression of 3-hydroxypropionic acid pathway genes and impact on its production in *Lactobacillus reuteri*. *Food Technol Biotechnol* **2015**, 53(3):331–6.
212. Dishisha T, Pyo SH, Hatti-Kaul R. Bio-based 3-hydroxypropionic- and acrylic acid production from biodiesel glycerol via integrated microbial and chemical catalysis. *Microb Cell Fact* **2015**, 14(200):1–11.

213. Vaidyanathan H, Kandasamy V, Ramakrishnan GG, Ramachandran KB, Jayaraman G, Ramalingam S. Glycerol conversion to 1, 3-propanediol is enhanced by the expression of a heterologous alcohol dehydrogenase gene in *Lactobacillus reuteri*. *AMB Express* **2011**, 1(37):1–8.
214. Chen L, Hatti-Kaul R. Exploring *Lactobacillus reuteri* DSM20016 as a biocatalyst for transformation of longer chain 1,2-diols: Limits with microcompartment. *PLoS One* **2017**, 12(9): 1–19.
215. Kang TS, Korber DR, Tanaka T. Bioconversion of glycerol to 1,3-propanediol in thin stillage-based media by engineered *Lactobacillus panis* PM1. *J Ind Microbiol Biotechnol* **2014**, 41(4):629–35.
216. Pflügl S, Marx H, Mattanovich D, Sauer M. Genetic engineering of *Lactobacillus diolivorans*. *FEMS Microbiol Lett* **2013**, 344(2):152–8.
217. Lindlbauer KA, Marx H, Sauer M. Effect of carbon pulsing on the redox household of *Lactobacillus diolivorans* in order to enhance 1,3-propanediol production. *N Biotechnol* **2017**, 34:32–9.
218. Lindlbauer KA, Marx H, Sauer M. 3-Hydroxypropionaldehyde production from crude glycerol by *Lactobacillus diolivorans* with enhanced glycerol uptake. *Biotechnol Biofuels* **2017**, 10(295):1–11.
219. Xu N, Wei L, Liu J. Biotechnological advances and perspectives of gamma-aminobutyric acid production. *World J Microbiol Biotechnol* **2017**, 33(64):1–11.
220. Zhao A, Hu X, Pan L, Wang X. Isolation and characterization of a gamma-aminobutyric acid producing strain *Lactobacillus buchneri* WPZ001 that could efficiently utilize xylose and corncob hydrolysate. *Appl Microbiol Biotechnol* **2015**, 99(7):3191–200.
221. Shi X, Chang C, Ma S, Cheng Y, Zhang J, Gao Q. Efficient bioconversion of l-glutamate to γ-aminobutyric acid by *Lactobacillus brevis* resting cells. *J Ind Microbiol Biotechnol* **2017**, 44:697–704.
222. Kook MC, Seo MJ, Cheigh CI, Lee SJ, Pyun YR, Park H. Enhancement of γ-amminobutyric acid production by *Lactobacillus sakei* B2-16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. *J Appl Biol Chem* **2010**, 53(6):816–20.
223. Lyu C, Zhao W, Peng C, Hu S, Fang H, Hua Y, Yao S, Huang J, Mei L. Exploring the contributions of two glutamate decarboxylase isozymes in *Lactobacillus brevis* to acid resistance and γ-aminobutyric acid production. *Microb Cell Fact* **2018**, 17(180):1–14.
224. Lyu CJ, Liu L, Huang J, Zhao WR, Hu S, Mei LH, Yao SJ. Biosynthesis of γ-aminobutyrate by engineered *Lactobacillus brevis* cells immobilized in gellan gum gel beads. *J Biosci Bioeng* **2019**, 128(2):123–8.
225. Lyu C, Hu S, Huang J, Luo M, Lu T, Mei L, Yao S. Contribution of the activated catalase to oxidative stress resistance and γ-aminobutyric acid production in *Lactobacillus brevis*. *Int J Food Microbiol* **2016**, 238:302–10.
226. Guan J, Han C, Guan Y, Zhang S, Yun J, Yao S. Optimizational production of phenyllactic acid by a *Lactobacillus buchneri* strain via uniform design with overlay sampling methodology. *Chinese J Chem Eng* **2019**, 27(2):418–25.
227. Li X, Jiang B, Pan B. Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus* sp. *Biotechnol Lett* **2007**, 29(4):593–7.
228. Wang H, Sun Y, Chen C, Sun Z, Zhou Y, Shen F, Zhang H, Dai Y. Genome shuffling of *Lactobacillus plantarum* for improving antifungal activity. *Food Control* **2013**, 32(2):341–7.
229. Li M, Meng X, Sun Z, Zhu C, Ji H. Effects of NADH Availability on 3-Phenyllactic Acid Production by *Lactobacillus plantarum* Expressing Formate Dehydrogenase. *Curr Microbiol* **2019**, 76(6):706–12.
230. Zheng Z, Xia M, Fang X, Jiang T, Ouyang J. Enhanced biosynthesis of chiral phenyllactic acid from L-phenylalanine through a new whole-cell biocatalyst. *Bioprocess Biosyst Eng* **2018**, 41(8):1205–12.

231. Speranza G, Corti S, Fontana G, Manitto P, Galli A, Scarpellini M, Chialva F. Conversion of meso-2,3-Butanediol into 2-Butanol by Lactobacilli. Stereochemical and Enzymatic Aspects. *J Agric Food Chem* **1997**, 45(9):3476–80.
232. Ghiaci P, Lameiras F, Norbeck J, Larsson C. Production of 2-butanol through meso-2,3-butanediol consumption in lactic acid bacteria. *FEMS Microbiol Lett* **2014**, 360:70–5.
233. Hviid AMM, Ruhdal-Jensen P, Kilstrup M. Butanol is cytotoxic to *Lactococcus lactis* while ethanol and hexanol are cytostatic. *Microbiol* **2017**, 163(4):453–61.
234. Petrova P, Tsvetanova F, Petrov K. Low cell surface hydrophobicity is one of the key factors for high butanol tolerance of lactic acid bacteria. *Eng Life Sci* **2019**, 19(2):133–42.
235. Berezina O V., Zakharova N V., Brandt A, Yarotsky S V., Schwarz WH, Zverlov V V. Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. *Appl Microbiol Biotechnol* **2010**, 87(2):635–46.
236. Russmayer H, Marx H, Sauer M. Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnol Biofuels* **2019**, 12(1):1–11.
237. Solopova A, van Tilburg AY, Foito A, Allwood JW, Stewart D, Kulakauskas S, Kuipers OP. Engineering *Lactococcus lactis* for the production of unusual anthocyanins using tea as substrate. *Metab Eng* **2019**, 54:160–9.
238. Martínez-Cuesta MC, Gasson MJ, Narbad A. Heterologous expression of the plant coumarate: CoA ligase in *Lactococcus lactis*. *Lett Appl Microbiol* **2005**, 40:44–9.
239. Song AAL, Abdullah JO, Abdullah MP, Shafee N, Rahim RA. Functional expression of an orchid fragrance gene in *Lactococcus lactis*. *Int J Mol Sci* **2012**, 13(2):1582–97.
240. Song AAL, Abdullah JO, Abdullah MP, Shafee N, Othman R, Tan EF, Noor NM, Raha AR. Overexpressing 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGR) in the Lactococcal Mevalonate Pathway for Heterologous Plant Sesquiterpene Production. *PLoS One* **2012**, 7(12):1–10.
241. Hernández I, Molenaar D, Beekwilder J, Bouwmeester H, Vlieg JETVH. Expression of plant flavor genes in *Lactococcus lactis*. *Appl Environ Microbiol* **2007**, 73(5):1544–52.
242. Song AA, Ong Abdullah J, Abdullah MP, Shafee N, Othman R, Noor NM, Rahim RA. Engineering the lactococcal mevalonate pathway for increased sesquiterpene production. *FEMS Microbiol Lett* **2014**, 355(2):177–84.
243. Dorau R, Chen L, Liu J, Jensen PR, Solem C. Efficient production of α-acetolactate by whole cell catalytic transformation of fermentation-derived pyruvate. *Microb Cell Fact* **2019**, 18(1):1–11.

7. Efficient Production of α-Acetolactate by Whole-Cell Catalytic Transformation of Fermentation-Derived Pyruvate

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Dorau R, Chen L, Liu J, Jensen PR, Solem C. Efficient production of α-acetolactate by whole-cell catalytic transformation of fermentation-derived pyruvate. *Microb Cell Fact* **2019**, 18(1):1–11.

7.1 Abstract

Background: Diacetyl provides the buttery aroma in products such as butter and margarine. It can be made via a harsh set of chemical reactions from sugarcane bagasse, however, in dairy products, it is normally formed spontaneously from α-acetolactate, a compound generated by selected lactic acid bacteria in the starter culture used. Due to its bacteriostatic properties, it is difficult to achieve high levels of diacetyl by fermentation. Here we present a novel strategy for producing diacetyl based on whole-cell biocatalysis, which bypasses the toxic effects of diacetyl.

Results: By expressing a robust α-acetolactate synthase (ALS) in a metabolically optimized *Lactococcus lactis* strain, we obtained a whole-cell catalyst (WCC) that efficiently converted pyruvate into α-acetolactate. After process optimization, we achieved a titer for α-acetolactate of 172 ± 2 mM. Subsequently, we used a two-stage production setup, where pyruvate was produced by an engineered *L. lactis* strain and subsequently used as the substrate for the biocatalysis. Using this approach, 122 ± 5 mM and 113 ± 3 mM α-acetolactate could be made from glucose or lactose in dairy waste, respectively. The WCC appeared robust and was fully active in crude fermentation broth containing pyruvate.

Conclusions: An efficient approach for converting sugar into α-acetolactate, via pyruvate, was developed and tested successfully. Due to the anaerobic conditions used for the biotransformation, little diacetyl was generated, and this allowed for efficient biotransformation of pyruvate into α-acetolactate, with the highest titers reported to date. The use of a two-step procedure for producing α-acetolactate, where non-toxic pyruvate first is formed and subsequently converted into α-acetolactate, also simplified the process optimization. We conclude that whole-cell biocatalysis is suitable for converting lactose in dairy waste into α-acetolactate, which favors resource utilization.

7.2 Background

Diacetyl, a generally recognized as safe (GRAS) compound with a pleasant buttery flavor, is widely used in the food industry (1,2). Diacetyl can be made by chemical or biological means. The chemical approach involves high-temperature treatment of sugarcane bagasse under oxygen limiting conditions, and through a series of radical reactions, diacetyl is formed (3). Interestingly, despite the harsh conditions used, the diacetyl recovered in this way is classified as natural.

In traditional dairy products, such as butter, diacetyl arises due to the activity of lactic acid bacteria (LAB). Different LAB, such as *Lactococcus lactis* (*L. lactis*) subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* spp. (4) and various *Lactobacillus* spp. (5,6) are able to generate diacetyl, or, more precisely, its precursor α-acetolactate, when citrate is present. By metabolizing citrate, the LAB achieve a growth advantage, since acid stress is relieved to some extent (7). In *L. lactis* subsp. *lactis* biovar *diacetylactis* citrate is transported into the cell via the citrate permease (CitP) and cleaved by citrate lyase (CL) into acetate and oxaloacetate. The latter is subsequently decarboxylated by oxaloacetate decarboxylase (OD) into pyruvate. Thus, the consumption of citrate increases the pyruvate pool, without increasing the NADH production in the cell. The excess pyruvate is subsequently consumed via the acetoin pathway, α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALD) (**Figure 7.1**). α-Acetolactate, the intermediate in this pathway, is inherently unstable and can either be enzymatically converted into acetoin or undergo spontaneous decarboxylation to diacetyl under aerobic conditions (4,8–11). As a result, α-acetolactate is mainly transformed into acetoin. However, small amounts of diacetyl can also form when oxygen is present. It has been found that the presence of metal ions accelerate the chemical conversion of α-acetolactate into diacetyl, which can be used for industrial diacetyl production (4,12). Even though diacetyl is a very potent flavor molecule, there are some advantages of using α-acetolactate instead, since diacetyl is highly volatile, which complicates its use in food products. To avoid this, α-acetolactate could be added to the products directly, and diacetyl is formed slowly when the product gets in contact with oxygen. Thereby a long-lasting taste is obtained, and high initial concentrations of diacetyl are avoided.

Metabolic engineering has been used to improve diacetyl formation (12–19). Hugenholtz et al. overexpressed Nox in an ALD deficient strain and managed to generate 5.7 mM α-acetolactate and 1.6 mM diacetyl using resting cells (14). We have previously reported the highest diacetyl titer ever achieved for LAB, 95 mM, which was attained using a metabolically rewired strain of *L. lactis,* blocked in all major catabolic pathways except for the one leading to α-acetolactate and growing under respiratory conditions (12). The α-acetolactate accumulated in the fermentation broth was subsequently converted into diacetyl using a metal ion catalyst. The chemical instability of α-acetolactate, however, resulted in diacetyl accumulation in the course of the fermentation, which ultimately affected growth and production of α-acetolactate. Previously it has been found that 10 mM of diacetyl completely inhibits the growth of *L. lactis* (12,14), and it was demonstrated

that the cytoplasmic NADH-oxidase (NOX) was one of the enzymes that is hampered the most by diacetyl (14). Diacetyl affects other enzymes as well, which may explain its strong antimicrobial activity (20). In the earlier work, we relied on respiration to regenerate NAD^+ and successfully generated large amounts of α-acetolactate and diacetyl (12). However, there are some drawbacks to this approach. Firstly, slow diacetyl accumulation took place during the fermentation, probably due to the presence of oxygen, which catalyzes the decomposition of α-acetolactate. Second, hemin adds additional costs to the fermentation process.

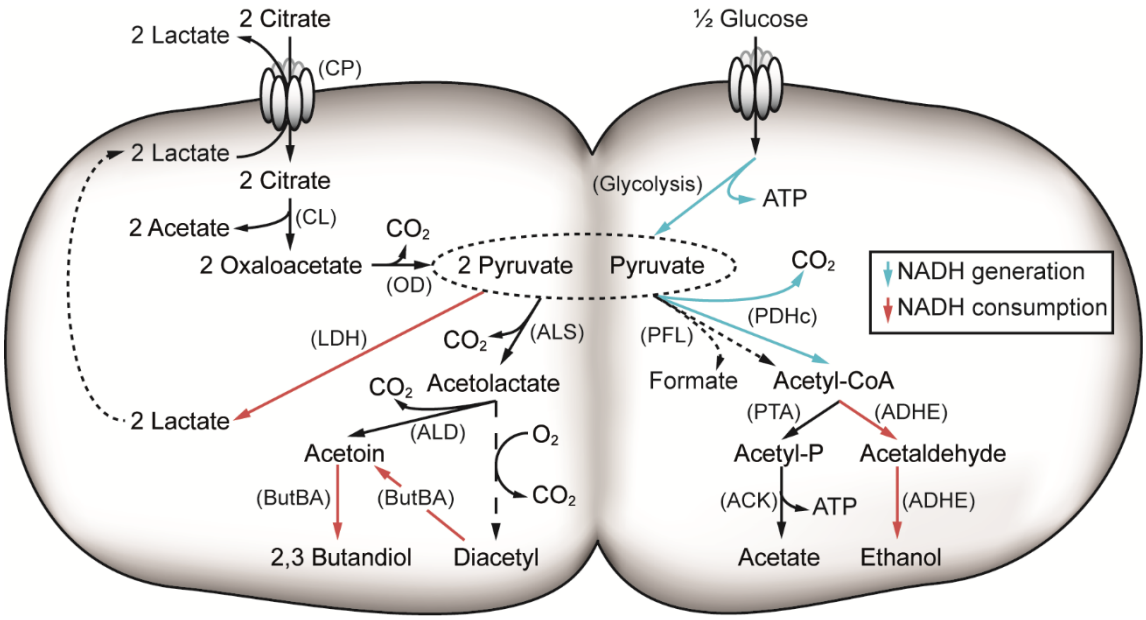
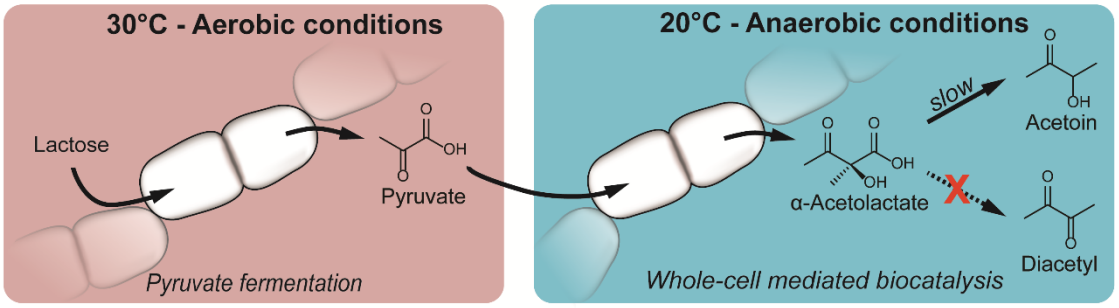


Figure 7.1 Central metabolism of *L. lactis* subsp. *lactis* biovar diacetylactis. Abbreviations for enzymes are CP: citrate permease (CitP); CL: Citrate lyase; OD: Oxaloacetate decarboxylase; ALS: α-Acetolactate synthase; ALD: α-Acetolactate decarboxylase; ButBA: Butanediol dehydrogenase; LDH: Lactate dehydrogenase; PFL: Pyruvate-formate lyase; PDHc: Pyruvate dehydrogenase complex; PTA: Phosphotransacetylase; ACK: Acetate kinase; ADHE: Alcohol dehydrogenase. The NADH generating reactions are illustrated in blue arrows, while the NADH consuming ones are shown in red arrows.

There are few reports describing the use of whole-cell biocatalysis for producing α-acetolactate from glucose or pyruvate (21,22). Using resting cells of an *L. lactis* strain with a deleted α-acetolactate decarboxylase (*aldB*) and an overexpressed NADH-oxidase (*noxE*), 5.7 mM α-acetolactate was obtained (14). In the patent literature, it has been claimed that higher α-acetolactate titers can be obtained from pyruvate using whole cells or cell lysates of *L. lactis* (21,22). It thus appears that the production of α-acetolactate by using whole-cell biocatalysis is possible. However, the existing processes do not seem to be commercially attractive due to low titers. It is, however, plausible that α-acetolactate production using a whole-cell biocatalysis process can be further improved, for example, by optimizing the ALS and by optimizing the production parameters.

Recently, we developed a pyruvate producing *L. lactis* strain, which can accumulate up to 49.7 g/l (564 mM) pyruvate from glucose or lactose under aerobic conditions (Suo et al., manuscript submitted). Under anaerobic conditions, α-acetolactate or pyruvate formation directly from sugar is normally not feasible as the NADH produced during glycolysis needs to be reoxidized, and this usually happens through either lactate formation or mixed-acid formation (23,24). Under aerobic conditions, however, NADH oxidase (NOX) or respiration can reconstitute the NADH/NAD^+ balance, thus allowing the cells to grow (12,23,24) and produce α-acetolactate, but with the drawback that inhibitory amounts of diacetyl can be formed during the fermentation. Here we explore a different strategy for producing α-acetolactate, which is based on whole-cell catalyzed transformation of pyruvate (**Figure 7.2**). We start out by constructing a suitable chassis based on *L. lactis,* then test the effectiveness of different ALS enzymes and finally optimize various parameters for the cell catalyst.

Presented approach: Pyruvate-fermentation and biocatalysis for producing α-acetolactate



Previous approaches: α-Acetolactate-fermentation

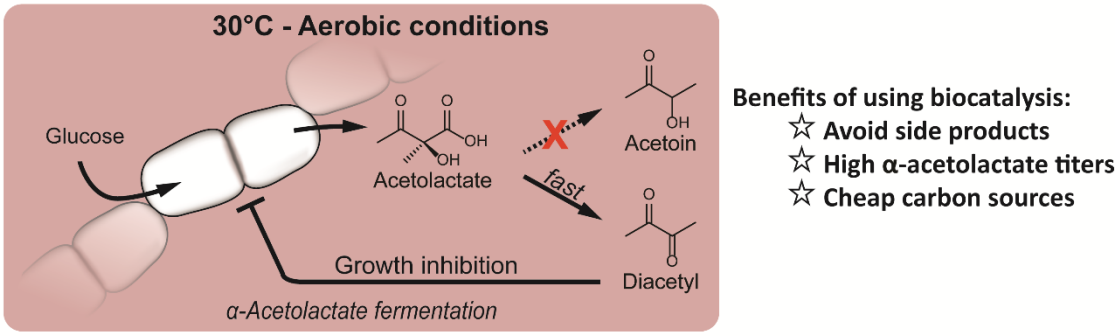


Figure 7.2 Overview of the whole cell catalyzed approach for producing α-acetolactate, which is explored in this study (upper row). Previous approaches for producing α-acetolactate directly via fermentation (lower row). Shifting the reaction environment from aerobiosis to anaerobiosis is advantageous for accumulating α-acetolactate, and higher titers can be reached.

7.3 Results

7.3.1 Strain Construction for Whole-Cell Biocatalysis

The inherent instability of α-acetolactate, causing it to decompose to growth-inhibiting diacetyl in the presence of oxygen, makes it a challenge to achieve high titers by aerated fermentation. We envisioned that using a production strategy based on whole cell-based biocatalysis might be a superior approach, for example, by using pyruvate as a substrate. Pyruvate can be obtained via fermentation using cheap carbon sources as described elsewhere (Suo et al., manuscript submitted). Diacetyl can easily be prepared from the resulting α-acetolactate by subsequent aeration, a reaction that can be further accelerated by metal-ion catalysts. In order to be efficient, the whole-cell catalyst (WCC) should possess a high ALS activity, and any genes encoding enzyme activities that could interfere with α-acetolactate production should be inactivated. *L. lactis* subsp. *cremoris* MG1363 is a well-characterized model strain, which we decided to use in this study. Starting from the previously described strain CS4525 (MG1363 *Δldh, ΔldhB ΔldhX Δpta ΔadhE ΔbutBA ΔaldB,* pCS4268) (12), we derived ll-cat01 (MG1363 *ΔldhB ΔldhX Δpta ΔadhE ΔbutBA ΔaldB*) by plasmid curing and selection for anaerobic growth, and in this strain, the chromosomal *ldh* had been restored after a recombination event with the plasmid-encoded *ldh* gene. ll-cat01 satisfied the requirements to be used as a chassis for our WCC.

7.3.2 α-Acetolactate Can Be Obtained from Pyruvate Using an *L. lactis* WCC

As proof of principle, we applied ll-cat01 in a solution containing 500 mM pyruvate buffered with citrate (100 mM) at different pH values in 1 ml volume. We assayed the α-acetolactate concentration during the first 5 h of the reaction using a colorimetric assay. This assay allows for the determination of the acetoin concentration after converting α-acetolactate to acetoin under acidic conditions. As α-acetolactate decarboxylase had been knocked out and there is no other cofactor independent metabolic pathway, we expected pyruvate to be solely converted into α-acetolactate. We did observe the formation of α-acetolactate, however, not at low pH (**Figure 7.3**). At pH 4.5, the catalyst appeared inactive, and no product was formed. At higher pH, α-acetolactate was formed, and the maximum titer was seen after 5 h at pH 5.5 (20.6 ± 0.3 mM). Since pH increases when two equivalents of acidic pyruvate (pKa = 2.5) are converted into one equivalent less acidic α-acetolactate (pKa = 3.45), it is likely that higher titers could have been obtained if the pH was controlled better, and this would explain the lower activity of the cell catalyst at pH 6.0.

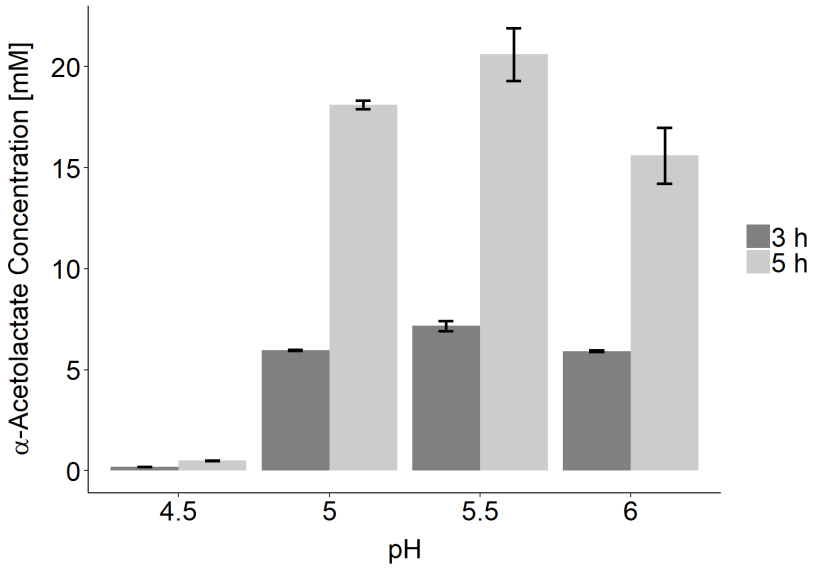


Figure 7.3 α-Acetolactate is obtained from pyruvate (500 mM) using ll-cat01 in citrate-buffered reaction solutions (pH 4.5, 5.0, 5.5 & 6.0). Concentrations after 3 h (dark) and 5 h (light) are shown.

7.3.3 Optimization of the Biocatalysis Process with Different ALS

The catalytic efficiency of ALS is likely to play a crucial role in the efficiency of the biocatalysis process. Therefore, enhancing the ALS activity is important for optimizing the biocatalytic process and can be done in different ways, for example, by overexpressing ALS or lowering the K_m by overexpressing foreign ALS. We decided to overexpress three different ALS enzymes, the native ALS (ll-ALS), ALS from *Bacillus licheniformis* WX-02 (bl-ALS) (25) and ALS from *Enterococcus faecalis* (ef-ALS) (26), where the latter two have been reported to be robust enzymes with high affinity towards pyruvate (**Table S 7.2**). The three enzymes were overexpressed individually in the strain ll-cat01 using the same expression cassette, resulting in strains RD04 (ll-ALS), RD05 (bl-ALS) and RD06 (ef-ALS). As a negative control, the empty plasmid (pLC0) was transformed into ll-cat01, resulting in RD03.

The optimal pH for the four strains RD03, RD04, RD05, and RD06, containing the four plasmids pLC0, pLC17, pLC02, pLC01, respectively, was determined using pH-monitoring (see **Section 7.6.5**), which was initially established and verified with ll-cat01 using different pH at the beginning of the reaction (See **Figure S 7.1**). All four strains showed “S”-shapes when monitoring pH over time, indicating the functional expression of the enzymes (**Figure 7.4, A**). Moreover, the curves differed significantly from each other: While the shape for RD03 and RD05 was rather flat, the shape for RD04 and RD06 showed a very sharp increase in activity early in the experiment. The difference in shape indicates altered reaction rates, which becomes more obvious when plotting the ΔpH versus either time or pH (**Figure 7.4, B** and **C**). The peaks in the corresponding curves show the time or pH when the consumption rate of pyruvate is highest, and the magnitude gives an indication of the overall maximum activity. The data indicates that RD06 performs best

under the given condition, followed by RD04, RD05, and RD03. This trend was confirmed in small scale biotransformations, where the pH was kept constant using a buffer (**Figure S 7.2**). Surprisingly, all four strains showed a very similar optimal pH between 5.5 and 5.6 (**Figure 7.4, C**). We chose RD06 for subsequent scale-up experiments because of its superior performance.

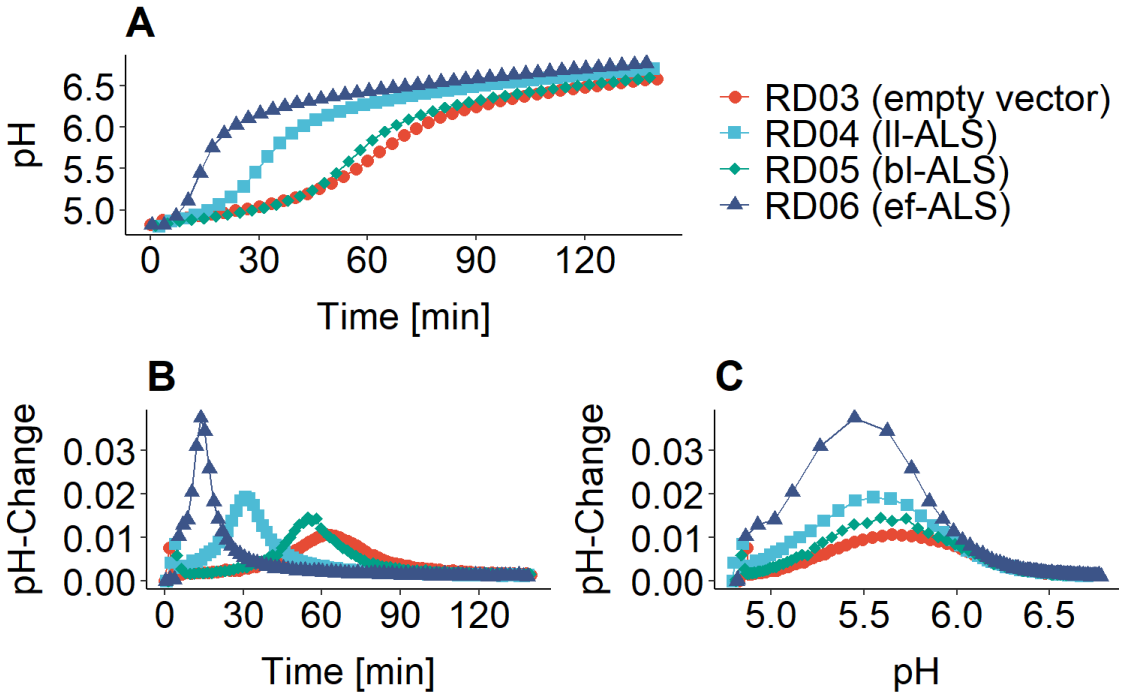


Figure 7.4 Comparison of the four strains RD03-RD06, derived from the same host ll-cat01 containing the plasmids pLC0, pLC17, pLC02, pLC01, as WCCs for the conversion of pyruvate to α-acetolactate. RD03-RD06 were incubated in an unbuffered solution containing 0.5 M pyruvate at pH 4.6 at OD_{600} = 1.0. RD03 contains the empty cloning vector as a negative control (circles). RD04-RD06 contain plasmids overexpressing different ALS enzymes from L. lactis (ll-ALS) (squares), B. licheniformis WX-02 (bl-ALS) (diamonds) and E. faecalis (ef-ALS) (triangles). A: pH-profile (pH against time [min]. B: pH-change as an indicator for the catalytic activity. Because of the generally small errors, the error bars are not indicated here. C: pH-change against pH.

7.3.4 Low Temperature and High Cell Density Are Beneficial for α-Acetolactate Accumulation

It is well known that α-acetolactate spontaneously decarboxylates in aqueous solution and forms acetoin or diacetyl depending on the presence or absence of oxygen, respectively. The process can be accelerated in an acidic environment and proceeds faster at high temperature (10,11). To determine the optimal temperature for α-acetolactate accumulation using RD06, the stability of α-acetolactate under different conditions was evaluated. The initial concentration of α-acetolactate was determined to be approximately 83 mM. Generally, it was observed that α-acetolactate mainly decarboxylates into acetoin, but other unknown side products are formed as well, especially at high temperatures. **Figure 7.5** shows that increasing temperatures and

prolonged reaction time are suboptimal for the accumulation of α-acetolactate. At 50°C, already after 6 h, only 19.1 ± 1.4 mM of the initial 83 mM α-acetolactate remained (23 ± 2%), and after 24 h at 40°C and 50°C only 8.6 ± 0.2 mM (10.4 ± 0.1%) and 2.0 ± 0.4 mM (2.5 ± 0.4%) remained, respectively. When comparing the lower temperatures, 20°C was found to be suitable since at 30°C after 24 h, already more than half of the α-acetolactate had been degraded. We decided to apply the WCC at 20°C at laboratory scale in order to stabilize α-acetolactate and increase the WCC concentration to compensate for decreased reaction rates. We found a linear correlation between cell density and α-acetolactate production (**Figure S 7.3**) and concluded that higher cell densities indeed could be applied to compensate for lower reaction rates caused by lower temperatures. To validate this hypothesis, we determined the α-acetolactate formation rate [mM/min] of RD06 at different temperatures from 15°C to 30°C (**Figure S 7.4**). In this temperature range, a nearly linear correlation was observed, and we demonstrated that increased cell density could compensate for the lower α-acetolactate formation rate when using lower temperatures (for example, 20°C). Higher cell densities might also compensate for increased α-acetolactate degradation at higher temperatures when shorter reaction times are applied.

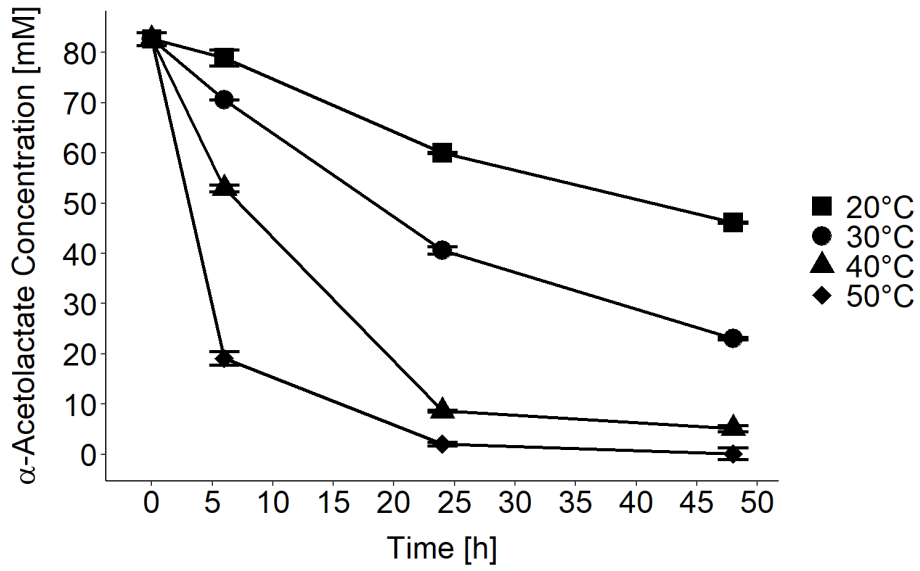


Figure 7.5 Stability of α-acetolactate under different temperatures (20°C (squares), 30°C (circles), 40°C (triangles) and 50°C (diamonds)) and in the presence of 500 mM pyruvate and 100 mM citrate at pH 5.5. α-Acetolactate decarboxylates to mainly form acetoin but also other side products.

7.3.5 Biotransformation under Optimized Conditions

As mentioned above, α-acetolactate rapidly decomposes into diacetyl in the presence of oxygen. Therefore, the following laboratory-scale biotransformations were carried out under anaerobic conditions. For the bioconversion, we applied the optimal conditions established above (pH 5.5, 20°C) and the most efficient catalyst (RD06). The consumption of pyruvate, as well as the production of α-acetolactate and acetoin, were followed over a period of 72 h (**Figure 7.6**). The

biotransformation reached its optimal state after 30 h when the α-acetolactate concentration was at a maximum (172 ± 2mM), and 472 ± 40 mM pyruvate had been consumed. We found that longer reaction times resulted in a loss of α-acetolactate because of spontaneous decarboxylation into acetoin. At the maximal α-acetolactate concentration the yield from pyruvate was 73 ± 9% (mol α-acetolactate/0.5*mol pyruvate consumed), and only smaller amounts of acetoin (23 ± 1 mM) and unknown side products were observed using HPLC (**Figure S 7.6** – **Figure S 7.8**). Furthermore, during the biotransformation, we observed a slight decrease in cell density, which might explain the decreased α-acetolactate formation rate in the later stages of the reaction (**Figure S 7.9**).

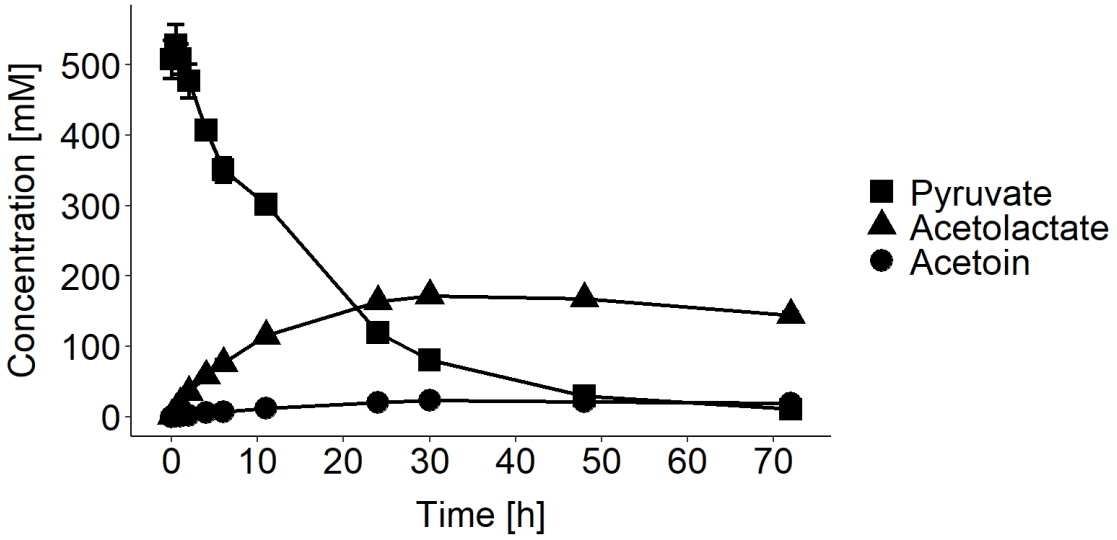


Figure 7.6 Production of α-acetolactate from pyruvate using RD06 as a whole-cell catalyst. The concentrations of pyruvate (squares), α-acetolactate (triangles), and acetoin (diamonds) are shown [mM].

7.3.6 Acetolactate Production Using a Dairy Side-Stream as Feedstock

We have demonstrated that our cell catalyst can transform a buffered solution of pure pyruvate efficiently into α-acetolactate. However, we do not know if the cell catalyst is active in spent fermentation broth containing pyruvate. Since we recently have generated *L. lactis* strains able to ferment either glucose or lactose into pyruvate, we decided to investigate this. The two strains FS1072 and FS1080, able to metabolize glucose or lactose, respectively, were used for the purpose. Two “dirty” pyruvate solutions containing 393 ± 30 mM pyruvate (GM17) and 380 ± 6 mM pyruvate (residual whey permeate, RWP) were produced (Phase I, **Figure 7.7** and **Figure 7.8**). The pyruvate titers obtained using these strains were somewhat lower than previously observed, but since the objective here was to demonstrate the functionality of the cell catalyst, we did not pursue optimization of the pyruvate fermentation, which is described elsewhere (Suo et al., manuscript submitted). Indeed, the cell catalyst turned out to be highly active in the presence of the various components present in the rich media used, which

demonstrates the robustness of the WCC. The α-acetolactate concentrations reached maxima after 30 h in phase II with 122 ± 5 mM or 113 ± 3 mM in GM17 or RWP, respectively. After 30 h, 15.2 ± 0.2 and 13.8 ± 0.9 mM acetoin had been formed in GM17 and RWP, respectively. However, in contrast to when using pure pyruvate, a nearly linear increase in acetoin concentration was observed for both reactions after 30 h. This indicates that α-acetolactate stability is compromised in GM17 and RWP, as the increase in acetoin concentration is directly ascribed to the decomposition of α-acetolactate. To summarize, α-acetolactate yield from pyruvate was 80 ± 3% (mol α-acetolactate/0.5*mol pyruvate consumed) for the reaction in GM17-broth and 76 ± 11% (mol α-acetolactate/0.5*mol pyruvate consumed) in RWP-broth. Compared to the biotransformation using pure pyruvate, the yields were similar. The overall yield from glucose or lactose was 44 ± 6% (mol α-acetolactate/mol glucose consumed) or 43 ± 14% (mol α-acetolactate/2*mol lactose consumed), which are both rather low, because of the non-optimized pyruvate fermentation.

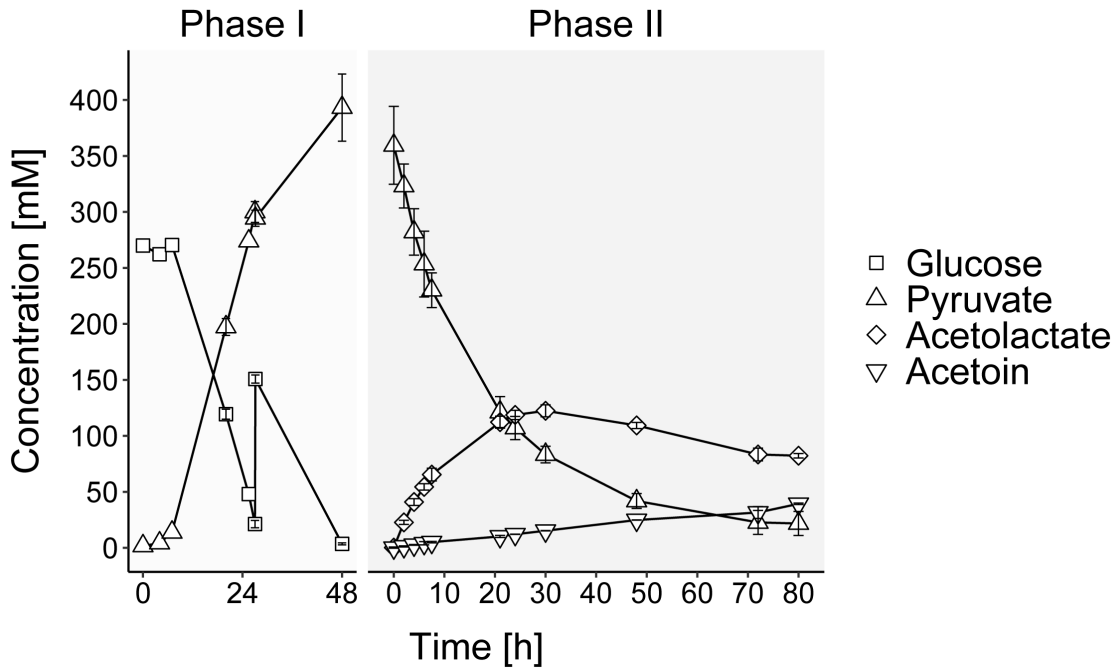


Figure 7.7 Production of α-acetolactate from glucose via pyruvate. Glucose is fermented using FS1072 in M17 broth, and pyruvate is obtained (Phase I). After 74 h, FS1072 was removed from the broth, and RD06 was applied as a whole-cell catalyst for converting pyruvate into α-acetolactate (Phase II). The concentrations of glucose (squares), pyruvate (triangles), α-acetolactate (diamonds), and acetoin (inverted triangle) are shown [mM]. Lactate and acetate were observed at different levels after phase I (81 ± 8 mM and 38 ± 8 mM, respectively), but they remained constant during phase II and are therefore not shown.

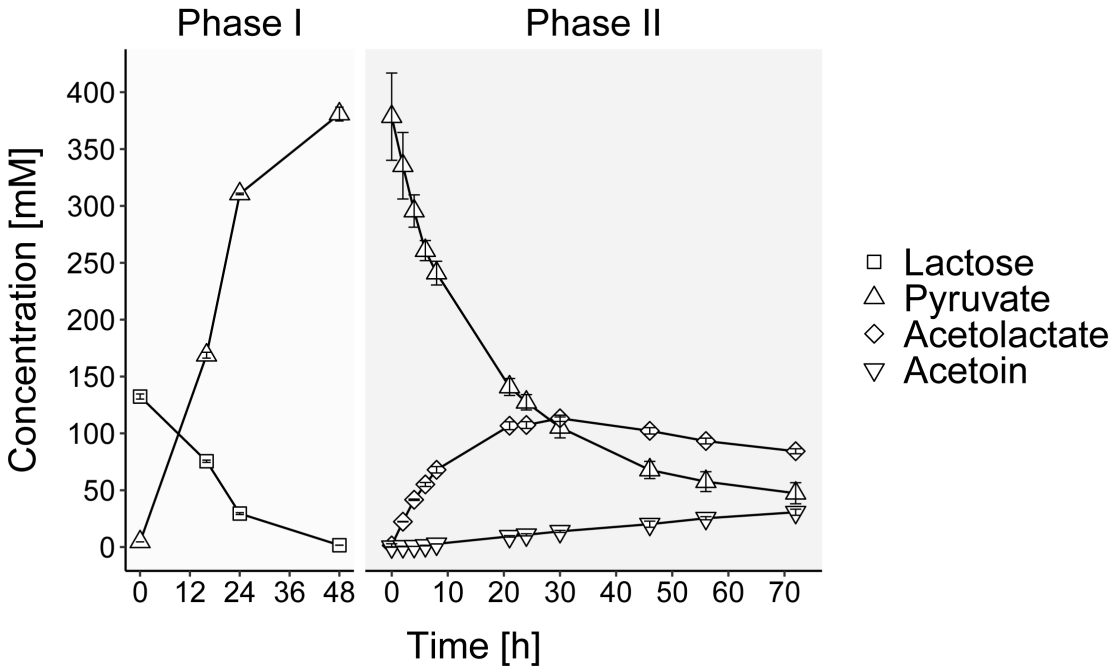


Figure 7.8 Production of α-acetolactate from lactose via pyruvate. Lactose is fermented using FS1080 in RWP, and pyruvate is obtained (Phase I). After 48h, FS1080 was removed from the broth, and RD06 was applied for converting pyruvate into α-acetolactate (Phase II). The concentrations of Lactose (squares), pyruvate (triangles), α-acetolactate (diamonds), and acetoin (inverted triangle) are shown [mM]. Lactate and acetate were observed at different levels after phase I (68 ± 4 mM and 31 ± 4 mM, respectively), but they remained constant during phase II and are therefore not shown.

7.4 Discussion

We have demonstrated that biocatalysis is applicable for producing high titers of α-acetolactate from pyruvate, either pure or crude fermentation-derived. Due to its unstable nature, it is difficult to obtain high titers of α-acetolactate by fermentation, and the best results thus far have been realized using aerated conditions (12,27). Using anaerobic conditions in combination with a cell catalyst, in this study, we managed to reach 172 ± 2 mM of α-acetolactate.

Normally, when producing so-called starter distillates, diacetyl in fermented broth is concentrated by steam distillation to prepare a potent flavoring agent, and the remaining liquid constitutes waste. In this study, we achieved higher concentrations of α-acetolactate, which can be quantitatively converted to diacetyl, for example, using Fe^{3+}-ions, and the obtained product potentially could be added directly to food products.

We find that, for all cell catalysts tested, the optimal pH is around 5.5. This is a little surprising since slightly different pH-optima have been reported previously for the different ALS enzymes used (**Table S 7.2**). It is likely that the low K_m of ef-ALS is responsible for the superior performance of RD06. As all three enzymes were overexpressed from the same expression cassette, similar expression values are expected, but potentially bl-ALS is expressed less efficiently due to the

lower phylogenetic relationship between lactococci and bacilli explaining the inferior performance of RD05. In future work, metabolic, protein, or process engineering will most certainly further enhance the efficiency and applicability of the presented process. As indicated above, biotransformation at higher temperatures such as 30°C with increased cell density (for example, OD_{600} = 5.0) would accelerate the conversion considerably. However, at the same time, α-acetolactate would be less stable, and side products would form more rapidly. Nevertheless, shorter biotransformation under those conditions might be desired in an industrial setting. For other cell catalysts, permeabilization was beneficial for improving efficiency (28,29). Unfortunately, our attempts to permeabilize the WCC using ethanol resulted in decreased reaction rates and reduced α-acetolactate yields (data not shown). It is possible that ALS requires a reduced environment for full functionality as present within intact cells. Another aspect of commercialization is the reusability of the cells because it is expensive to produce biomass. Reusability would ease large scale applications of the presented technology. Surprisingly, when cells, which had been used for biotransformation in pyruvate solution, were reused under the same reaction conditions, the formation of a different product was observed, which we have not identified yet. Further attempts for reusing the cells are ongoing.

7.5 Conclusion

In this study, we have demonstrated the production of α-acetolactate from pyruvate (172 ± 2 mM), and a dairy-derived waste stream (113 ± 3 mM). The reported product titers are exceeding previously published results. In our experiments, we used bioreactors for the biotransformation of pyruvate into α-acetolactate, which makes a subsequent upscaling easier because the conditions can be directly transferred to larger vessels. The presented process further provides a solution for the recycling of a dairy waste stream into a desired food ingredient.

7.6 Methods

7.6.1 Bacterial Strains and DNA Techniques

ll-cat01 (MG1363 *ΔldhB ΔldhX Δpta ΔadhE ΔbutBA ΔaldB*) is a plasmid-free strain derived from *L. lactis* MG1363 (30). Gene deletions were achieved using the method developed by Solem et al. (31). The vector pLC0 is a size-reduced derivative of pTD6 (32) lacking the *gusA* gene. The ALS genes from *Enterococcus faecalis* ATCC 29212 (ef-ALS), *Bacillus licheniformis* WX-02 (bl-ALS) (25), and *L. lactis* MG1363 (ll-ALS) were cloned into pLC0 resulting in the plasmids pLC01, pLC02, and pLC17, respectively. To overexpress these genes, they were put under the control of the strong constitutive promoter P8 (33). FS1072 (**Table 7.1**) was constructed based on the strain CS4363 (32) by deleting the *als* gene. To make the strain able to use lactose as a carbon source, the plasmid pLP712 was extracted from the strain NCDO712 and transformed into the strain FS1072, resulting in the strain FS1080 (**Table 7.1**) (Suo et al., manuscript submitted).

Table 7.1 Bacterial strains and plasmids used in this study

Designation	Genotype or description	Reference
***L. lactis* strains**		
MG1363	Plasmid free *L. lactis* strain	(30)
FS1072	MG1363 *⊿³ldh ⊿pta ⊿adhE ⊿als*	(Suo et al.)
FS1080	FS1072 containing the pLP712 plasmid	(Suo et al.)
ll-cat01	MG1363 *ΔldhB ΔldhX Δpta ΔadhE ΔbutBA ΔaldB*	This work
RD03	pLC0 in ll-cat01	This work
RD04	pLC17 in ll-cat01	This work
RD05	pLC02 in ll-cat01	This work
RD06	pLC01 in ll-cat01	This work
Plasmids		
pLP712	Plasmid from *L. lactis* NCDO712 encoding lactose utilization gene cluster	(30)
pLC0	pTD6 derived plasmid	This work
pLC01	pLC0 inserted with the *als* gene from *Enterococcus faecalis* ATCC 29212 (ef-ALS)	This work
pLC02	pLC0 inserted with the *als* gene from *Bacillus licheniformis* WX-02 (bl-ALS)	This work
pLC17	pLC0 inserted with the *als* gene from *L. lactis* MG1363 (ll-ALS)	This work

7.6.2 Construction of the Plasmids pLC0, pLC01, pLC02, and pLC17

PCR products *pLC0-L* amplified from the plasmid pTD6 with the primers *pTD-CF1* and *pTD-CR1* (**Table S 7.1**) were circularized using T4 DNA ligase after phosphorylation to construct the plasmid pLC0. The DNA fragment *P8-F* containing the P8 promoter (33) was obtained by PCR using primers *KF147-P8F* and *KF147-P8R* (**Table S 7.1**) and genomic DNA of *L. lactis* subsp. *lactis* KF147 as a template. The DNA fragment *efALS-F* was amplified from *Enterococcus faecalis* ATCC 29212 (purchased from German Collection of Microorganisms and Cell Cultures GmbH, DSM No.: 2570) with the primers *ALS-EF-CF1* and *ALS-EF-CR1* by PCR (**Table S 7.1**). The DNA fragment *wxALS-F* was obtained by PCR, using the primer pair *WX02-ALS-F/ WX02-ALS-R* (**Table S 7.1**) and synthetic DNA as a template (see **Section 7.9.1**). The DNA fragment *llALS-F* was amplified from the genomic DNA of *L. lactis* MG1363 using primers *ALS-Ll-CF1* and *ALS-Ll-CR1* (**Table S 7.1**). The fragments P8-F and *efALS*-F were inserted into the vector *pLC0-L* simultaneously by using the In-Fusion® HD Cloning Kit (https://www.takarabio.com) to generate the plasmid pLC01. The plasmids pLC02 and pLC17 were constructed in the same way but with the fragments *wxALS-F* and *llALS-F*, respectively.

7.6.3 Fermentation for Producing Pyruvate and Broth Treatment

Pyruvate was produced from glucose or lactose contained in RWP, as described by Suo et al. (manuscript submitted) with minor modifications. Fermentations were carried out in Biostat A bioreactors equipped with 1 l vessels. For glucose fermentations, M17 medium was used. The seed culture (20 ml) was inoculated 1/100 from an overnight culture in a shake flask using M17 medium containing 1% (w/v) glucose and incubated with strong shaking at 30°C for 16 h. Subsequently, the seed culture was used to inoculate 800 ml M17-medium 1/100. Initially, 5% glucose was added to the medium, and another 2% was added subsequently when the sugar concentration decreased below 3% (after approximately 24 h). Temperature and pH were kept constant at 30°C and pH 7.0, respectively. The fermenters were aerated using air at 1000 ccm/min and stirring at 500 rpm. Glucose was fully depleted after 72 hours and the medium harvested. When fermenting RWP, the same conditions were applied. RWP was diluted two-fold to lower lactose concentration to 5-6% lactose and was supplemented with 2% yeast extract (autoclaved separately). The fermentation was terminated after 48 h when lactose was depleted. Cells were removed from the fermentation broth by centrifugation (10.000 x g, 10 min), and the pH of the resulting pyruvate-rich broth was adjusted to 5.5 using 1 M HCl.

7.6.4 WCC Preparation and Biotransformation

Small-scale reactions were carried out in 1 ml scale using 1.5 ml reaction tubes. Cells were grown in GM17 broth, containing tetracycline (5 µg/ml) when required, at 30°C for 16 h overnight. The cells were harvested by centrifugation (10.000 x g, 10 min), washed once with cold Tris/NaCl-buffer (100 mM/150 mM, pH 6.5), and suspended in H_2O. The optical density (OD_{600}) was determined in duplicate and set to approximately OD_{600} = 50. During harvesting, cells were kept on ice. For starting the reactions, the cell suspensions were centrifuged (10.000 x g, 10 min), and cells were resuspended in solutions containing pyruvate, either pure or contained in fermentation broth. The cell suspensions were incubated at the desired temperatures in a ThermoMixer with shaking. Negative control reactions were carried out in the same way but without any pyruvate.

For larger scale biotransformation, strains RD03-RD06 and ll-cat01 were grown anaerobically in 5 ml GM17 broth at 30°C for overnight. Subsequently, 500 ml cultures in 1L shake flasks were inoculated 1:1000. After grown for 14-16 h at 30°C with shaking, cells were harvested as described above. Laboratory scale biotransformations were carried out in 1-L fermenters (Sartorius “Biostat-A” equipped with a “UniVessel Glass”). The reaction vessel was filled with 350 mL 0.5 M pyruvate solution or pyruvate containing fermentation broth. Before adding the cells, the reaction solution was flushed with N_2 and cooled to 20°C. During the reaction, the pH was kept constant at 5.5 using 1 M HCl. The acid consumption was recorded during the process (**Figure S 7.5**), and samples were taken regularly for analysis. Dilution factors were applied to the results.

7.6.5 Optimization of the Reaction Conditions

Determination of the optimal pH for the reaction and comparison of different WCC was carried out using an iCinac instrument. For this purpose, 10 ml of non-buffered pyruvate solution (0.5 M) was prepared, and the pH was set to 4.6, which is the lowest pH, allowing ALS reaction. This solution was equipped with a pH-electrode, and after stabilization of pH and temperature, cells were added to reach a final OD_{600} 2.5. Immediately after the addition of the cells, the pH recording was started. The resulting pH-shift allowed the determination of the optimal pH for the reaction and comparison of different WCC, as described below.

For determining the optimal temperature for the biotransformation, α-acetolactate stability was investigated. In this case, α-acetolactate was obtained by hydrolyzing ethyl-2-acetoxy-2-methyl acetoacetate using an equimolar amount of NaOH and incubating at room temperature for 1 h. The resulting hydrolysate was added to a solution containing 500 mM pyruvate and 100 mM citrate at pH 5.5. The mixture was incubated at 20°C, 30°C, 40°C and 50°C, and the concentrations of α-acetolactate and side products were followed over time.

7.6.6 Analytical Methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). Determination of glucose, lactose, pyruvate, lactate, and acetoin was carried out by HPLC equipped with the Aminex HPX-87H column (Bio-Rad, Hercules, USA) and RI detector. Additionally, pyruvate was determined by a UV detector at 210 nm. The column oven temperature was set at 60°C and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.5 ml/min. The α-acetolactate was quantified after conversion to acetoin, which was subsequently analyzed by HPLC, whereby the conditions for the conversion were adapted from (10). Briefly, the samples were diluted three-fold in water (reference sample) and three-fold in 0.5 M HCl (treated sample). Subsequently, the treated sample was incubated 30 min at 44°C to allow complete decarboxylation of α-acetolactate to acetoin, while the reference sample was stored at 4°C in order to prevent this reaction. Finally, both samples were analyzed using HPLC. The α-acetolactate concentration was obtained by subtracting the acetoin concentration of the reference sample from the acetoin concentration of the treated sample and dividing by the factor 0.62 (10,34).

Alternatively, acetoin and α-acetolactate concentrations were determined using a colorimetric assay. 50 µl of reaction mixtures were added in a 96-well microtiter-plate (MTP). 5 µl 2 M H_2SO_4 were added to the mixtures for decarboxylation of α-acetolactate to acetoin, which was completed by incubation for 15 min at 60°C. Subsequently, 45 µl 0.5% creatine in H_20 and 45 µl 5% 1-Naphthol in 2M NaOH were added and mixed by pipetting several times. The color formation was completed by incubating for 15 min at 60°C and 15 min at 20°C. Finally, the absorbance at 530 nm was measured using a plate reader. As a blank value, negative control reactions without pyruvate were used. A standard curve was measured each time using acetoin (0 – 1 mM) dissolved in the respective reaction solution. The procedures above are based on the

method developed by W. Westerfeld for detecting acetoin and were adapted and modified as previously described (26,35,36).

7.7 Authors' Contributions

RD, JL, CS, and PRJ designed and conceived the project. RD and LC performed all the experiments. All the authors analyzed the results, prepared the manuscript, and finally approved the manuscript.

7.8 References

1. Clark S, Winter CK. Diacetyl in Foods: A Review of Safety and Sensory Characteristics. *Compr Rev Food Sci Food Saf* **2015**, 14(5):634–43.
2. Wijaya I, Rankin SA. Diacetyl levels and volatile profiles of commercial starter distillates and selected dairy foods. *J Dairy Sci* **2012**, 95(3):1128–39.
3. Zeitsch KJ. 16. Diacetyl and 2,3-pentanedione. In: Sugar Series. Editor: Zeitsch KJ. Elsevier Ltd **2000**, 120–49.
4. de Man JC. The formation of diacetyl and acetoin from α-acetolactic acid. *Recl des Trav Chim des Pays-Bas* **1959**, 78(7):480–6.
5. Medina de Figueroa R, Oliver G, Benito de Cárdenas IL. Influence of temperature on flavour compound production from citrate by *Lactobacillus rhamnosus* ATCC 7469. *Microbiol Res* **2001**, 155(4):257–62.
6. Branen AL, Keenan TW. Diacetyl and acetoin production by *Lactobacillus casei*. *Appl Microbiol* **1971**, 22(4):517–21.
7. Laëtitia G, Pascal D, Yann D. The Citrate Metabolism in Homo- and Heterofermentative LAB: A Selective Means of Becoming Dominant over Other Microorganisms in Complex Ecosystems. *Food Nutr Sci* **2014**, 5(10):953–69.
8. Starrenburg MJC, Hugenholtz J. Citrate fermentation by *Lactococcus* and *Leuconostoc* spp. *Appl Environ Microbiol* **1991**, 57(12):3535–40.
9. Verhue WM, Tjan FSB. Study of the Citrate Metabolism of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis by Means of 13C Nuclear Magnetic Resonance. *Appl Environ Microbiol* **1991**, 57(11):3371–7.
10. Jordan KN, Cogan TM. Production of acetolactate by *Streptococcus diacetylactis* and *Leuconostoc* spp. *J Dairy Res* **1988**, 55(2):227–38.
11. Ronfags E, Stien G, Germain P, Marc I. Kinetic Study of the Chemical Reactivity of α-Acetolactate as a Function of pH in Water, and in Fresh and Fermented Culture Media Used for *Lactococcus lactis* spp. *lactis* bv. diacetylactis. *Biotechnol Lett* **1996**, 18(7):747–52.
12. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. *Metab Eng* **2016**, 36:57–67.
13. E. W. Seitz, W. E. Sandine, E. A. Day PRE. Studies on factors affecting diacetyl production by *Streptococcus diacetilactis*. *J Dairy Sci* **1961**, 44(6):1159–.
14. Hugenholtz J, Kleerebezem M, Starrenburg M, Delcour J, De Vos W, Hols P. *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Appl Environ Microbiol* **2000**, 66(9):4112–4.
15. Guo T, Kong J, Zhang L, Zhang C, Hu S. Fine-tuning of the lactate and diacetyl production through promoter engineering in *Lactococcus lactis*. *PLoS One* **2012**, 7(4):1–10.
16. Goupil N, Corthier G, Ehrlich SD, Renault P. Imbalance of leucine flux in *Lactococcus lactis* and its use for the isolation of diacetyl-overproducing strains. *Appl Environ Microbiol* **1996**, 62(7):2636–40.

17. Aymes F, Monnet C, Corrieu G. Effect of α-acetolactate decarboxylase inactivation on α-acetolactate and diacetyl production by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. *J Biosci Bioeng* **1999**, 87:87–92.
18. Monnet C, Aymes F, Corrieu G. Diacetyl and α-acetolactate overproduction by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis mutants that are deficient in α-acetolactate decarboxylase and have a low lactate dehydrogenase activity. *Appl Environ Microbiol* **2000**, 66(12):5518–20.
19. Monnet C, Schmitt P, Divies C. Development and Use of a Screening Procedure for Production of α-Acetolactate by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis Strains. *Appl Environ Microbiol* **1997**, 63(2):793–5.
20. Jay JM. Antimicrobial properties of diacetyl. *Appl Environ Microbiol* **1982**, 44(3):525–32.
21. Verhue WMM, Tjan SB, Verrips CT, van Schie BJ. Process for preparing an aroma product containing α-acetolactic acid. European Patent Application, EP0483888A2, **1991**.
22. Marugg JD, Toonen MY, Verhue WMM, Verrips CT. Process for the preparation of α-acetolactic acid. European Patent Application, EP0500188A2, **1992**.
23. Liu J, Wang Z, Kandasamy V, Lee SY, Solem C, Jensen PR. Harnessing the respiration machinery for high-yield production of chemicals in metabolically engineered *Lactococcus lactis*. *Metab Eng* **2017**, 44:22–9.
24. De Felipe FL, Starrenburg M, Hugenholtz J. The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis* subsp. *lactis* MG1363. *FEMS Microbiol Lett* **1997**, 156:15–9.
25. Huo Y, Zhan Y, Wang Q, Li S, Yang S, Nomura CT, Wang C, Chen S. Acetolactate synthase (AlsS) in *Bacillus licheniformis* WX-02: enzymatic properties and efficient functions for acetoin/butanediol and L-valine biosynthesis. *Bioprocess Biosyst Eng* **2018**, 41(1):87–96.
26. Lee SC, Kim J, La IJ, Kim SK, Yoon MY. Characterization of recombinant FAD-independent catabolic acetolactate synthase from *Enterococcus faecalis* V583. *Enzyme Microb Technol* **2013**, 52(1):54–9.
27. Zhao L, Bao Y, Wang J, Liu B, An L. Optimization and mechanism of diacetyl accumulation by *Enterobacter aerogenes* mutant UV-3. *World J Microbiol Biotechnol* **2009**, 25(1):57–64.
28. Somkuti GA, Dominiecki ME, Steinberg DH. Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with Ethanol. *Curr Microbiol* **1998**, 36(4):202–6.
29. Shin KC, Sim DH, Seo MJ, Oh DK. Increased Production of Food-Grade D-Tagatose from D-galactose by Permeabilized and Immobilized Cells of *Corynebacterium glutamicum*, a GRAS Host, Expressing D-Galactose Isomerase from *Geobacillus thermodenitrificans*. *J Agric Food Chem* **2016**, 64(43):8146–53.
30. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **1983**, 154(1):1–9.
31. Solem C, Defoor E, Jensen PR, Martinussen J. Plasmid pCS1966, a New Selection / Counterselection Tool for Lactic Acid Bacterium Strain Construction Based on the oroP Gene, Encoding an Orotate Transporter from *Lactococcus lactis*. *Appl Environ Microbiol* **2008**, 74(15):4772–5.
32. Solem C, Dehli T, Jensen PR. Rewiring *Lactococcus lactis* for ethanol production. *Appl Environ Microbiol* **2013**, 79(8):2512–8.
33. Zhu D, Liu F, Xu H, Bai Y, Zhang X, Saris PEJ, Qiao M. Isolation of strong constitutive promoters from *Lactococcus lactis* subsp. *lactis* N8. *FEMS Microbiol Lett* **2015**, 362(16):1–6.
34. Benson KH, Godon JJ, Renault P, Griffin HG, Gasson MJ. Effect of ilvBN-encoded α-acetolactate synthase expression on diacetyl production in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **1996**, 45(1–2):107–11.

35. Nguyen DMN, Lipscomb GL, Schut GJ, Vaccaro BJ, Basen M, Kelly RM, Adams MWW. Temperature-dependent acetoin production by *Pyrococcus furiosus* is catalyzed by a biosynthetic acetolactate synthase and its deletion improves ethanol production. *Metab Eng* **2016**, 34:71–9.
36. Westerfeldt WW. A colorimetric Determination of Blood Acetoin. *J Biol Chem* **1945**, 161:495–502.

7.9 Supplementary Material

7.9.1 Supplementary Methods

Table S 7.1 Primers used in this study

Primer	Sequence (5'→3')
pTD-CF1	TAGTAGCTAGCTAGTAAAAAAAAGAACCCGAGTGG
pTD-CR1	GCATGCGGCCGCATATGCCATG
KF147-P8F1	TGGCATATGCGGCCGCATGCgataaaatttctaatgatttttttaggacaatTatttctcataa aaagcagattttag
KF147-P8R	CATTATATCTCTCCATTTCTGCTGC
ALS-EF-CF1	GCAGAAATGGAGAGATATAAtgagtaaaaaaggatcagatatcatagtagaaag
ALS-EF-CR1	TTTTTACTAGCTAGCTACTActaataaagttgatctggtaacaatgttttacc
WX02-ALS-F	AGCAGAAATGGAGAGATATAATGAATAATGTAGCCGCTAAAAATGAAA CTC
WX02-ALS-R	TTTTTACTAGCTAGCTACTATCAAGATTGCTTAGAGGCTTCTTTAT
ALS-LI-CF1	GCAGAAATGGAGAGATATAatgtctgagaaacaatttggggcg
ALS-LI-CR1	TTTTTACTAGCTAGCTACTAtcagtaaaattcttctggcaataatttttctgc

gBlock Sequence of *Bacillus licheniformis* WX-02 ALS (bl-ALS)

TGTAGCTGAATGAAATTGAAAGGAATTAAATTGAATAATGTAGCCGCTAAAAATGAAACTCT
TACTGTAAGAGGAGCAGAGCTTGTGGTGGATAGTCTCATTCAGCAAGGTGTCACTCATGTT
TTCGGTATTCCGGGAGCGAAAATCGATGCGGTGTTTGACGTATTGAAAGACAAGGGGCCT
GAATTGATCGTTTGCCGTCACGAGCAGAATGCAGCATTTATGGCGGCGGCAGTCGGACGA
TTGACTGGAAAGCCCGGTGTTTGCCTGGTGACTTCAGGTCCGGGAGCGTCTAATTTAGCG
ACCGGTCTTGTAACAGCCAATACAGAAGGAGATCCGGTTGTTGCCCTGGCGGGTGCTGTA
AAAAGAGCGGATCGTCTCAAAAAAACTCATCAATCGATGGATAATGCGGCGTTGTTTCAGC
CGATTACGAAATATAGCGCAGAAGTGGAAGATGCGAACAACATACCTGAGGCTGTAACCAA
TGCATTCAGAGCGGCGGCTTCTGGACAGGCTGGCGCAGCGTTTCTCAGCTTTCCGCAGGA
CGTTACGGCCGGTCCGGCAACTGCCAAGCCGGTGAAAACCATGCCGGCGCCGAAGCTGG
GCGCGGCTTCGGACGAACAAATCAGCGCGGCCATCGCCAAAATTCACAATGCGAACCTTC
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TGCTAAGGAAAGTGAAACTGCCGTTTGTTGAAACATACCAAGCAGCGGGTACGCTGTCTCA
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GCTATTGGAAAAAGCGGATGTCGTTTTGACGGTCGGCTATGATCCGATTGAATACGATCCG

GTCTTTTGGAATGGAAAAGGCGAACGAAGCGTGATTCATCTTGACGAAATACAAGCCGATA
TCGATCATGACTATCAGCCCGAAATCGAGTTGATCGGCGATATCGCAGAGACGTTAAACCA
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TGCTGATTTCCAACGGCATGCAGACGCTTGGGGTGGCTTTGCCGTGGGCGATTGCAGCAA
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7.9.2 Supplementary Results

ll-cat01 – pH Shift at Different Starting pH

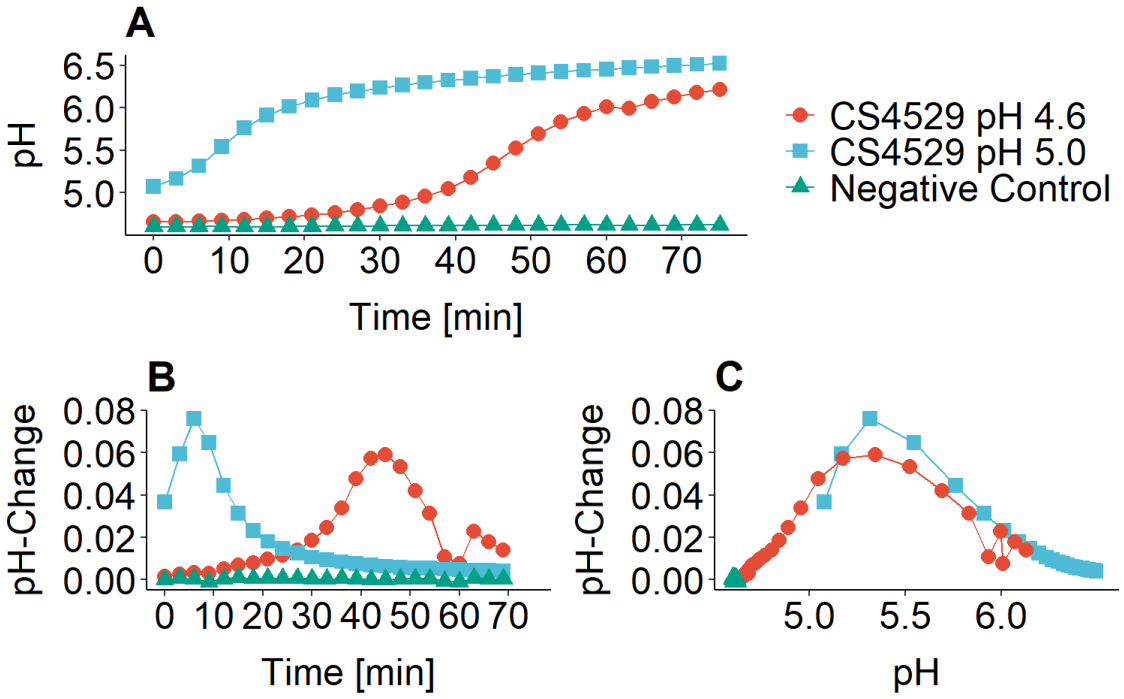

Figure S 7.1 ll-cat01 was incubated in an unbuffered solution containing 0.5 M pyruvate at pH 4.6 and pH 5.0 at OD_{600} = 2.5. As a negative control, ll-cat01 was incubated in a solution without pyruvate. A: pH-profile (pH against time [min]). B: pH-change as an indicator for the catalytic activity over time. C: pH-change against pH.

α-Acetolactate Formation Rate of the Four WCCs (RD03 - RD06)

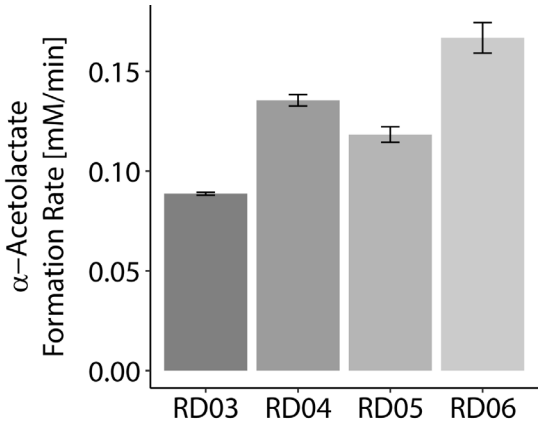

Figure S 7.2 Comparison of RD03 – RD06 using small-scale biotransformations. The α-acetolactate formation rate [mM/min] was determined at constant pH. The WCCs were applied at a cell density corresponding to OD_{600} = 2.5 at 20°C.

Catalytic Properties of Different Catabolic ALS Used in This Study

Table S 7.2 Comparison of the catalytic properties of different catabolic ALS.

Strain	Specific Activity (U/mg)	K_m for Pyruvate (mM)	K_{cat} (1/s)	Optimum pH	References
B. licheniformis WX-02	35.4	3.96	514	6.5	(1)
L. lactis	103†	50	NR	6.5-7.0	(2)
E. faecalis	5.36	1.37	NR	6.8	(3)

†: The method for purifying the enzyme differs in comparison to the other two enzymes. NR: not reported

Linear Correlation Between OD_{600} and α-Acetolactate Production Using RD06

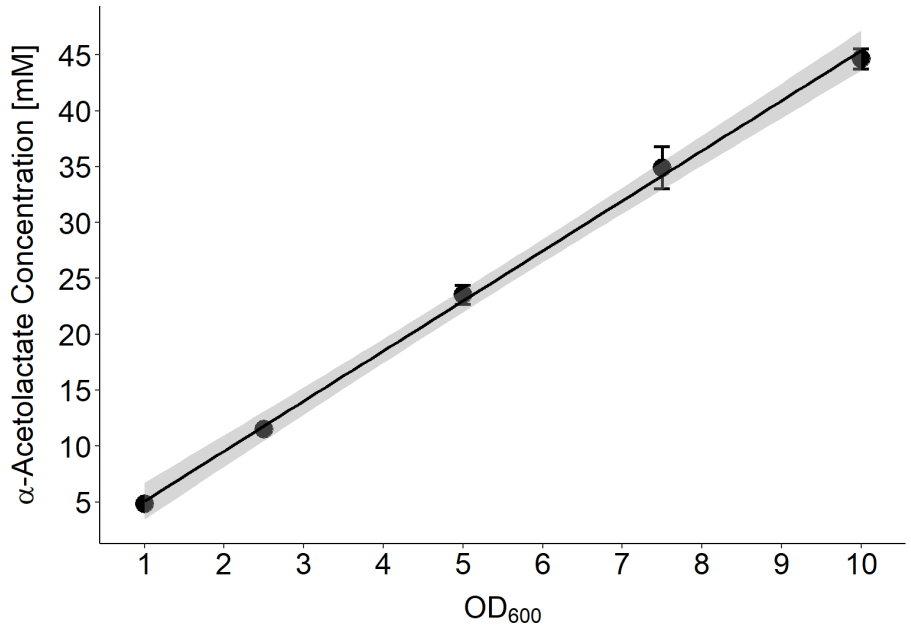

Figure S 7.3 Linear correlation between OD_{600} and α-acetolactate concetration produced by RD06 after 2 h in 500 mM pyruvate buffered with 100 mM citrate at pH 5.5. The regression follows: y = 4.478 * x + 0.598 (R^2 = 0.9986).

Linear Correlation Between Temperature and α-Acetolactate Formation Rate Using RD06

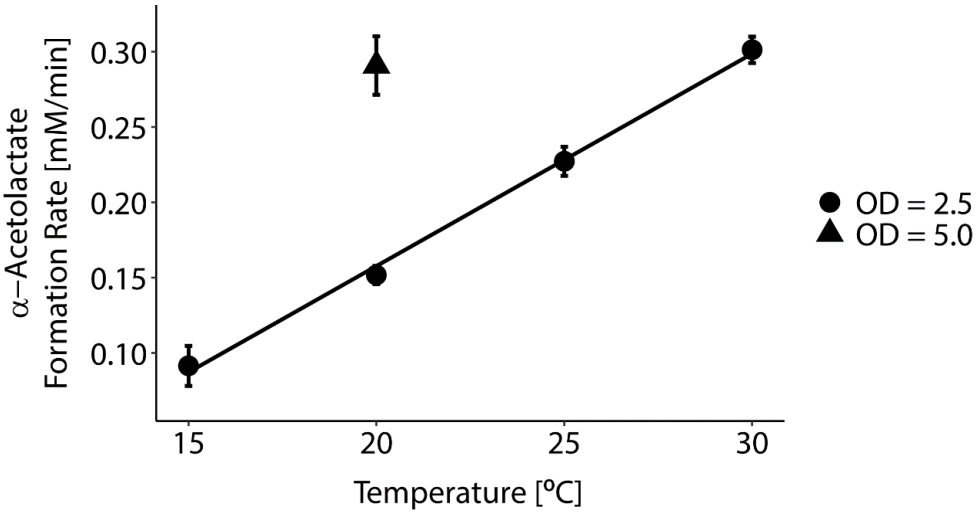

Figure S 7.4 α-Acetolactate formation rate [mM/min] for RD06 at different temperatures and cell densities (OD = 2.5 (circles) and OD = 5.0 (triangles). A nearly linear correlation between temperature and formation rate was observed. Increased cell density can compensate for decreased activity at lower temperatures, as indicated at 20°C.

Acid Consumption during Laboratory-Scale Biotransformation

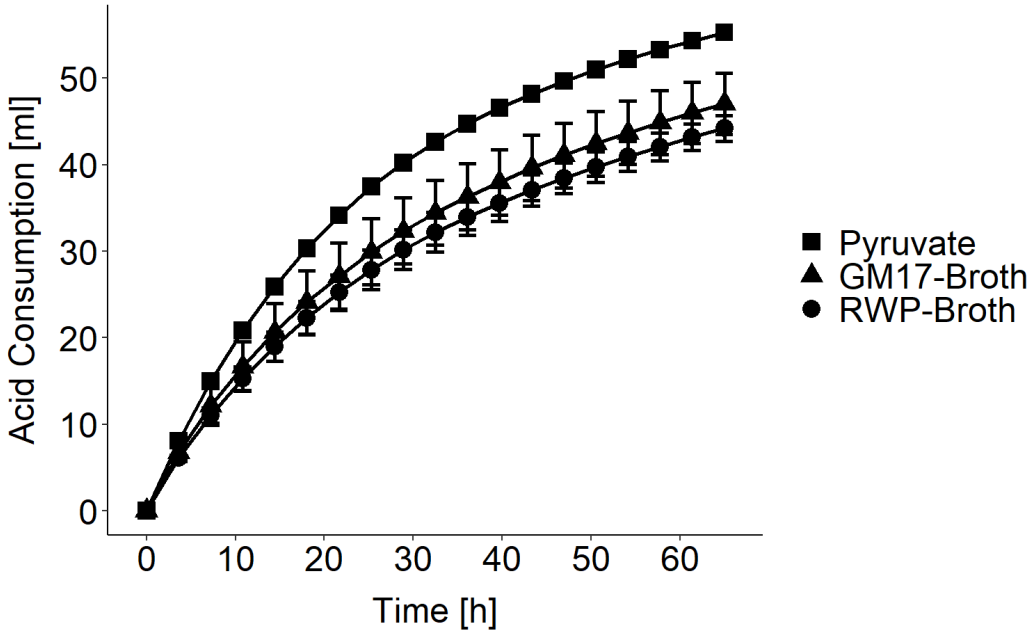

Figure S 7.5 Acid consumption during the laboratory-scale biotransformation. Data is the average of two independent experiments.

Side Products Observed in HPLC

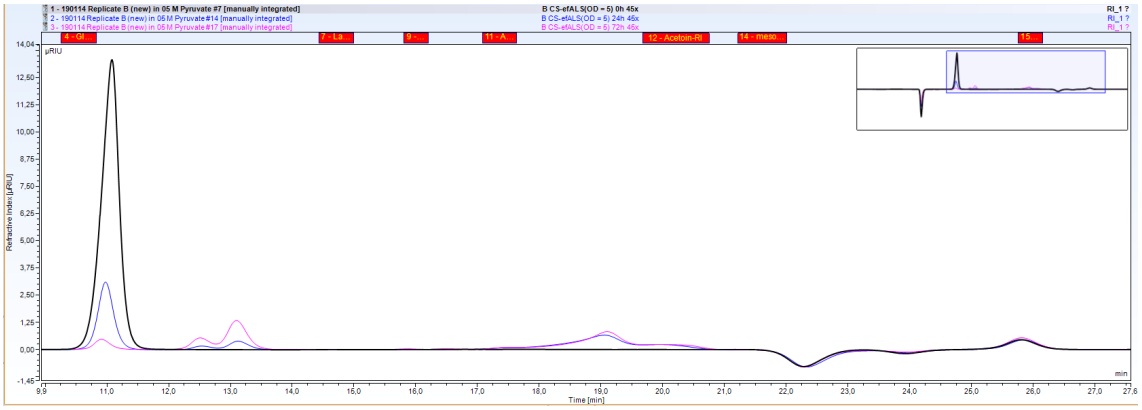

Figure S 7.6 HPLC-spectrum of reactions of RD06 in pyruvate solution with 45x dilution. Results after 0 h (black), 24 h (blue) and 72 h (pink) are shown. Retention times are as follows: Pyruvate: 10.8 min, α-Acetolactate: 19.0 min, Acetoin: 20.7 min, Unknown Side product: 13.1 min.

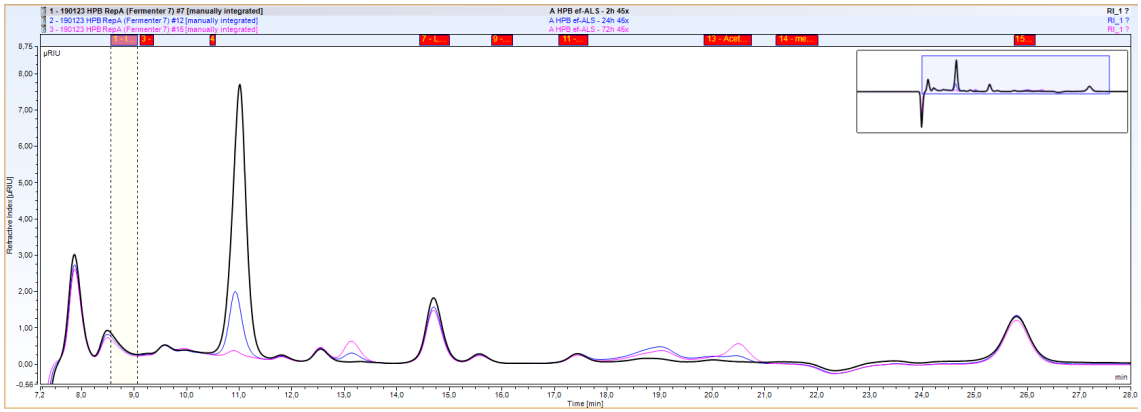

Figure S 7.7 HPLC-spectrum of reactions of RD06 in pyruvate-containing GM17-broth with 45x dilution. Results after 0 h (black), 24 h (blue) and 72 h (pink) are shown. Relevant retention times are as follows: Pyruvate: 10.7 min, α-Acetolactate: 18.9 min, Acetoin: 20.5 min, Unknown Side product: 13.1 min.

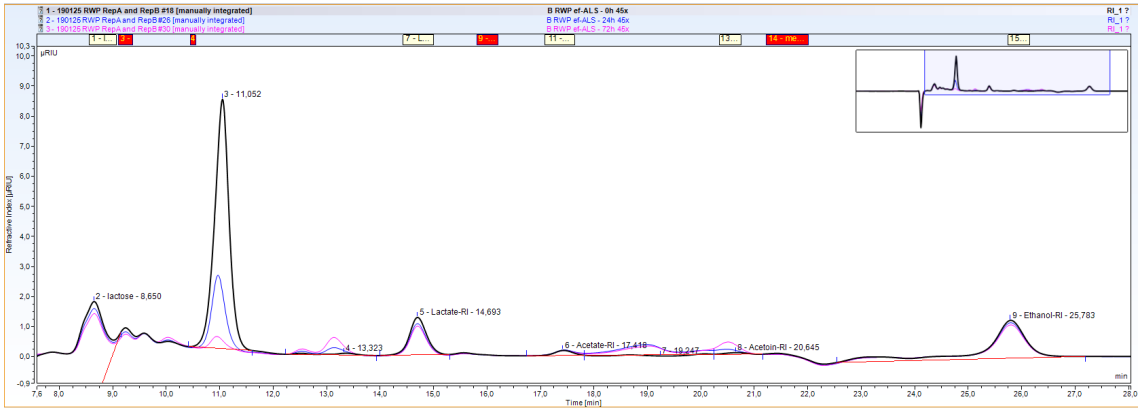

Figure S 7.8 HPLC-spectrum of reactions of RD06 in pyruvate-containing RWP with 45x dilution. Results after 0 h (black), 24 h (blue) and 72 h (pink) are shown. Relevant retention times are as follows: Pyruvate: 10.7 min, α-Acetolactate: 19.0 min, Acetoin: 20.5 min, Unknown Side product: 13.2 min.

Development of OD during a Representative Biotransformation (Second Phase)

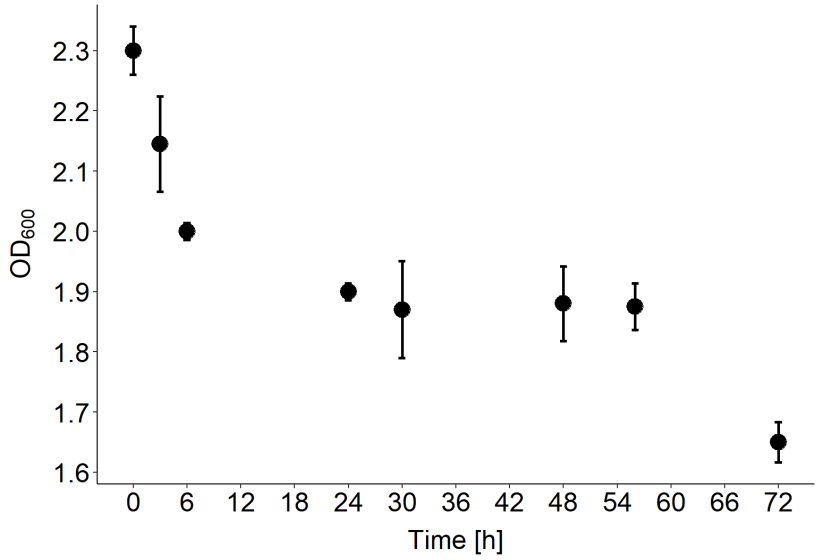

Figure S 7.9 Change in cell density during biotransformation (second phase) in a solution containing 500 mM pyruvate. The pH was kept constant using a pH-control with 0.5 M HCl.

7.9.3 References for Supplementary Material

1. Huo Y, Zhan Y, Wang Q, Li S, Yang S, Nomura CT, Wang C, Chen S. Acetolactate synthase (AlsS) in *Bacillus licheniformis* WX-02: enzymatic properties and efficient functions for acetoin/butanediol and L-valine biosynthesis. *Bioprocess Biosyst Eng*. 2018;41(1):87–96.
2. Snoep JL, Mattos MJTDE, Starrenburg MJC. Isolation, Characterization, and Physiological Role of the Pyruvate Dehydrogenase Complex and α-Acetolactate Synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. *J Bacteriol*. 1992;174(14):4838–41.
3. Lee SC, Kim J, La IJ, Kim SK, Yoon MY. Characterization of recombinant FAD-independent catabolic acetolactate synthase from *Enterococcus faecalis* V583. *Enzyme Microb Technol*. 2013;52(1):54–9.

8. Boosting α-Acetolactate Production from Pyruvate and Waste-Derived Citrate using Permeabilized Cells

8.1 Abstract

In this chapter, the optimization of the whole-cell based transformation of pyruvate into α-acetolactate is described. Several *Lactococcus lactis* (*L. lactis*) strains were studied, which have previously been optimized by metabolic engineering to overexpress different α-acetolactate synthases. Transport across the cell wall was identified as a bottleneck, which was then overcome by permeabilization using the antimicrobial peptide nisin. Permeabilized cells were shown to have a higher α-acetolactate formation rate in buffers containing the cofactor thiamine pyrophosphate. Furthermore, the activity of permeabilized cells in pyruvate-containing fermentation broth based on a lactose-rich dairy waste stream was demonstrated.

We attempted to utilize whole-cell catalysis for producing α-acetolactate from another dairy-derived waste material, which is rich in citrate. In *L. lactis* subsp. *lactis* biovar diacetylactis, citrate is a natural precursor of α-acetolactate, which is obtained through a redox-cofactor independent three enzyme cascade. We tested two citrate metabolizing strains as whole-cell biocatalysts for their ability to convert citrate into α-acetolactate and observed that full conversion is possible within several hours. The strains tested, however, possessed α-acetolactate decarboxylase (ALD) activity, so that acetoin was accumulated instead of α-acetolactate. Nonetheless, these experiments show that it should, in principle, be possible to produce α-acetolactate directly from citrate in dairy waste using strains deficient in ALD.

8.2 Introduction

Biocatalysis comprises three branches: fermentation, whole-cell-based bioconversions, and enzyme-based bioconversions. Commonly, in all three branches, a biological unit, a cell, or an enzyme, is used as catalyst, the so-called biocatalyst, for producing the desired product (1). In the food industry, fermentation is most established and has been used for millennia to produce safe beverages or foods, such as beer or cheese (2,3). For instance, in cheese manufacturing, lactic acid bacteria (LAB) are grown in milk, where lactose is converted into biomass and lactic acid. Whole-cell based bioconversions are distinct from fermentation processes, as biomass and product formation are separated. Usually, such a process relies on a single or handful of enzymes when in comparison, in a fermentation process, typically, hundreds of enzymes act in synergy for achieving cellular growth (4–6).

Consequently, whole-cell based bioconversions are more similar to enzyme-based bioconversions in which one or several purified enzymes are used in an artificial environment (7). Enzyme-based bioconversions for modulating texture or flavor are widely used in the food and

flavor industry (8,9). Similarly, whole-cell-based bioconversions are gaining a foothold in the food and flavor industry and are used by several companies (10). This is not surprising as the application of whole-cell catalysts (WCCs) holds many superior characteristics compared to fermentation or enzyme-based bioconversions. First, biomass production can be carried out under optimal conditions, allowing fast growth and high yields of the WCC (11,12). Second, the cell density during the bioconversion can easily be increased by removing the fermentation medium, which allows higher productivities (1). Third, biomass can be reused, thereby reducing the overall cost for producing the WCC and allowing higher overall yields per catalyst unit (1). Forth, the biotransformation can be performed under conditions typically restricting cellular growth, such as high substrate concentrations or the presence of toxic compounds, as the cell envelope shields the enzymes (4,11–13). Fifth, cofactor and enzymes for regeneration are supplied by the cell, which simplifies the process and reduces costs (4,13).

In summary, compared to fermentations, whole-cell-based bioconversions are more flexible by allowing wider substrate and product scopes, cell recycling, and high cell densities. Compared to enzyme-based bioconversions, WCCs are cheaper, more robust, and easier to implement due to saved costs for purifying and immobilizing an enzyme. Biotransformations based on more than one enzyme further benefit from using whole-cell biocatalysis, as the individual enzymes do not need to be purified separately and are naturally nearby within the cell. Therefore, the application of WCCs has excellent potential for producing natural food ingredients, such as aroma compounds or vitamins from biobased materials. Furthermore, whole-cell-based bioconversions may facilitate the upcycling of waste materials, which often contain high concentrations of inhibitory substances and thereby restrict fermentation or enzyme-based bioconversions. One such a waste stream is the so-called mother liquor (ML), also called delactosed whey permeate, which is obtained in large quantities in the dairy industry (14). Due to the high salt and comparably low protein content, fermentation of this waste stream is troublesome, but biocatalysis can help to produce valuable ingredients as it has been demonstrated previously (15).

Besides those numerous advantages of whole-cell-based bioconversions, limited substrate, or product transport over the membranes, which is particularly challenging for charged or polar molecules, might reduce the productivity of a WCC. Permeabilization has been shown in many cases to benefit conversion rates (16). Permeabilization can be achieved by several means, including chemical treatment with organic solvents, surfactants or salts, and physical treatments such as freeze and thaw cycles, heat shock, electroporation, or ultrasound (16,17). Permeabilization can also be achieved by applying or expressing lytic enzymes or antimicrobial peptides (18–20). Nisin is such an antimicrobial peptide, which can serve as a permeabilizing agent by facilitating the formation of stable pores in the cell membranes of Gram-positive bacteria (21). It has been shown by our research group that LAB permeabilized with nisin are efficient food-grade cell catalysts for hydrolyzing lactose in dairy products (22).

α-Acetolactate is the precursor of diacetyl, which provides a pleasant buttery aroma, for instance, in dairy products, and is generally recognized as safe (GRAS) (23,24). Producing diacetyl by chemical means involves harsh production conditions, which are energy demanding (25). Conversely, biotechnological production offers higher sustainability and safety. Nevertheless, like many other comparable processes, the biotechnological α-acetolactate production currently still lacks sufficiently high yield and productivity (7). Only a few reports about using whole-cell biocatalysis for producing α-acetolactate exist and systematic optimization of the process has not been reported to date. So far, only a handful of practical examples for using untreated whole cells or crude cell lysates in milk or whey were described in the patent literature, not revealing extensive details (26,27). Recently, we reported the scalable high-yield production of α-acetolactate using a WCC and achieved the highest α-acetolactate product titers so far. Those high product titers were possible because the bioconversion was carried out under anaerobic conditions, which was not possible for fermentative production as described in detail below (28–30).

In bacteria, such as *Lactococcus lactis* (*L. lactis*) subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* spp., or *Lactobacillus* spp., α-acetolactate is formed from citrate via a three-enzyme cascade-(31-33). In the first step, citrate is converted into acetate and oxaloacetate by citrate lyase (CL). Then, oxaloacetate is decarboxylated into pyruvate and CO_2 by oxaloacetate decarboxylase (OD). The last step is catalyzed by α-acetolactate synthase (ALS), which forms α-acetolactate from two pyruvate molecules while CO_2 is released. In *L. lactis* subsp. *lactis* biovar *diacetylactis*, all three enzymes are naturally induced during acidic growth, whereby CL and OD are transcribed as single polycistronic mRNA of 8.6 kb (34), and ALS is transcribed as monocistronic mRNA (35). The CL complex is composed of three subunits: *citF*, *citE,* and *citD*, which are complemented by *citG, citX,* and *citC,* and a rather broad pH optimum around pH 7 was reported (36,37). CL requires Mn^{2+} or Mg^{2+} to be functional and has to be activated by acetylation in the presence of ATP and acetate (37–39). OD, encoded by the *citM* gene, belongs to the malic enzyme family, but its activity is independent of any redox-cofactors. The enzyme also requires Mn^{2+} or Mg^{2+} for activity and has a pH optimum at pH 4.4 (40). ALS, encoded by the *als* gene, requires thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors and has a pH optimum at pH 6.0 (41). Both Mg^{2+} and TPP are located in the active center of ALS (42). During the catalytic cycle, one pyruvate molecule binds TPP and is decarboxylated, thereby forming 2-hydroxyethy-TPP. This intermediate reacts with the second pyruvate molecule to form α-acetolactate, which is released, and TPP is recovered (43,44). In *L. lactis*, the high K_m for pyruvate (K_m = 50 mM) is remarkable in comparison to ALS from other organisms (45). Due to the low affinity of ALS towards pyruvate, during growth on glucose or lactose, naturally, only little α-acetolactate is formed. Only when the pyruvate pool increases, for example, by metabolizing citrate, α-acetolactate is produced (46). Furthermore, pyruvate formation via glycolysis provides NADH, and for balancing the redox state of the cell, pyruvate and NADH need to be consumed equivalently. In most LAB, this is achieved by forming lactic acid, ethanol, acetic acid, or mixtures

thereof (47). In contrast, pyruvate produced from citrate is formed without changing the cellular redox cofactor balance and consequently, nearly all citrate-derived pyruvate is processed by ALS (48).

Usually, in LAB, α-acetolactate is not accumulated but directly converted into acetoin by α-acetolactate decarboxylase (49). Furthermore, α-acetolactate is unstable and quickly decomposes into diacetyl or acetoin under aerobic or anaerobic conditions, respectively (33,50-53). Overproduction of diacetyl or α-acetolactate has been targeted by several research groups using metabolic engineering (29,49,54-59). Most efficiently, in a metabolically engineered *L. lactis* strain, 95 mM α-acetolactate were obtained by blocking all significant pathways through gene deletions except for the one leading to α-acetolactate (29). Oxygen was required during the fermentation for balancing redox-cofactors by NADH oxidase action, which unavoidably resulted in α-acetolactate conversion to diacetyl. As diacetyl has antimicrobial activity by inhibiting several crucial enzymes, including NADH oxidase, ultimately growth and production of α-acetolactate were affected (29,55,60). Anaerobic production of α-acetolactate using a WCC is therefore advantageous because diacetyl formation is avoided during the bioconversion, which allows higher product yields (see **Chapter 7**, (28)). Afterward, the chemical conversion of α-acetolactate into diacetyl can be initiated just when desired, for example, by adding metal ions in the presence of oxygen (29,33). Alternatively, the addition of α-acetolactate to food products instead of diacetyl has the advantage that diacetyl is formed slowly, thereby creating a long-lasting taste.

Here, the optimization of α-acetolactate production using whole-cell biocatalysis was attempted. By using permeabilized cells and waste-derived citrate, the process was both accelerated and prepared for greener diacetyl production.

8.3 Results

8.3.1 ALS Activity in Whole Cells and Crude Cell Extracts

All strains used in this study have been described previously (see **Chapter 7**, (28)). RD03 is a metabolically engineered mutant of *L. lactis* subsp. *cremoris* MG1363 in which the following genes had been inactivated: *ldhB*, *ldhX*, *pta*, *adhE*, *butBA,* and *aldB*. In RD04-RD06, different ALS were overexpressed, namely ALS from *L. lactis*, *Bacillus licheniformis* WX-02, and *Enterococcus faecalis*, under the control of a strong constitutive promoter in the same cellular background, while RD03 contained the empty vector. For all enzymes, the same expression cassette was used, which theoretically allows 6- to 8-fold overexpression of the target gene (61). Consequently, an increase of ALS activity within the same range could be expected. However, when comparing α-acetolactate formation rates using intact cells, a rather low increase was observed, which was strongest for RD06 (1.9 ± 0.1-fold increase) (**Figure 8.1**).

To validate whether the rather low increase of ALS activity was caused by a limited overexpression or other factors such as transport limitations through the cell membranes, ALS activity in crude cell extracts was determined. In contrast to the experiments using intact cells, a

strong increase of ALS activity was observed. Most prominently in RD04, overexpressing the native lactococcal ALS, a 7.8 ± 0.5-fold increase in ALS activity was observed, which is in agreement with the expected overexpression. Interestingly, the fold change for RD06 was lower than that observed for RD04 (7.1 ± 0.3-fold). Furthermore, it was surprising to find no activity increase for RD05, expressing ALS from *Bacillus licheniformis* WX-02 (45), in cell extracts, even though in whole cells, an increase was observed. Next, the effect of permeabilization on pyruvate to α-acetolactate conversion was investigated for validating whether the limited effect of ALS overexpression was caused by transport limitations.

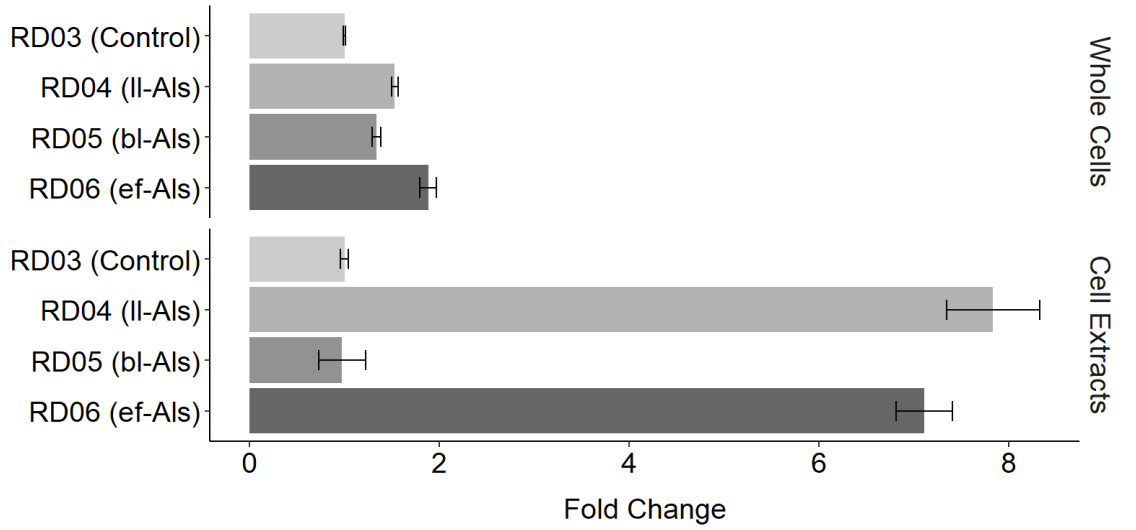

Figure 8.1 Comparison of strains expressing different ALS using the same expression cassette. Fold change compared to RD03 is shown for the biotransformation of pyruvate to α-acetolactate using either whole cells (upper row) or crude cell extracts (lower row). RD03 was the control strain harboring an empty expression vector, RD04 overexpressed native lactococcal ALS (ll-ALS), RD05 expressed ALS from Bacillus licheniformis WX-02 (bl-ALS), RD06 expressed ALS from Enterococcus faecalis (ef-ALS).

8.3.2 Permeabilization Using Nisin Boosts α-Acetolactate Production from Pyruvate

Plenty of methods for permeabilizing bacteria have been reported. Here, only methods employing food-grade agents were considered. Recently, our laboratory developed a food-grade method for mild and efficient permeabilization of LAB based on the antimicrobial peptide nisin. Nisin is a food-approved bacteriocin, which is naturally produced by food grade *L. lactis* strains. In contrast to using, for example, organic solvents such as ethanol, which is also approved for food applications, nisin exclusively targets the cell wall of bacteria and does not affect enzyme activities. In order to test whether this method was suitable for accelerating α-acetolactate production using whole-cell biocatalysis, the WCCs RD03 and RD04 were incubated with different concentrations of nisin. As RD04 showed the biggest fold change compared to RD03 (**Figure 8.1**), the permeabilization

method was validated using only this strain and RD03 as control. Permeabilization of RD03 only resulted in a minor activity increase, whereas the increase for RD04 was strong (**Figure 8.2**). Under the given conditions (pH 6.0, 100 mM pyruvate), which are not optimal for whole cells, the untreated RD04 cells showed only marginal ALS activity. In contrast to that, already 1 µg/ml nisin increased ALS activity. With increasing concentrations of nisin, the ALS activity increased accordingly, roughly following a logarithmic curve. The α-acetolactate formation rate seemed to saturate, eventually using 500 µg/ml at 0.56 ± 0.09 mM/min. Higher nisin concentrations were not tested as those were considered impractical for later use.

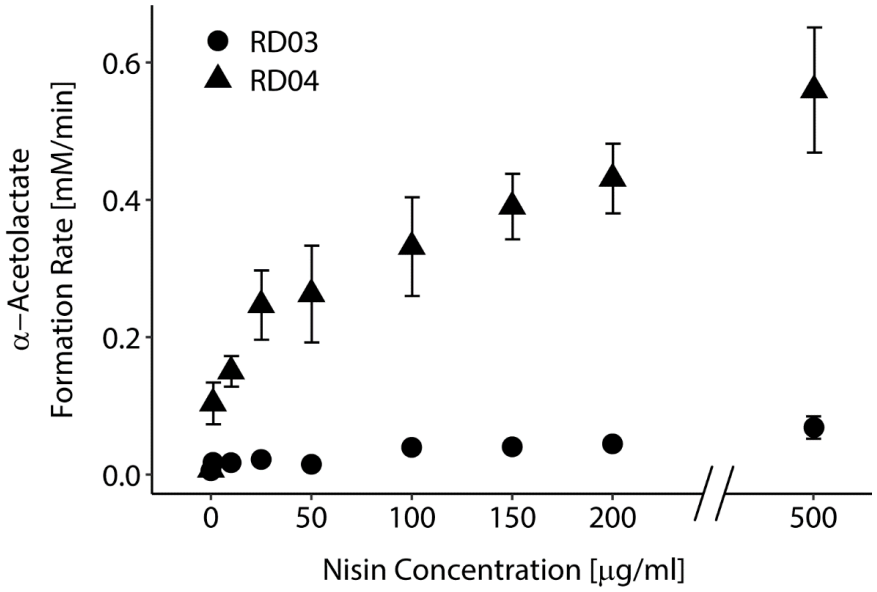

Figure 8.2 Permeabilization using increasing concentrations of the permeabilizing agent nisin. Cells equivalent to OD_{600} = 1 in 1 ml were treated with the following concentrations [µg/ml]: 0, 1, 10, 25, 50, 100, 150, 200, 500. The axis was broken between 200 and 500 µg/ml for better visualization. Symbols are used as follows: RD03: circle, RD04, triangle.

8.3.3 Permeabilized Cells Are Active over a Broad pH-Range and at Increasing Cell Densities

Through permeabilization, not only substrate and product transport over the cell membrane are affected, but also other small molecules can freely diffuse, which eventually equalizes the intra- and extracellular environments. In the following experiments, 100 µg/ml nisin with slightly longer incubation time compared to 8.3.2 were used to achieve maximal permeabilization. The pH dependence of the pyruvate to α-acetolactate conversion using permeabilized cells was determined in this way. In this comparison, the differences between the four strains, and thus the three ALS used, become clear (**Figure 8.3**).

RD03 expressing the native ALS from its chromosome, showed unremarkable changes over the whole pH-range with a maximum at pH 5.5 (0.045 ± 0.006 mM/min). The weak effect of permeabilization is most likely due to the low expression level of ALS under the given conditions.

Quite the opposite is observed in RD04, which overexpressed the native ALS from an expression plasmid using a strong constitutive promoter. RD04 was active over a broad pH-range from pH 4.5 to 7.0 with a maximum at pH 6.5 (0.58 ± 0.06 mM/min), which was close to the maximal activity determined in 8.3.2.

Consequently, the longer incubation time during permeabilization could compensate for lower nisin concentrations. Similarly, RD06 showed activity between pH 5.0 and 7.0 with a flat plateau between pH 5.0 and 6.5. Maximal activity was found at pH 6.5 (0.436 ± 0.004 mM/min). Noteworthy, at all pH values, the activity of RD06 was lower compared to RD04. RD05 expressing ALS from *Bacillus licheniformis* WX-02, showed maximum activity at pH 6.0 (0.158 ± 0.007 mM/min), which was quite low compared to RD04 or RD06, but slightly higher than RD03.

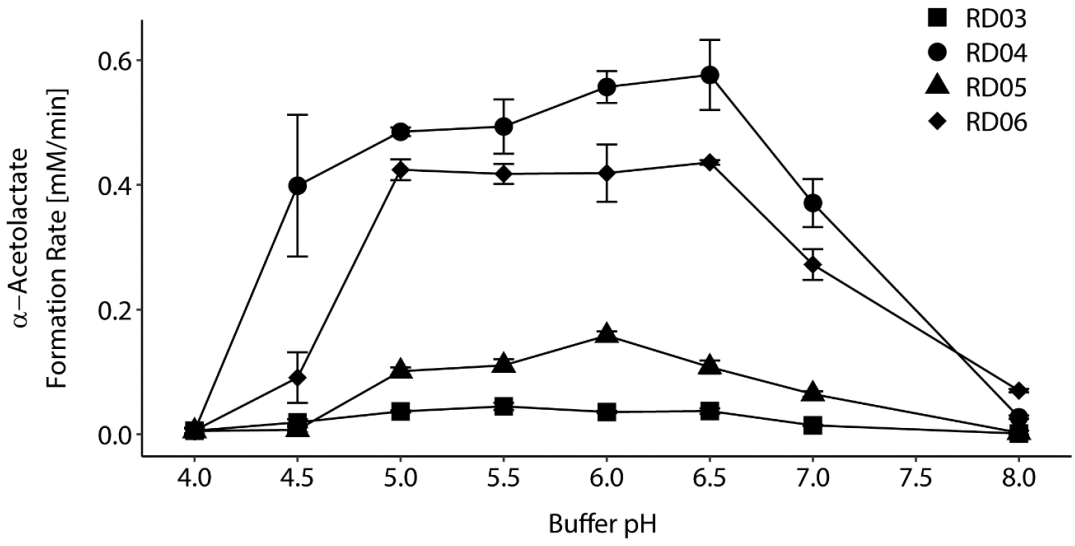

Figure 8.3 Activity of RD03-RD06 between pH 4.0 and 8.0. For determining the activity of the WCCs, the initial α-acetolactate formation rate [mM/min] was determined. Symbols are used as follows: RD03: square, RD04, circle, RD05, triangle, RD06: diamond.

One of the significant advantages of whole-cell biocatalysis is the possibility of using high cell densities in bioconversions. For testing the feasibility of that, the permeabilized cells were employed at high cell densities, and as expected, the α-acetolactate formation rate scaled linearly with cell density (**Figure S 8.1**). At the highest cell density used (OD_{600} = 10), the α-acetolactate formation rate was 4.2 ± 0.6 mM/min, allowing high-level α-acetolactate production from pyruvate within hours. Especially for producing α-acetolactate, this is promising, as α-acetolactate is unstable, and therefore short reaction times would be desired.

8.3.4 Addition of Thiamine Pyrophosphate is Required for Full ALS Activity in Buffered Solution

The cofactor requirement was tested for the best performing strain RD04. In all assay presented above, a reaction solution containing sodium-phosphate buffer, pyruvate, $MgCl_2$, and TPP was used as suggested in the literature (62). When using whole-cell biocatalysis at a larger scale, the addition of expensive cofactors is not desired, and therefore the effect of omitting of the cofactors was investigated (**Figure 8.4**). The absence of TPP and Mg^{2+} resulted in an approximately two-fold decreased activity compared to the control conditions (TPP & Mg^{2+}) (0.24 ± 0.04 mM/min versus 0.58 ± 0.04 mM/min). The addition of Mg^{2+} alone could not restore the activity but had a small positive effect (0.32 ± 0.06 mM/min). Contrarily, the addition of TPP alone nearly restored full ALS activity (0.49 ± 0.09 mM/min). It still seems most beneficial, when both Mg^{2+} and TPP are present in the reaction mixture.

In residual whey permeate (RWP) or M17 broth, which had both been enriched with pyruvate through lactose fermentation as described previously (see **Section 7.7.3**), the WCC seems to find beneficial conditions. The activity in RWP was comparable to that in buffer supplemented with TPP (0.51 ± 0.04 mM/min), and, to our surprise, in M17 broth, the activity was enhanced quite strongly (1.13 ± 0.08 mM/min) by approximately two-fold. It should be noted that the pyruvate concentrations in buffer, RWP, and M17 were different (100 mM, approx. 200 mM, and 350 mM, respectively), which might be a cause for the different activities.

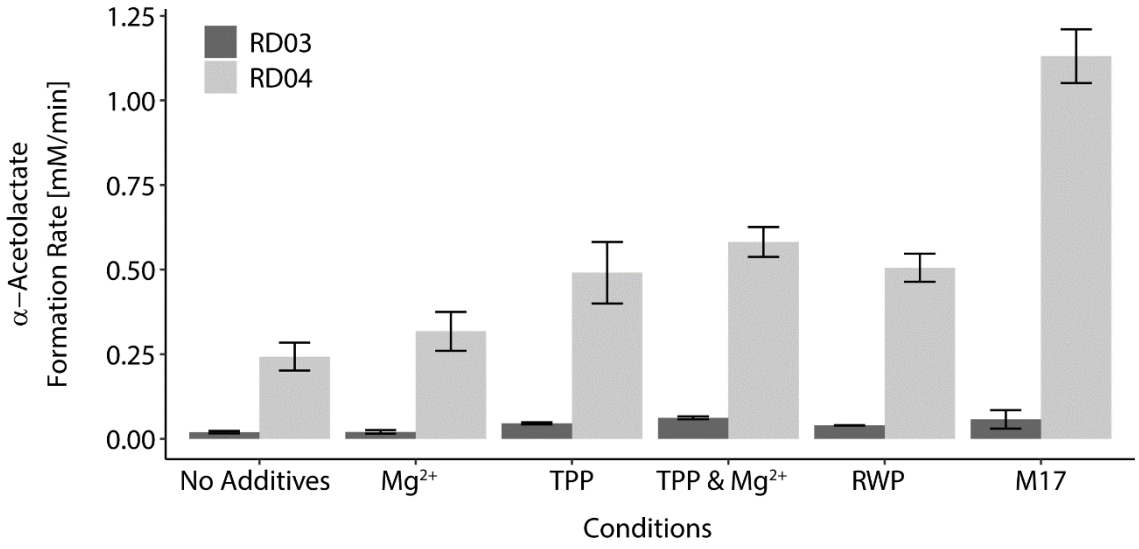

Figure 8.4 Influence of different additives or reaction media containing fermentation-derived pyruvate on α-acetolactate formation rate [mM/min]. Data for RD03 (dark grey) and RD04 are shown (light grey). Abbreviations are as follows: TPP: thiamine pyrophosphate, RWP: residual whey permeate enriched with pyruvate through fermentation, M17: LM17-broth enriched with pyruvate through fermentation.

8.3.5 Towards WCC-Based α-Acetolactate Production using a Cheap Feedstock rich in Citrate

Nowadays, one of the most concerning waste streams obtained during cheese production is collected after whey protein and lactose removal from cheese whey, the so-called mother liquor (ML) or delactosed whey permeate (14). This waste stream contains 10-20% (w/v) lactose but is not readily fermentable due to its high salt and low protein content (63–65). Diluting the ML and adding limiting nutrients can result in a fermentable substrate (15,63,66). The composition of ML varies between producers, and we obtained ML from Arla Food Ingredients (Viby J, Denmark) containing approximately 440 mM lactose, 120 mM citrate, 100 mM lactate, and 10 mM acetate.

We speculated that the citrate in ML could serve as a substrate for producing α-acetolactate using a WCC using the natural three-enzyme pathway described above. For producing α-acetolactate from citrate in *L. lactis*, a citrate-converting strain with low α-acetolactate decarboxylase (ALD) activity would be required. Such as strain was unfortunately not available in our strain collection when this project was carried out. Until such an ALD-deficient strain was available, optimization of acetoin production from ML was targeted.

As proof of concept, one random citrate fermenting strain was isolated from a starter culture and designated F102. This strain was cultivated in a low-pH medium containing citrate for inducing citrate utilization genes, and whole cells were incubated in untreated and undiluted ML at OD_{600} = 5 under anaerobic conditions (**Figure 8.5**). As expected, the citrate was nearly fully consumed (111 ± 10 mM) within 21 h, and similar acetate production (129 ± 1 mM) was observed deriving from CL activity. Acetoin, the main product from the citrate pathway, was detected at slightly lower concentrations as expected (46 ± 1 mM). Theoretically, 55.5 mM acetoin could be expected, and as no pyruvate was detected, it could be assumed that approx. 10 mM of the intermediates oxaloacetate or α-acetolactate were produced. Lactose consumption was observed (25 ± 8 mM), and accordingly, 81 ± 4 mM lactate and a surplus of approximately 17 ± 1 mM acetate were produced by the cells. This experiment demonstrated that citrate in ML could be converted into acetoin using whole-cell biocatalysis. Thus it would indeed be feasible to produce α-acetolactate from raw ML using an ALD-deficient strain of *L. lactis* subsp. *lactis* biovar diacetylactis.

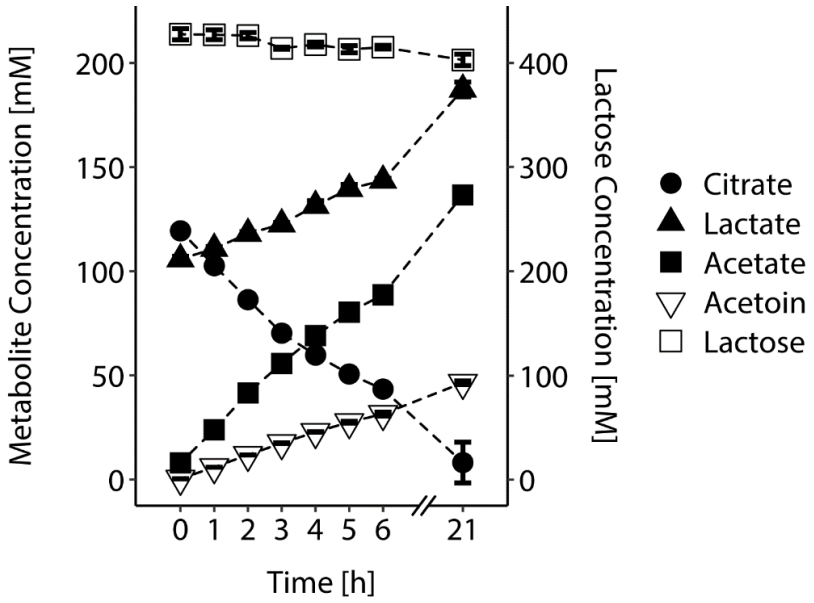

Figure 8.5 Citrate consumption in mother liquor (ML) using the wildtype isolate F102, which served as an example for an *L. lactis* subsp. *lactis* biovar diacetylactis strain. The bioconversion was carried out anaerobically using cells according to OD_{600} = 5.0. Metabolite concentrations are shown in different shapes (citrate: circles, lactate: triangles, acetate: squares, acetoin: crosses, lactose: open squares). The x-axis was broken for better visualization. The experiment was carried out in duplicate, and the standard deviations are shown.

For increasing the flux through ALS, further experiments were initiated, and preliminary results are reported. As lactose consumption and corresponding lactate formation were observed, the reaction conditions were changed from anaerobic to aerobic conditions to evaluate the effects on lactate formation. Furthermore, a natural lactate dehydrogenase (LDH) deficient mutant of *L. lactis* subsp. *lactis* biovar diacetylactis SD96, RD1M5, was employed. This mutant is an efficient cell factory for producing acetoin from lactose in ML (63). Here, higher cell densities (OD_{600} = 10.0) compared to the experiment shown in

Figure 8.5 were used to speed up the reaction. Within 5-6 h, both strains consumed all citrate and produced similar amounts of acetoin (approx. 35 mM) and acetate (approx. 100 mM) but nearly no lactate (**Figure 8.6**). In conclusion, the application of aerobic conditions seems to be a suitable method for reducing flux through LDH for whole-cell biocatalysis.

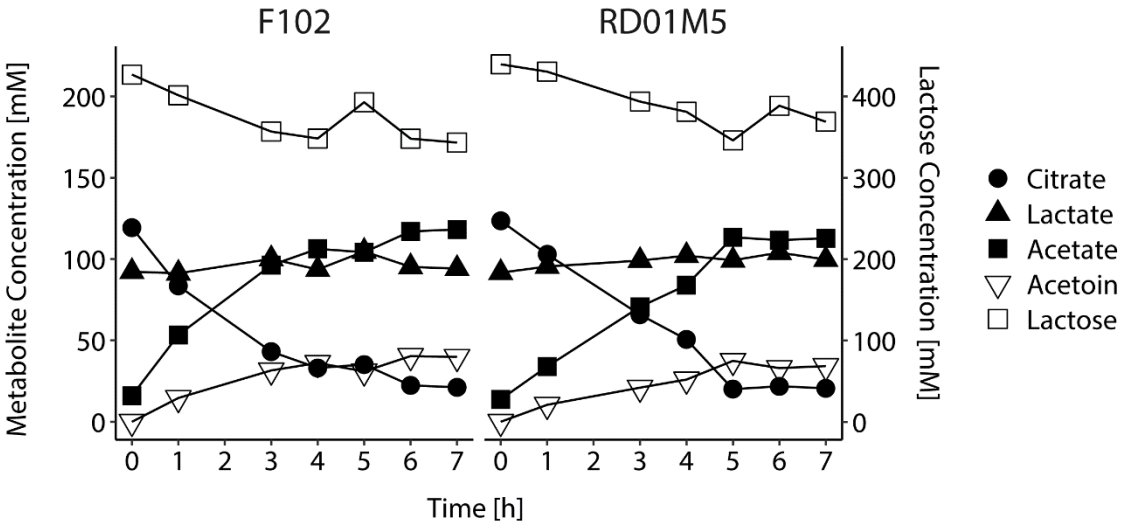

Figure 8.6 Initial results for the bioconversion of citrate in mother liquor (ML) using F102, a wildtype dairy strain, and RD1M5, a lactate dehydrogenase deficient mutant. Aerobic conditions were used with cells according to OD_{600} = 10.0. Metabolite concentrations are shown in different shapes (citrate: circles, lactate: triangles, acetate: squares, acetoin: crosses, lactose: open squares). Results are derived from single experiments and should, therefore, be treated with caution.

8.4 Discussion

8.4.1 Permeabilization Accelerates Whole-Cell Biocatalytic Production of α-Acetolactate

In this study, the positive effect of permeabilization on the bioconversion of pyruvate to α-acetolactate was presented. Previously, we reported whole-cell biocatalysis using several mutants of *L. lactis* expressing different ALS enzymes and observed a positive effect when using ALS from *E. faecalis*. Theortically, based on the constitutive promoter used, overexpression by 6- to 8-fold was expected, but the effect was smaller than that. Thus, we determined the ALS activity in cell extracts and found that RD04 and RD06, expressing ALS from *L. lactis* and *E. faecalis*, respectively, showed the expected 7- to 8-fold increased activity. Unexpectedly, RD05 expressing ALS from *B. licheniformis* WX-02 did not show increased ALS activity in cell extracts. In whole cells, the expression of this enzyme was shown to have a weak but positive effect on the α-acetolactate formation rate. Potentially, cell disruption negatively affected the enzymatic activity.

Based on these results, we hypothesized that transport over the cell membrane was responsible for the low increase in activity when using whole cells. Therefore, permeabilization seemed to be a straightforward solution for overcoming transport limitations. Since enzyme activity might be affected by the permeabilization procedure, we chose to permeabilize the WCCs with the antimicrobial peptide nisin. The choice of permeabilization method is essential because, for example, ALS from *E. faecalis* is sensitive to organic solvents such as ethanol, which is a common

permeabilizing agent (67). Nisin permeabilization does not affect enzyme activity because nisin acts exclusively on the cell wall and creates small pores (2 - 2.5 nm), which hold proteins within the cell envelope (21). Nonetheless, indirect effects, either positive or negative, are expected due to the increased exchange between intra- and extracellular space after permeabilization. In our experiments, permeabilization accelerated the bioconversion of pyruvate to α-acetolactate when using a reaction solution containing TPP, $Mg^{2+,}$ and pyruvate at pH 6.0. Permeabilization using the reaction solution, which was used for whole-cell based bioconversion, resulted in lower activities (data not shown). Because of that, we investigated the influence of pH, cofactors, and reaction medium on the bioconversion.

As described previously, whole-cell bioconversion of pyruvate to α-acetolactate was optimal at pH 5.5 for all strains investigated (RD03-RD06) (see **Chapter 7**, (28)). Furthermore, the addition of cofactors in the reaction was not required for maximal activity (data not shown). Additionally, permeabilized cells had broader pH optima than whole cells, which differed depending on the ALS expressed (**Figure 8.3**). Noteworthy, the here determined pH optima correspond well to the values reported by other researchers (45). Moreover, for obtaining maximal activity with permeabilized cells, the addition of TPP was required. So far, two conclusions can be drawn from the presented experiments. First, when using whole cells for the bioconversion, the pH optimum of pH 5.5 represents the optimal pH for substrate transport, and the external pH does not strongly affect the ALS enzymes. Contrarily, when cells are permeabilized, transport is not limiting, and substrates freely enter the cells, which also balances the intra- and extracellular pH, so that the optimal pH-values for permeabilized cells represent the optimal pH-values for the overexpressed ALS. Second, the TPP supply by *L. lactis* is not sufficient to equip all overexpressed enzymes with cofactors. Since the addition of TPP increased the activity of permeabilized cells overexpressing ALS, it is expected that without external TPP supply, a fraction of the ALS enzymes lacks the cofactor and is thus inactive. This effect was not observed in whole cells, potentially because TPP might not be taken up by whole cells. Interestingly, also the addition of neither thiamine nor TPP in the growth medium resulted in increased activities after premeabilization.

Both conclusion combined allow further speculation. Since in whole cells the substrate transport was shown to be limiting, the intracellular pyruvate concentration was potentially not sufficient to saturate all ALS, resulting in suboptimal activity. This could be underlined by the observation that in whole cells, overexpression of ALS from E. faecalis (ef-ALS) was most beneficial, while in permeabilized cells ALS from *L. lactis* (ll-ALS) was superior. The main difference between these two enzymes is the affinity for pyruvate, which is high for ef-ALS (K_m = 1.37 mM) and low for ll-ALS (K_m = 50 mM). Consequently, ef-ALS is expected to be more productive at low pyruvate concentrations compared to ll-ALS. Since pyruvate can freely enter permeabilized cells, here, the low affinity of ll-ALS towards pyruvate is not limiting.

8.4.2 Whole-Cell Biocatalysis Facilitates the Utilization of Citrate from Dairy Waste

Regarding the conversion of dairy waste-derived citrate into α-acetolactate and finally diacetyl, important preliminary experiments were presented. Nevertheless, an ALD-deficient strain able to utilize citrate is required for preventing acetoin formation from α-acetolactate. Such a strain would be crucial for developing a useful process. Still, we demonstrated that citrate is converted mainly into acetoin using a random strain from a starter culture and another industrial strain. In conclusion, potentially any citrate converting strain might be suitable to produce α-acetolactate, which is advantageous for screening purposes to isolate an ALD-deficient strain. Those strains could be obtained either by random mutagenesis or directly from natural biodiversity. For both approaches, different screening methods are available, which might increase the chances of finding a suitable candidate (57,68,69).

In this chapter, it was demonstrated how citrate from ML, a low-cost dairy waste, could be converted into acetoin using the randomly chosen *L. lactis* subsp. *lactis* biovar diacetylactis strain F102. Under anaerobic conditions, citrate was converted into acetoin, and residual lactose was converted into lactate and acetate. Aerobic conditions seemed to be beneficial for avoiding flux from lactose into lactate, but acetoin titers remained unaltered. Furthermore, as α-acetolactate formation is preferably carried out anaerobically for avoiding diacetyl formation, aerobic conditions are not ideal. As ML already contains lactate, further lactate production might potentially not considered problematic. In initial experiments, an LDH-deficient mutant strain RD1M5 was applied both aerobically and anaerobically (data not shown) for converting citrate into acetoin, and as expected lactate formation was not observed. Unexpectedly, the acetoin concentration using the LDH-deficient strain RD01M5 did not exceed those obtained using F102. When comparing anaerobic (**Figure 8.5)** and aerobic conditions (**Figure 8.6**), pyruvate was accumulated in the latter condition. Further experiments are planned for answering open questions regarding the metabolite flux during the biocatalytic transformation of citrate and lactose in ML using a WCC.

8.4.3 Permeabilization Negatively Affects the Activity of Citrate Lyase

Permeabilization of RD1M5 and other *L. lactis* subsp. *lactis* biovar diacetylactis strains (SD96 and RD07, see **Chapters 2-4**) resulted in activity loss for citrate conversion, while pyruvate conversion remained active (data not shown). The activity loss was observed both in ML and in a buffer containing citrate.

As citrate concentrations remained unchanged, it is expected that CL was affected. CLs can be inactivated by deacylation, which could have occurred during permeabilization. Such inactivation is common among CLs but has been shown to proceed slowly in *L. lactis* (39). Furthermore, it has been demonstrated that Mg^{2+} competitively inhibits CL, and it was concluded that CL recognizes citrate only when complexed with Mg^{2+} (70). Thus a balanced concentration of Mg^{2+}

and citrate must be present for optimal activity. In intact cells, the ratio of Mg^{2+} and citrate might be maintained by controlled transport, but in permeabilized cells, the ratio is entirely dependent on the media composition. Possibly, CL activity was negatively affected by the high citrate concentration present in ML because the Mg^{2+} concentration was too low. On a dry-weight basis, citrate in ML is present approximately in 1.5-fold excess (65), but the availability of free Mg^{2+} might be much lower due to salt formation with other ions in the ML. In buffered solutions containing 50 mM sodium phosphate, 50 mM citrate, Mn^{2+} (1 mM), Mg^{2+} (10 mM), and TPP (1 mM), citrate consumption was also not observed, which could be due to the unbalanced citrate and Mg^{2+} concentrations. Using balanced concentrations of citrate and Mg^{2+} in buffer or the addition of Mg^{2+} to ML could be an easy and straightforward option for achieving citrate formation in permeabilized cells.

8.5 Conclusion

Based on our previous work on using whole-cell biocatalysis for producing α-acetolactate from fermentation-derived pyruvate (see **Chapter 7**, (28)), we identified and eliminated several bottlenecks, which resulted in increased conversion rates of the WCC. Most importantly, we showed that transport over the cell membrane was limiting, which was overcome by permeabilization with the antimicrobial peptide nisin. This method provided efficient and mild permeabilization and resulted in excellent whole-cell biocatalysts. In the WCCs, the conversion of pyruvate to a-acetolactate is mediated by ALS, which was overexpressed 7- to 8-fold. TPP, an essential cofactor for ALS, was found to be limiting in reactions employing buffered pyruvate solutions, possibly due to the increased abundance of ALS. Interestingly, dairy-waste- or M17-based broths containing pyruvate, which were produced by fermentation as described previously (see **Chapter 7**, (28)), seemed to contain sufficient TPP and allowed fast reaction rates.

Furthermore, we showed that *L. lactis* subsp. *lactis* biovar diacetylactis is well suited for utilizing citrate, a natural precursor for pyruvate, to produce α-acetolactate. Citrate is present in high amounts (approx. 120 mM) in ML, a problematic waste stream obtained in immense quantities from the dairy industry. We showed that all the citrate could be converted into approximately 40 mM acetoin within several hours using nothing else than pure and undiluted ML. Unfortunately, for producing α-acetolactate in such a process, an ALD-deficient strain would be required, which was not available at the time, when this work was carried out. With further optimization of the whole-cell biocatalytic process, we expect that yield and productivity increase even more. Utilizing waste streams, such as ML, for producing chemicals or food ingredients is critical for achieving a circular economy in the dairy industry, which would limit the need for waste disposal and thereby reduce both costs and greenhouse gas emissions.

8.6 Methods

8.6.1 Bacterial Strains and Plasmids

Most strains and plasmids used in this study have been created and described previously (see **Chapter 7**, (28)). Briefly, ll-cat01 is a mutant strain based on MG1363, with deletions in *ldhB*, *ldhX*, *pta*, *adhE*, *butBA*, and *aldB*. RD03 contains pLC0, which is an empty expression vector. RD04-RD06 contain pLC0-based plasmids for overexpression of different ALS from *L. lactis* MG1363 (ll-ALS), *Bacillus licheniformis* WX02 (bl-ALS) and *Enterococcus faecalis* (ef-ALS), respectively, as summarized in **Table 8.1**. F102 was isolated randomly from a cheese starter culture by screening for citrate utilizing strains. Isolation of RD1M5 was described previously (63). Strains carrying pLC0-based plasmids (RD03-RD06) were cultivated at 30°C in M17 medium containing 1% glucose and 5 µg/ml tetracycline using shake flasks (250 rpm). F102 and RD1M5 were cultivated at 30°C as static cultures in M17 medium containing 0.5% lactose and 20 mM citrate at pH 6.0.

8.6.2 Preparation of Crude Cell Extracts

RD03-RD06 were cultivated as mentioned above, and cells from the early stationary phase were harvested by centrifugation at 5.000 x g for 10 min at 4°C. Afterward, the cells were always kept on ice. The supernatant was removed, and the cells were washed with 0.2% KCl solution. The washed cells were resuspended in extraction buffer (45 mM Tris, 1.5 mM Tricarballylate, 20% (v/v) glycerol, 4.5 mM $MgCl_2$, and 1 mM DTT at pH 7.2) and transferred into 2 ml safe-lock tubes containing approx. 500 µl glass beads (< 106 µm). Lysis was achieved using a FastPrep beat grinder (MP Biomedicals) at maximum speed for 5 min. The mixture was centrifuged at 20.000 x g for 5 min, and the supernatant was used for determining ALS activity as described below. Before the application in bioconversions, protein concentration in the lysates was determined using the Coomassie Plus (Bradford) Protein Assay (Thermo Fischer Scientific) with standard conditions.

Table 8.1 Bacterial strains and plasmids used in this study.

Designation	Genotype or description	Reference
***L. lactis* strains**		
MG1363	Plasmid free *L. lactis* subsp. *cremoris* strain	(71)
ll-cat01	MG1363 *ΔldhB ΔldhX Δpta ΔadhE ΔbutBA ΔaldB*	(28)
RD03	pLC0 in ll-cat01	(28)
RD04	pLC17 in ll-cat01	(28)
RD05	pLC02 in ll-cat01	(28)
RD06	pLC01 in ll-cat01	(28)
F102	*L. lactis* subsp. *lactis* biovar diacetylactis, random isolate from a starter culture	This work
RD1M5	*L. lactis* subsp. *lactis* biovar diacetylactis derived from SD96 by adaptive laboratory evolution and mutagenesis	(63)
Plasmids		
pLC0	pTD6 derived plasmid	(28)
pLC01	pLC0 inserted with the *als* gene from *Enterococcus faecalis* ATCC 29212 (ef-ALS)	(28)
pLC02	pLC0 inserted with the *als* gene from *Bacillus licheniformis* WX-02 (bl-ALS)	(28)
pLC17	pLC0 inserted with the *als* gene from *L. lactis* MG1363 (ll-ALS)	(28)

8.6.3 Whole-Cell Catalyst Preparation, Permeabilization, and Biotransformation

Cells were always harvested during the early stationary phase after diluting a fully-grown culture 1000-fold in fresh medium and cultivating for 16 h. Before harvesting, the cultures were put on ice, and the optical density at 600 nm (OD_{600}) was determined. Between cultivation and reaction, cells were always kept on ice and were only treated with cold solutions.

For small-scale reactions in 1 ml scale, the cells were harvested by centrifugation in appropriate amounts to reach OD_{600} = 1 in 1 ml. Then, the cells were suspended in 1 ml sterile POM buffer (50 mM K_2HPO_4/ KH_2PO_4,1 mM $MgCl_2$, pH 7.4) containing 100 µg/ml nisin or other concentrations as indicated in the text. For permeabilization, the cell suspensions were incubated at 37°C for 15 min. For initial tests using varying amounts of nisin, slightly shorter incubation times of 10 min were used. After incubation with nisin, the cells were washed once with POM buffer and suspended in 1 ml reaction solution. The reaction solution contained 50 mM sodium phosphate buffer (pH 6.0), 100 mM sodium pyruvate, 10 mM $MgCl_2$ and 1 mM TPP. For measuring the pH-range of the permeabilized cells, sodium phosphate buffer was used for pH 5.5, 6.0 and 6.5, acetate-buffer for lower pH (4.0 and 5.0), and Tris-buffer for higher pH (7.0 and 8.0). The reactions

were incubated at 30°C in a ThermoMixer with shaking, and samples were withdrawn regularly within the first 2 h, usually with 30 min intervals. The α-acetolactate formation rate was derived by linear regression using at least three data points. All small scale reactions were carried out at least in duplicates. Negative control reactions were carried out in the same way but without any pyruvate. Fermentation-broth was obtained, as described previously (see **Section 7.7.3**).

For conversion of citrate in ML, ML was autoclaved, and solids were removed by sedimentation. The pH was set to pH 5.5 using HCl for reactions involving whole cells. Cells from the early stationary phase were harvested and directly suspended in ML. The pH was monitored and controlled with 1 M HCl using a Biostat A unit from Sartorius. Aerobic or anaerobic conditions were set by flushing the medium with air or N_2, respectively.

8.6.4 Analytical Methods

Cell growth was measured by determining the optical density at 600 nm (OD_{600}). Glucose, lactose, pyruvate, lactate, and acetoin were measured by HPLC using the Aminex HPX-87H column at 60°C (Bio-Rad, Hercules, USA) and an RI detector at 30°C. Pyruvate was measured using UV detection at 210 nm during the same HPLC run. 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.5 ml/min.

For determining ALS activity or α-acetolactate formation rate, samples were taken regularly during the biotransformation reaction. A small volume of the reaction mixture was transferred to a new reaction vial and centrifuged to remove the cells. The supernatant was transferred to a 96-well microtiter plate, diluted with H_2O, and immediately mixed with 5 µl 2 M H_2SO_4 for quenching the reaction. The 96-well MTP was stored at 4°C until acetoin and α-acetolactate were measured using the method developed by W. Westerfeld (72). For this, the plate was incubated at 60°C for 15 min to allow full decarboxylation of α-acetolactate to acetoin. Then, 45 µl 0.5% creatine in H_2O and 45 µl 5% α-naphthol in 2 M NaOH were added. The resulting solution was mixed by pipetting and incubated first at 60°C for 15 min and then at room temperature for another 15 min. A standard curve using acetoin was prepared in the same way as the samples. The α-acetolactate formation rate was determined after measuring the absorbance at 530 nm using a plate reader.

8.7 References

1. Woodley JM. Advances in biological conversion technologies: new opportunities for reaction engineering. *React Chem Eng* **2020**, 5(4):632–40.
2. Kindstedt PS. The history of cheese. In: Global Cheesemaking Technology: Cheese Quality and Characteristics. Editors: Papademas P, Bintsis T. John Wiley & Sons Ltd. **2017**, 3–19.
3. Meussdoerffer FG. A Comprehensive History of Beer Brewing. In: Handbook of Brewing: Processes, Technology, Markets. Editor: Eßlinger HM. Wiley-VCH Verlag GmbH **2009**, 1–42.
4. Lin B, Tao Y. Whole-cell biocatalysts by design. *Microb Cell Fact* **2017**, 16(1):1–12.
5. Ladkau N, Schmid A, Bühler B. The microbial cell-functional unit for energy dependent multistep biocatalysis. *Curr Opin Biotechnol* **2014**, 30:178–89.

6. Orth JD, Conrad TM, Na J, Lerman JA, Nam H, Feist AM, Palsson B. A comprehensive genome-scale reconstruction of *Escherichia coli* metabolism-2011. *Mol Syst Biol* **2011**, 7(535):1–9.
7. Sheldon RA, Woodley JM. Role of Biocatalysis in Sustainable Chemistry. *Chem Rev* **2018**, 118(2):801–38.
8. Feijoo-Siota L, Blasco L, Rodríguez-Rama JL, Barros-Velázquez J, Miguel T De, Sánchez-Pérez A, Villa TG. Recent patents on microbial proteases for the dairy industry. *Recent Adv DNA Gene Seq* **2014**, 8(1):44–55.
9. Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, Madhavan A, Rebello S, Pandey A. Applications of microbial enzymes in food industry. *Food Technol Biotechnol* **2018**, 56(1):16–30.
10. Sales A, Paulino BN, Pastore GM, Bicas JL. Biogeneration of aroma compounds. *Curr Opin Food Sci* **2018**, 19:77–84.
11. Burg JM, Cooper CB, Ye Z, Reed BR, Moreb EA, Lynch MD. Large-scale bioprocess competitiveness: the potential of dynamic metabolic control in two-stage fermentations. *Curr Opin Chem Eng* **2016**, 14:121–36.
12. de Carvalho CCCR. Whole cell biocatalysts: essential workers from nature to the industry. *Microb Biotechnol* **2017**, 10(2):250–63.
13. Wachtmeister J, Rother D. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. *Curr Opin Biotechnol* **2016**, 42:169–77.
14. Oliveira D, Puri R, Fenelon MA, O'Mahony JA. Delactosed permeate as a dairy processing co-product with major potential value: a review. *Int J Food Sci Technol* **2018**, 54(4):1–10.
15. Shen J, Chen J, Jensen PR, Solem C. Development of a novel, robust and cost-efficient process for valorizing dairy waste exemplified by ethanol production. *Microb Cell Fact* **2019**, 18(1):1–10.
16. Chen RR. Permeability issues in whole-cell bioprocesses and cellular membrane engineering. *Appl Microbiol Biotechnol* **2007**, 74(4):730–8.
17. Ewe JA, Wan Abdullah WN, Bhat R, Karim AA, Liong MT. Enhanced growth of lactobacilli and bioconversion of isoflavones in biotin-supplemented soymilk upon ultrasound-treatment. *Ultrason Sonochem* **2012**, 19:160–73.
18. Fallico V, McAuliffe O, Fitzgerald GF, Hill C, Ross RP. The presence of pMRC01 promotes greater cell permeability and autolysis in lactococcal starter cultures. *Int J Food Microbiol* **2009**, 133(3):217–24.
19. Martínez-Cuesta MC, Requena T, Peláez C. Cell membrane damage induced by lacticin 3147 enhances aldehyde formation in *Lactococcus lactis* IFPL730. *Int J Food Microbiol* **2006**, 109(3):198–204.
20. Chen RR, Guo X. Methods and Compositions for Increasing Membrane Permeability. **2008**.
21. Zendo T, Yoneyama F, Sonomoto K. Lactococcal membrane-permeabilizing antimicrobial peptides. *Appl Microbiol Biotechnol* **2010**, 88(1):1–9.
22. Wang Q, Lillevang SK, Rydtoft SM, Xiao H, Fan M, Solem C, Liu J, Jensen PR. No more cleaning up - Efficient lactic acid bacteria cell catalysts as a cost-efficient alternative to purified lactase enzymes. *Appl Microbiol Biotechnol* **2020**.
23. Clark S, Winter CK. Diacetyl in Foods: A Review of Safety and Sensory Characteristics. *Compr Rev Food Sci Food Saf* **2015**, 14(5):634–43.
24. Wijaya I, Rankin SA. Diacetyl levels and volatile profiles of commercial starter distillates and selected dairy foods. *J Dairy Sci* **2012**, 95(3):1128–39.
25. Zeitsch KJ. 16. Diacetyl and 2,3-pentanedione. In: Sugar Series. Editor: Zeitsch KJ. **2000**, 120–49.
26. Verhue WMM, Tjan SB, Verrips CT, van Schie BJ. Process for preparing an aroma product containing α-acetolactic acid. EP0483888A2, **1991**. 1–14.
27. Marugg JD, Toonen MY, Verhue WMM, Verrips CT. Process for the preparation of α-acetolactic acid. EP0500188A2, **1992**. 1–23.

28. Dorau R, Chen L, Liu J, Jensen PR, Solem C. Efficient production of α-acetolactate by whole cell catalytic transformation of fermentation-derived pyruvate. *Microb Cell Fact* **2019**, 18(1):1–11.
29. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. *Metab Eng* **2016**, 36:57–67.
30. Wang Y, Sun W, Zheng S, Zhang Y, Bao Y. Genetic engineering of *Bacillus* sp. and fermentation process optimizing for diacetyl production. *J Biotechnol* **2019**, 301(2):2–10.
31. Medina de Figueroa R, Oliver G, Benito de Cárdenas IL. Influence of temperature on flavour compound production from citrate by *Lactobacillus rhamnosus* ATCC 7469. *Microbiol Res* **2001**, 155(4):257–62.
32. Branen AL, Keenan TW. Diacetyl and acetoin production by *Lactobacillus casei*. *Appl Microbiol* **1971**, 22(4):517–21.
33. de Man JC. The formation of diacetyl and acetoin from α-acetolactic acid. *Recl des Trav Chim des Pays-Bas* **1959**, 78(7):480–6.
34. Martín MG, Sender PD, Peirú S, De Mendoza D, Magni C. Acid-inducible transcription of the operon encoding the citrate lyase complex of *Lactococcus lactis* biovar diacetylactis CRL264. *J Bacteriol* **2004**, 186(17):5649–60.
35. García-Quintáns N, Repizo G, Martín M, Magni C, López P. Activation of the diacetyl/acetoin pathway in *Lactococcus lactis* subsp. *lactis* bv. diacetylactis CRL264 by acidic growth. *Appl Environ Microbiol* **2008**, 74(7):1988–96.
36. Cogan TM. Constitutive nature of the enzymes of citrate metabolism in *Streptococcus lactis* subsp. *diacetylactis*. *J Dairy Res* **1981**, 48(3):489–95.
37. Singh M, Srere PA. Purification and properties of citrate lyase from *Streptococcus diacetilactis*. *J Biol Chem* **1975**, 250(15):5818–25.
38. HARVEY RJ, COLLINS EB. Role of citritase in acetoin formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. *J Bacteriol* **1961**, 82(1956):954–9.
39. Kümmel A, Behrens G, Gottschalk G. Citrate lyase from *Streptococcus diacetilactis* - Association with its acetylating enzyme. *Arch Microbiol* **1975**, 102(1):111–6.
40. Sender PD, Martín MG, Peirú S, Magni C. Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family. *FEBS Lett* **2004**, 570:217–22.
41. Snoep JL, Mattos MJTDE, Starrenburg MJC. Isolation, Characterization, and Physiological Role of the Pyruvate Dehydrogenase Complex and α-Acetolactate Synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. *J Bacteriol* **1992**, 174(14):4838–41.
42. Sommer B, Moeller H Von, Haack M, Qoura F. Detailed Structure – Function Correlations of *Bacillus subtilis* Acetolactate Synthase. *ChemBioChem* **2015**, 16:110–8.
43. Pang SS, Duggleby RG, Schowen RL, Guddat LW. The Crystal Structures of *Klebsiella pneumoniae* Acetolactate Synthase with Enzyme-bound Cofactor and with an Unusual Intermediate. *J Biol Chem* **2004**, 279(3):2242–53.
44. Liu Y, Li Y, Wang X. Acetohydroxyacid synthases: evolution, structure, and function. *Appl Microbiol Biotechnol* **2016**, 100:8633–49.
45. Huo Y, Zhan Y, Wang Q, Li S, Yang S, Nomura CT, Wang C, Chen S. Acetolactate synthase (AlsS) in *Bacillus licheniformis* WX-02: enzymatic properties and efficient functions for acetoin/butanediol and l-valine biosynthesis. *Bioprocess Biosyst Eng* **2018**, 41(1):87–96.
46. Quintans NG, Blancato V, Repizo G, Magni C, Lopez P. Citrate metabolism and aroma compound production in lactic acid bacteria. In: Molecular Aspects of Lactic Acid Bacteria for Traditional and New Applications. Editors: Mayo B, López P, Pérez-Martínez G. **2008**, 65–88.
47. Kleerebezem M, Hols P, Hugenholtz J. Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. *Enzyme Microb Technol* **2000**, 26(9):840–8.

48. Curic M, Richelieu M De, Henriksen CM, Nilsson D. Glucose/Citrate Cometabolism in *Lactococcus lactis* subsp. *lactis* biovar diacetylactis with Impaired α-Acetolactate Decarboxylase. *Metab Eng* **1999**, 298:291–8.
49. Aymes F, Monnet C, Corrieu G. Effect of α-acetolactate decarboxylase inactivation on α-acetolactate and diacetyl production by *Lactococcus lactis* subsp. lactis biovar diacetylactis. *J Biosci Bioeng* **1999**, 87:87–92.
50. Starrenburg MJC, Hugenholtz J. Citrate fermentation by *Lactococcus* and *Leuconostoc* spp. *Appl Environ Microbiol* **1991**, 57(12):3535–40.
51. Verhue WM, Tjan FSB. Study of the Citrate Metabolism of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis by Means of 13C Nuclear Magnetic Resonance. *Appl Environ Microbiol* **1991**, 57(11):3371–7.
52. Jordan KN, Cogan TM. Production of acetolactate by *Streptococcus diacetylactis* and *Leuconostoc* spp. *J Dairy Res* **1988**, 55(2):227–38.
53. Ronfags E, Stien G, Germain P, Marc I. Kinetic Study of the Chemical Reactivity of α-Acetolactate as a Function of pH in Water, and in Fresh and Fermented Culture Media Used for *Lactococcus lactis* spp. *lactis* bv. diacetylactis. *Biotechnol Lett* **1996**, 18(7):747–52.
54. E. W. Seitz, W. E. Sandine, E. A. Day PRE. Studies on factors affecting diacetyl production by *Streptococcus diacetilactis*. *J Dairy Sci* **1961**, 44(6):1159.
55. Hugenholtz J, Kleerebezem M, Starrenburg M, Delcour J, De Vos W, Hols P. Lac*tococcus lactis* as a cell factory for high-level diacetyl production. *Appl Environ Microbiol* **2000**, 66(9):4112–4.
56. Guo T, Kong J, Zhang L, Zhang C, Hu S. Fine tuning of the lactate and diacetyl production through promoter engineering in *Lactococcus lactis. PLoS One* **2012**, 7(4):1–10.
57. Goupil N, Corthier G, Ehrlich SD, Renault P. Imbalance of leucine flux in *Lactococcus lactis* and its use for the isolation of diacetyl-overproducing strains. *Appl Environ Microbiol* **1996**, 62(7):2636–40.
58. Monnet C, Aymes F, Corrieu G. Diacetyl and α-acetolactate overproduction by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis mutants that are deficient in α-acetolactate decarboxylase and have a low lactate dehydrogenase activity. *Appl Environ Microbiol* **2000**, 66(12):5518–20.
59. Monnet C, Schmitt P, Divies C. Development and Use of a Screening Procedure for Production of α-Acetolactate by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis Strains. *Appl Environ Microbiol* **1997**, 63(2):793–5.
60. Jay JM. Antimicrobial properties of diacetyl. *Appl Environ Microbiol* **1982**, 44(3):525–32.
61. Zhu D, Liu F, Xu H, Bai Y, Zhang X, Saris PEJ, Qiao M. Isolation of strong constitutive promoters from *Lactococcus lactis* subsp. *lactis* N8. *FEMS Microbiol Lett* **2015**, 362(16):1–6.
62. Singh BK, Stidham MA, Shaner DL. Assay of acetohydroxyacid synthase. *Anal Biochem* **1988**, 171(1):173–9.
63. Liu J, Chen L, Dorau R, Lillevang SK, Jensen PR, Solem C. From waste to taste – Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) *Lactococcus lactis* dairy isolate. *J Agric Food Chem* **2020**, 68(21):5891–9.
64. Durham RJ. Modern approaches to lactose production. Woodhead Publishing Limited **2009**, 103–144 p.
65. Friend MA, Kaiser AG, Piltz JW, Sillence MN, Jolliffe SK. Use of delactose whey permeate as a supplement for cattle on a cereal straw based diet. *Aust J Exp Agric* **2004**, 44(9):833–40.
66. Liu J, Dantoft SH, Würtz A, Jensen PR, Solem C. A novel cell factory for efficient production of ethanol from dairy waste. *Biotechnol Biofuels* **2016**, 9:33.

67. Lee SC, Kim J, La IJ, Kim SK, Yoon MY. Characterization of recombinant FAD-independent catabolic acetolactate synthase from *Enterococcus faecalis* V583. *Enzyme Microb Technol* **2013**, 52(1):54–9.
68. Monnet C, Corrieu G. Selection and Properties of *Lactobacillus* Mutants Producing α-Acetolactate. *J Dairy Sci* **1998**, 81(8):2096–102.
69. Curic M, Stuer-lauridsen B, Renault P, Nilsson DAN. A General Method for Selection of a-Acetolactate Decarboxylase-Deficient *Lactococcus lactis* Mutants To Improve Diacetyl Formation. *Appl Environ Microbiol* **1999**, 65(3):1202–6.
70. Harvey RJ, Collins EB. The Citritase of *Streptococcus*. *J Biol Chem* **1963**, 238(8):2648–53.
71. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **1983**, 154(1):1–9.
72. Westerfeldt WW. A colorimetric Determination of Blood Acetoin. *J Biol Chem* **1945**, 161:495–502.

8.8 Supplementary Material

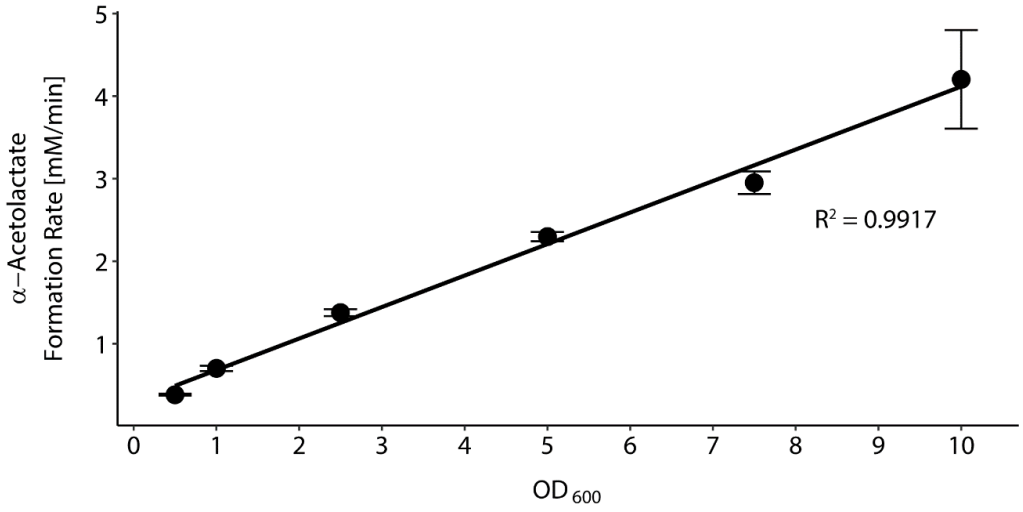

Figure S 8.1 Linear relation between cell density and α-acetolactate formation rate [mM/min]. RD04 was used for determining the effect of increased cell densities.

9. Summary and Conclusion of Part II

Summary

Lactic acid bacteria (LAB) are promising candidates for producing natural food ingredients or bulk chemicals from renewable resources, and metabolic engineering is becoming increasingly feasible for optimizing LAB for such purposes. This topic is thoroughly reviewed in **Chapter 6**, which serves as an introduction for the subsequent two chapters. In **Chapter 7**, the application of metabolically optimized *Lactococcus lactis* (*L. lactis*) as a whole-cell catalyst (WCC) is demonstrated for producing α-acetolactate from a dairy waste stream. α-Acetolactate can be readily converted into diacetyl, a potent flavor compound, naturally providing buttery aroma in dairy products. The developed two-step process combining fermentation for obtaining large amounts of pyruvate and its subsequent biocatalytic transformation into α-acetolactate was shown to be superior to previous production processes. Further successful optimization of this process is reported in **Chapter 8**. For this, the WCC was permeabilized using the food approved antimicrobial peptide nisin. Initial experiments towards utilizing a low-value waste stream rich in citrate, which is a natural precursor for producing α-acetolactate in *L. lactis*, were successful.

Conclusion

Metabolic engineering of LAB has experienced a significant advancement during the last years due to the development of new genome editing techniques. Until recently, only selected LAB, which had been adapted to the laboratory environment, could be genetically modified, and applications were limited. Nowadays also wild strains become amenable for genome editing and metabolic engineering, which allows the construction of more efficient and robust cell factories, biocatalysts, or novel probiotics. The review in **Chapter 6**, which will be published in the renowned *Advanced Biotechnology* book series by Wiley, will guide students and researchers through the field of metabolic engineering of LAB.

Using whole-cell biocatalysis for producing α-acetolactate from dairy waste (**Chapters 7 & 8**) has shown to be more efficient, scalable, and flexible compared to fermentation processes. Dairy waste is a challenging substrate for LAB due to the high salt and sugar concentrations. Therefore, product titers were often limited, particularly when toxic side products, such as diacetyl, may be formed during the process, adding yet another constraint on the bacteria. Consequently, the here described two-step bioprocess is an excellent example of how to overcome challenges associated with salt- and nutrient-rich waste streams from the food industry. Such biocatalytic processes are not widely established in the dairy industry, and therefore this work might serve as inspiration for upcycling industrial waste material in the future. This work is in line with the need to establish circular economy principles and bio-based production of bulk or fine chemicals at a larger scale to achieve more sustainable industrial food and chemical production in the future.